STUDIES ON *VIBRIO* SPP. IN JUVENILES OF *PENAEUS INDICUS* IN CULTURE SYSTEMS

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BY

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DECLARATION

I hereby declare that this Thesis entitled "Studies on Vibrio spp. in juveniles of Penaeus indicus in culture systems" is based on my own research and has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

Cochin 682 014,
July 1996.

(Sini Joys Mathew)

CERTIFICATE

This is to certify that the Thesis entitled "Studies on Vibrio spp. in juveniles of Penaeus indicus in culture systems" is the bonafide record of the work carried out by Shri. Sini Joys Mathew under my guidance and supervision and that no part thereof has been presented for the award of any other Degree or Diploma.

15.7.1996

Cochin 682 014,
July 1996.

(Dr. K. Rengarajan)
Senior Scientist,
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Dedicated to

The Sovereign God,
The Origin of all Knowledge, Wisdom and Goodness

For
The betterment of His precious Mankind

In loving memory of my
beloved sister Mrs. Mini Mathews

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PREFACE

PREFACE

When God almighty created this earth, He made every thing so perfect, well balanced and enriched with natural resources. But when man became the dominating creature of this planet, he became greedy and his indiscriminate exploitation of natural resources ultimately resulted in reduction in the natural resources.

As the supply of marine living resources mainly in the form of fishes from the sea has dwindled to meet the needs of the growing human population, man started farming different aquatic animals in inshore, coastal, lagoon and estuarine waters.

As the understanding of the factors that play important role in the survival and growth of farming organisms in the culture systems expanded, it has become evident that diseases, among other environmental variables drastically affect captive and cultured populations.

Use of weak or unhealthy seeds, nutritional imbalance in the supplementary diets, deterioration in the water quality are some of the important factors that predispose the animals to diseases of infectious or non-infectious aetiology. Many pathogenic organisms are already distributed widely in culture systems and the disease outbreaks are often precipitated by adverse environmental factors. Hence, many diseases encountered in aquaculture can very well be prevented by strict, timely and scientific management. Knowledge of the factors that induce physiological stress in the animal and that influence the survival and growth of the cultured animal, is essential for the success and profitability of aquaculture.

The major penaeid shrimp diseases are caused by viruses, bacteria, fungi and protozoans. Among the bacterial diseases the most prevalent and wide spread is 'vibriosis'

caused by different species of *Vibrio*. They also cause severe secondary bacterial infection along with many other diseases which lead to high rate of mortality in the cultured shrimp population.

In order to develop a viable technology to combat 'vibriosis' in shrimps in the culture systems, adequate information on the impact on environmental factors on the occurrence, abundance and species diversity of *Vibrio* spp. is highly essential. Such comprehensive studies are very meagre from the Indian Coast. Further the nature and incidence of associated vibrios to cause infection on the juveniles of shrimps at different habitats have also not been documented sufficiently yet.

This paucity of information which has been remaining as the major impediment in developing suitable and effective management measures to combat 'vibriosis' in shrimps during culture, prompted the candidate to take up the **"Studies on *Vibrio* spp. in juveniles of *Penaeus indicus* in culture systems"** for the doctoral research.

The Thesis begins with an "Introduction" followed by "A review of literature" on diseases of penaeid shrimps with particular reference to 'vibriosis' and "Material and methods" which details with the methods and procedures followed in the experiments and analyses of data. This Thesis consists of three chapters.

Chapter 1deals with the incidence and ecology of *Vibrio* spp. in water, sediment and in juveniles of the Indian white prawn *Penaeus indicus* in the culture systems.

In Chapter 2, characteristics of vibrio isolates including growth response to various levels of temperature, salinity and pH, sensitivity to 40 antibiotics and minimal inhibitory concentration tests are detailed.

The Chapter 3 discusses the role of physico-chemical parameters in the incidence, seasonal abundance of *Vibrio* spp. and in 'vibriosis'. A summary of the whole work and list of references are also included at the end.

This study gives a detailed information regarding the incidence and ecology of vibrios in the culture systems, their characteristics and pathogenicity.

The salient findings of this study are enumerated below.

1. The incidence of vibrios in the culture systems was significantly correlated to the environmental parameters such as temperature, salinity, total hardness and alkalinity of the pond water.

2. The incidence of vibrios and their species diversity was lowest during Monsoon and it increased to the maximum in Summer through Postmonsoon and Transition.

3. Juvenile shrimps seem to provide a suitable habitat for vibrios and the digestive system was found most ideal for their growth and multiplication.

4. In culture ponds incidence of vibrios was higher in water than in the sediment.

5. *V. anguillarum* was predominant in the culture systems which accounts for 24.40% of the total, followed by *V. fischeri* like bacteria (19.05%), *V. parahaemolyticus* (17.26%), *V. neries* (11.90%), *V. ordalii* (9.52%), *V. fluvialis* biovar II (8.33%), *V. cholerae* non-O1 (5.36%) and *V. proteolyticus* (4.17%).

6. A sudden decline in the vibrio population leading to their disappearance in the culture systems, immediately after the unprecedented and sudden rain

was a significant finding. Environmental changes in the system and the entry of the alien bacteria (*Bacillus* sp.) into the culture systems by the rain water is probably a causative factor for this unusual phenomenon.

7. All the vibrio isolates registered good growth in the temperature range 25 °-35°C, pH 7.0-8.5 and salinity 15-30 ppt.

8. Multiple drug resistance was exhibited by isolates of all *Vibrio* spp.

9. Among 40 antibiotics tested, Co-trimoxazole was the most effective against vibrios and all the species were highly resistant to Bacitracin, Methicillin, Oxacillin and Penicillin.

10. The minimum dose of intramuscular injection required to induce mortality in juvenile shrimps was 22.5 x 10^6 cells/animal for *V. anguillarum* and 30.5 x 10^7 cells/animal for *V. parahaemolyticus*.

11. Pathogenicity test in stress conditions proved that the opportunistic pathogens which form part of normal gut flora of the juvenile shrimps could infect a host which is under prolonged and specific physiological stress due to physico-chemical parameters.

12. It was found that the ideal condition for the growth and multiplication of pathogen is not the factor that governs the infection, but the physiological stress suffered by the host has the crucial role in infection.

13. 35 ppt salinity was found to make the shrimps able to resist against vibrio infection.

It is earnestly hoped that the results of this research work will definitely provide information to some of the problems faced by the prawn farmers and pave way to new ideas, ultimately leading to the solution of the enigma of the pandemic of 'vibriosis' in shrimps.

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ACKNOWLEDGEMENTS

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INTRODUCTION

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Aquaculture is a fast developing and established industry in many countries and it has been considered as an extreme focus sector for development. In recent years, aquaculture has substantially increased to include new species with specific nutritional characteristics, pharmaceuticals, chemical and bio-active compounds.

In addition to this, aquaculture (i) also generates many employment opportunities in the rural sector, (ii) augments local, regional and international trade to the inputs and outputs of aquaculture and (iii) helps to earn valuable foreign exchange through exports to improve the socio-economic condition of the farmers. Intensification of aquaculture practices have also added an array of ancillary industries such as feed industry, aquaculture machinery, chemicals, pharmaceuticals, food processing, etc.

Shrimp is given prime importance in aquaculture by virtue of its ever-increasing demand in international markets. There are mainly 4 types of culture practices employed in shrimp culture systems namely extensive, semi-intensive, intensive and ultra-intensive (Fast, 1991). This grouping is based on the stocking density and management practices employed in the culture systems.

(i) In extensive culture practice, natural seeds are stocked in low density with minimal management attention. (ii) In semi-intensive system, both wild and hatchery seeds are stocked with continuous management attention. (iii) In the case of intensive culture systems, seeds produced in the hatchery are stocked in high density with continuous and skilled management. (iv) In ultra-intensive systems, seeds from the hatchery are stocked in very high densities in concrete tanks or raceway systems with continuous and highly skilled management attention.

Unfortunately shrimp culture all around the world is facing serious problems with

sudden out-break and spread of diseases. Disease out-breaks are frequent and have become a serious problem with intensive and ultra-intensive culture practices (Fast, 1991).

The diseases are caused by different agents such as viruses, bacteria, fungi, protozoans, etc. In addition to this, diseases due to nutritional imbalance and deficiencies. toxicity and environmental stress also cause damage to the shrimp population in ponds. Viral diseases are the most serious disease which often result in total loss of the crop. But in most of the diseases, secondary bacterial infections are invariably reported from the diseased animal which actually lead the animal to death.

To face the challenge of diseases in shrimp culture farms and to adopt control measures, adequate knowledge of various aspects on pathology particularly interaction between host, pathogen and environment is essential. Among the bacterial diseases the most prevalent and wide spread in Indian waters is 'vibriosis' caused by different species of *Vibrio*. In addition to this, they cause severe secondary bacterial infections in many diseases.

Vibrios are gram negative asporogenous, motile, short or curved rods, which are widely distributed in aquatic habitats with a wide range of salinity and are very common in marine and estuarine environments. Several species are pathogenic to man as well as to marine vertebrates and invertebrates (Baumann *et al.*, 1984). Their incidence, distribution and abundance are governed mainly by the prevailing environmental parameters.

Vibrios are reported to be predominant in natural microbial flora of the penaeid shrimps which provide them a suitable environment for growth. They play both beneficial and harmful role in the life of the animal by enhancing the digestive process on one side and at the same time infecting the host during prolonged adverse environmental conditions. Since vibrios form part of normal bacterial flora of shrimps and their environment, and at

the same time cause infection when the host is under physiological stress, they are considered as facultative or opportunistic pathogens.

Colwell *et al.* (1984) state "*It is clear that unless the ecology of the vibrios is not fully understood, complete control of the disease caused by these micro-organism will not be possible*". This indicates the significant role played by the environmental parameters in the vibrio infection.

Though many chemotherapeutics including antibiotics were reported effective against 'vibriosis', development of drug resistance in the pathogens has been and continue to be a serious problem in involving human health effects.

Absolute removal of all pathogens is practically impossible in a semi-intensive type of shrimp culture system. Maintenance of a healthy environment and prevention of stress condition in culture system are considered effective in reducing the chances of disease out-breaks.

With these in mind, the investigation was carried out to understand (i) the incidence and occurrence of species of *Vibrio* in different culture systems in and around Cochin, (ii) characteristics of vibrios isolates, their ecology including growth response to various hydrological parameters, sensitivity to about 40 antibiotics, and (iii) role of physico-chemical parameters in pathogenicity of vibrios, etc. and the results emerged from the investigations are important and encouraging for better understanding of the 'vibriosis' in the culture systems to develop remedial measures to control disease out-breaks.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Penaeid shrimp culture is fast expanding in many countries as an important segment of their economy. But this industry has always been threatened with the occurrence of diseases of different aetiologies. The incidence of disease is almost dependent on the culture practice adopted. The incidence of disease was reported serious with the intensive and ultra-intensive culture practices, but with the extensive and semi-intensive practices it was not reported as a problem (Fast, 1991).

Information regarding the diseases in penaeid shrimps were reviewed by several authors (Overstreet, 1973; Lightner, 1977,1983, 1985, 1993; Johnson, 1978; Ruangpan, 1982; Baticados, 1988 a, 1988 b; Boonyaratpalin, 1990; Nash, 1990; Sindermann, 1990 a).

Penaeid shrimp diseases can be broadly classified into three : (i) Infectious diseases, (ii) Non-infectious diseases and (iii) Nutritional, Toxic and Environmental diseases. Diseases caused by viruses, pathogenic bacteria, fungi and protozoans are included under infectious diseases. Diseases caused by epicommensals and algae are included under non-infectious diseases and abnormalities due to nutritional imbalance in diet, toxins and environmental factors are included under the third group (Lightner, 1985).

INFECTIOUS DISEASES

At present there are 15 viruses known to infect penaeid shrimps in the culture systems and in the wild (Lightner *et al.*, 1994). *Baculovirus penaei* (BP), *Penaeus monodon* baculovirus (MBV), Baculoviral midgut gland necrosis (BMN), Infectious hypodermal and hematopoietic necrosis virus (IHHNV), Hepato-pancreatic parvo like virus (HPV) and Reo like virus (REO) are the important viral agents causing diseases in penaeids (Couch, 1974; Sano *et al.*, 1981; Lightner *et al.*, 1983; Lightner, 1985; Baticados, 1988 a; Lightner

and Redman, 1991; Tsing and Bonami, 1984; Overstreet, 1994). Some other diseases in penaeid shrimps are suspected of the possible viral aetiology, caused by Picorna virus or Parvo virus (Foster *et al.*, 1981), Togavirus (Bonami *et al.*, 1992) and Nodavirus (causing Taura syndrome) (Chamberlain, 1994).

'Ricketsia' and 'chlamidia' are also reported recently among the agents that cause disease in the cultured shrimp (Lightner, 1993).

Bacterial species causing disease out-break in penaeid shrimps are considered as "opportunistic pathogens" which affect shrimps both as primary and secondary invaders (Baticados, 1988 a). They have been implicated as cause of diseases and mortality in cultured penaeids especially in larval, postlarval and juvenile stages (Johnson, 1978; Lightner, 1983). Necrosis of appendages, shell disease, filamentous bacterial disease, luminous bacterial disease and septicemia are caused mainly by pathogenic bacteria belonging to genus *Vibrio*, *Aeromonas*, *Pseudomonas* and *Flavobacterium* (Lightner, 1975, 1983, 1985, 1993; Lightner and Lewis, 1975; Delves-Broughton and Poupard, 1976; Ruangpan, 1982; Khan *et al.*, 1984; Lightner *et al.*, 1984; Chong and Chao, 1986; Sano and Fukuda, 1987; Anderson *et al.*, 1988; Boonyaratpalin, 1990; Nash, 1990; Sindermann, 1990 a). Necrotising Hepatopancreatitis (NHP) caused by an intracellular gram negative pleomorphic bacterium have been recently reported causing sever damages to cultured penaeid shrimp population (Krol *et al.*, 1991; Frielier *et al.*, 1992, 1993, 1994; Lightner and Redman, 1994).

Different fungal pathogens infect penaeid shrimps at different life stages. One of the most important fungal diseases of penaeid larvae is larval mycosis caused by *Lagenidium* spp. (Barkate *et al.*,1974; Lightner, 1975, 1985; Ruangpan, 1982; Lightner *et al.*, 1984; Baticados, 1988 a; Boonyaratpalin, 1990). *Sirolpedium* spp. and *Haliphthoros* spp. are the other species infect the penaeid larvae (Gacutan, 1979; Hatai

et al., 1980; Lightner, 1985; Aquacop, 1983; Baticados, 1988 a; Boonyaratpalin, 1990). Fusarium infection have been observed among larvae to adult shrimps causing white cotton like out growth in larvae and blackening of gill tissue in adults (Johnson, 1974; Lightner, 1975, 1985; Laramore *et al.*, 1977; Ruangpan, 1982; Lightner *et al.*, 1984).

Parasitic protozoan diseases such as Microsporidiosis and Gregarine diseases in penaeid shrimps (Johnson, 1978; Lightner, 1975, 1983; Ruangpan, 1982; Nash, 1990; Sindermann, 1990 a) have been reported as of infectious nature (Lightner, 1985).

NON-INFECTIOUS DISEASES

Epicommensal infestation in penaeids by filamentous bacteria, peritrichous protozoans and pinnate diatoms are reported as non-infectious (Lightner, 1985).

Bacterial epicommensals like *Lucothrix mucor*, *Thiothrix* spp. *Cytophaga* spp. and *Flexibacter* spp. have been implicated in bacterial gill diseases and surface fouling (Delves-Broughton and Poupard, 1976; Lightner, 1975, 1983; Baticados, 1988 a; Boonyaratpalin, 1990). The important peritrichous protozoan epicommensals of penaeid shrimps included species such as *Epistylis* spp., *Zoothamnium* spp., *Vorticella* spp. and *Acineta* spp. (Lightner, 1975, 1983, 1985, 1993; Johnson, 1978; Ruangpan, 1982; Boonyaratpalin, 1990; Nash, 1990). A number of blue green algae and diatoms have been reported to be among the epicommensal organism causing surface fouling and gill diseases in cultured and captive penaeids (Lightner, 1983, 1993).

Parasitic worms (Trematodes, Cestodes and Nematodes) also have been reported occasionally from penaeid shrimps (Johnson, 1978).

NUTRITIONAL, TOXIC AND ENVIRONMENTAL DISEASES

The only reported nutritional disease syndrome of cultured penaeids is black-death or shrimp scurvy. It occurs in penaeids receiving diets with insufficient ascorbic acid content (Lightner *et al.*, 1977, 1979; Lightner, 1985).

The important toxic diseases include Haemocytic entritis, dinoflagellate poisoning and Aflotoxicosis (Liao *et al.*, 1977; Lightner, 1978, 1983, 1985, 1993; Lightner *et al.*,1982, 1984; Wiseman *et al.*, 1982; Baticados 1988 a).

Environmental diseases such as gas-bubble disease, cramped tail, spontaneous muscle necrosis and chronic soft shell syndrome have been reported in cultured penaeid shrimps (Venkataramaiah, 1971; Johnson *et al.*, 1975; Johnson 1978; Lakshmi *et al.*, 1978; Rajamani, 1982; Lightner, 1983, 1985; Rao, 1983; Brison, 1985; Anon., 1987; Baticados *et al.*, 1986, 1987).

VIBRIOS

Vibrios are straight or curved rod-shaped, gram negative, motile, facultatively anaerobic chemo-organotrophs, capable of both fermentative and respiratory metabolism and found in aquatic habitat with a wide range of salinities (Baumann *et al.*, 1984). Twenty species of vibrios are listed in Bergiey's (1984) manual of systematic bacteriology. The species are

Vibrio cholerae,
V. harveyi,
V. parahaemolyticus,
V. alginolyticus,
V. neries,
V. metschnikovii,
V. campbelli,
V. vulnificus
V. natriegens,
V. fluvialis biovar I and II,

V. splendidus,	*V. pelagius* biovar I and II
V. nigripulchritudo,	*V. anguillarum* biovar I and II,
V. fischeri,	*V. logei*,
V. proteolyticus,	*V. gazogenes*,
V. marinus	*V. costicola*.

Taxonomy of vibrios is still in a state of uncertainities (West and Colwell, 1984) and taxonomists disagree with the taxonomic position of many *Vibrio* isolates from shrimps (Lightner, 1977).

Members of the Family Vibrionaceae can be isolated from freshwater, estuarine and seawater environment as well as from alimentary tract of man and warm blooded animals. Some species are pathogenic to man and others are pathogenic for marine animals. Some species comprise the ectocommensal flora of finfish and shellfish, and some others participate in recycling of the organic matter (West and Colwell, 1984). Vibrios associated with marine animals have been reviewed by Seethalakshmi (1978).

OCCURRENCE OF VIBRIOS IN NATURE

The occurrence, distribution, abundance and the diversity of the bacterial species are greatly influenced by the prevailing environmental parameters (Sochard *et al.*, 1979; Ramamirtham *et al.*, 1987; Alavandi, 1989). Vanderzant *et al.* (1971) had reported that the salinity fluctuation in the water due to evaporation and rainfall cause difference in microbial flora of the Gulf Coast, Pacific and pond reared shrimp. Straub *et al.* (1993) also have reported the direct correlation between the changes in salinity and diversity of the bacterial flora in association with brine shrimp.

Alavandi (1989) has observed low count of bacterial population in the coastal waters of Cochin during Monsoon which was suggested to be due to the discharge of flood waters from Vembanad Lake and the reason for the major peak observed during January-February was related to the factors such as suitable temperature, evaporation of surface water and low variation in salinity.

Vanderzant *et al.* (1971) and Vanderzant and Nichelson (1973) agree that changes in number and type of micro-organism are related to some extent to changes in water characteristics such as temperature, salinity, oxygen level phytoplankton activity and pH.

But Christopher *et al.* (1978), could not establish any relationship between change in number and type of microorganism in pond reared shrimps or pond water and changes in pond water characteristics such as temperature, salinity, dissolved oxygen and pH.

Alavandi (1989) has reported that vibrios share 5% of the total heterotrophic bacteria in the coastal waters of Cochin whereas dominance of *Vibrio* spp. in the pond water was reported by Christopher *et al.* (1978). From shrimp farms Leangphibul *et al.* (1986) could isolate *V. parahaemolyticus*, *V. alginolyticus*, *V. fluvialis* and *V. cholerae* non-O1.

Nair *et al.* (1980) have identified *V. alginolyticus*, *V. parahaemolyticus*, *V. nitriegens* and *Beneckea harveyi* from Vellar Estuary of South India, and Seethalakshmi (1978) has studied *V. parahaemolyticus* associated with the plankton of the same estuary. Sajeev and Stephen (1993) have found higher densities of *V. parahaemolyticus* in the estuarine (Cochin) shellfish when compared with those from the Arabian Sea.

The incidence of vibrios in the environment is influenced by various factors like temperature, pH, incidence of zooplankton, availability of nutrients and other unidentified factors (Kaneko and Colwell, 1973; Varga and Hirtle, 1975; Colwell, 1984; West and Lee, 1984; Singh *et al.*, 1989; de la Pena *et al.*, 1992).

Vibrios were reported to be the predominant bacteria in the natural microbial flora of the penaeid shrimp (Vanderzant *et al.*, 1971; Lightner, 1975; Christopher, *et al.*, 1978; Singh, 1986; Singh *et al.*, 1985, 1989; Hammed, 1993). Microbial flora of *P. indicus* with reference to its digestive system has been studied by Palaniappan (1982). Mitra (1987) also has studied intestinal microflora of *P. indicus*. The digestive tract or the intestine of the shrimps and larvae were found to harbour large numbers of vibrios by providing them a very suitable environment for growth and multiplication (Yasuda and Kitao, 1980; Colorni, 1985; Dempsey and Kitting,1987; Dempsey *et al.*, 1989; Ninawe and Banik, 1987; Singh *et al.*, 1991).

Some other marine and estuarine crustaceans were also found to harbour vibrios in their body. Lee and Pfeifer (1975) had reported the occurrence of *Vibrio* spp. in the intestine of Dugness crab. An unique gut flora with predominant vibrios was reported in marine copepods by Sochard *et al.* (1979). Incidence of vibrios in the haemolymph, stomach and gill of Blue crab *Callinectes sapidus* was reported by Davis and Sizemore (1982) and in the haemolymph of the Mangrove crab *Scylla serrata*. *V. alginolyticus*, *V. fluvialis* and *V. parahaemolyticus* were reported by Chong and Chao (1986). Mahadevan (1977) has studied *V. parahaemolyticus* associated with shellfish of Proto Novo, India and a quantitative study on the same bacteria in finfish and crustaceans has been conducted by Nair (1985).

Occurrence of vibrios in the intestine of estuarine and marine crustaceans suggests their role in the process of digestion. High incidence of vibrios in the intestine of penaeid shrimps suggests their prominent role in the digestive process (Dempsey and Kiting, 1987; Dempsey *et al.*, 1989; Singh *et al.*, 1989, 1991).

Vibrios inhabiting the gut of *P. indicus* was reported playing dual role, both beneficial and harmful in the life of the animal by enhancing the digestive process and at the same

time infecting the host (by invading into other body parts and resulting in disease) during adverse environmental conditions (Singh *et al.*, 1989, 1991). Hameed (1993) suggested that vibrios may be harmful to larvae and postlarvae of shrimps when present in large numbers.

STRESS AND DISEASE

Many micro-organisms which form part of the natural flora of cultured shrimps were reported turning pathogens when the animals face prolonged physiological stress.

Stress is a *"strain or burden placed on an animal as a result of extreme stimulation of external or internal origin"* (Sindermann, 1990 a). As 'vibriosis' is considered as a stress related disease (Viveres *et al.*, 1992) and inorder to minimise the damage due to this disease by modifying culture techniques or manipulating the environmental conditions, it is essential to determine the exact stress factors which predispose prawn to the acquisition of infection or produce overt infection in carriers (de la Pena *et al.*, 1992). Delves-Broughton and Poupard (1976) and Sindermann (1990 a) also suggest the probable role of environmental parameters in the vibrio infections of penaeid shrimps.

Various factors such as intensive culture methods, stocking density (over crowding), handling, sudden changes in the environmental conditions, environmental deterioration, nutritional deficiency, H_2S, toxins and wounds had been described as major stress factors to the cultured aquatic animal population, which consequently predispose to disease out-breaks (Overstreet, 1973; Vanderzant and Nickelson, 1973; Lightner, 1975, 1977, 1983; Smith and Ratcliffe, 1978; Pillai, 1981; Johnson, 1983; Baticados, 1988 a; Boonyaratpalin, 1990; Nash, 1990; Sindermann, 1990a; Anon., 1993; de la Pena *et al.*, 1993; Mathew and Sanilkumar, 1995). The effect of an Environmental stress on out-breaks of infectious diseases of lobsters was studied by Snieszko (1974).

Stress can lead to the suppression of immune response (resistance), increased susceptibility to facultative pathogens and the development of latent infection into potency (Sindermann, 1990 b).

VIBRIOSIS

'Vibriosis' an infection caused by bacterial species of the genus *Vibrio* (Egidius, 1987) is a universal problem in coastal and estuarine animal population.

Vibrios are considered as facultative pathogens or opportunistic pathogens since they form part of the normal flora of the environment and cultured animals and produce a disease condition in stressed host population (Lightner, 1977, 1985; Bowser *et al.*, 1981; Nash, 1990; Sindermann, 1990 a; Pizarro and Alfaro, 1990).

Vibrio infections have been implicated as a major cause of mortality in juvenile penaeids in the culture system. The infection may be chronic, sub-acute or acute and mortality may be 100% in some cases (Sindermann, 1971). 'Vibriosis' in *P. monodon*, *P. setiferus*, *P. orientalis* and *P. merguensis* was reported with 60-70% mortality by Delves-Broughton (1976). Ruangpan (1982) had reported 70-80% mortality in *P. monodon* larvae in hatchery. Sano and Fucuda (1987) had reported a loss of 231.4 million Yen in the year 1984 due to 'vibriosis' in Kuruma shrimp *P. japonicus* in Japan, which formed 49.65% of the total loss due to all diseases (both known and unknown) in shrimp (*P. japonicus*) culture.

Secondary vibrio infection

Vibrio spp. can be of primary aetiology or can cause secondary infections depending upon the circumstances (Boonyaratpalin, 1990). Nash (1990) had reported high disease incidence in combination infection of *Peaneus monodon* type baculovirus and vibrios. Secondary vibrionic infections were reported in Haemocytic entritis (Lightner

et al., 1984) and Peru necrotising hepatopancreatitis (Lightner and Redman, 1994) of penaeid shrimp.

Species involved in 'vibriosis'

The important species causing 'vibriosis' in culture systems are *V. anguillarum*, *V. parahaemolyticus* and *V. alginolyticus* (Lightner, 1975, 1977, 1983, 1985, 1993; Delves-Broughton and Poupard, 1976; Mahadevan *et al.*, 1978; Lightner *et al.*, 1984; Anderson *et al.*, 1988; Boonyaratpalin, 1990; Nash, 1990; Sindermann, 1990 b; Anon., 1993). Other opportunistic vibrios such as *V. algosus*, *V. vulnificus*, *V. damsella*, *V. fischeri*, *V. fluvialis*, *V. panulirus* were occasionally reported from shrimps (Kusuda and watada, 1969; Pillai, 1984; Nash, 1990; Lightner, 1993).

Certain other gram negative rods including *Pseudomonas* spp. and *Aeromonas* spp. were reported for their occasional involvement in disease out-breaks along with *Vibrio* spp. (Lewis, 1973; Lightner, 1975, 1977; Lightner and Levis, 1975; Khan *et al.*, 1984; Nash, 1990)

V. harveyi and *V. splendidus* are the two important species causing the serious luminous bacterial disease in the penaeid hatcheries (Pitago, 1988; Boonyaratpalin, 1990; Lightner, 1993; Karunasagar *et al.*, 1994). *V. harveyi* infecting pond reared black tiger prawn (*P. monodon*) in Thailand have been reported by Jiravnichpaisal *et al.* (1994). Abraham and Manley (1995) have recently reported the involvement of luminous and non-luminous *V. harveyi* in shell disease of cultured *P. indicus*.

Shrimps affected with 'vibriosis'

Vibrios were found associated with shell disease, appendage necrosis, septicemia, brown-spot disease and infected wounds (Lightner, 1983) in penaeid shrimps.

Different penaeids which were reported to be affected with vibrios include *P. monodon*, *P. indicus*, *P. japonicus*, *P. vennamei*, *P. merguiensis*, *P. setiferus*, *P. orientalis*, *P. aztecus* and *P. duorarum* (Vanderzant *et al.*, 1970; Vanderzant and Nickelson, 1973; Lightner, 1975, 1993; Lightner and Lewis, 1975; Delves-Broughton and Poupard 1976; Lakshmanaperumalsamy *et al.*, 1982; Takahashi *et al.*, 1984, 1985; Chong and Chao, 1986; Anderson *et al.*, 1988; Boonyaratpalin, 1990; Nash, 1990; de la Pena *et al.*,1992, 1993; Alfaro *et al.*, 1993; Anon., 1993).

'Vibriosis' is also reported in other crustaceans such as lobsters (*Homerus americanus* and *Panulirus* spp.), Blue crab *Callinectes sapidus*, Swimming crab *Portunus trituberculatus*, Red-swamp craw fish *Procambarus clarkii*, Mangroove crab *Scylla serrata* (Bowser *et al.*, 1981; Davis and Sizemore, 1982; Sizemore and Davis, 1985; Chong Chao, 1986; Thune *et al.*, 1991; Muroga *et al.*, 1994)

'Vibriosis' in fishes

'Vibriosis' in fishes is known officially since 1909 (Bergman, 1909) and is the most important disease in the marine fish culture affecting a large number of species (Egidius, 1987).

In their review of vibrio diseases of marine fish, Anderson and Conroy (1970) listed more than 40 species of fishes, both from the wild and culture systems, from all over the world, in which vibrio-associated diseases are described. Eight species of vibrios are listed as fish pathogens by Colwell and Grimes (1984). The commonly known and wide spread of the fish pathogenic vibrios is *V. anguillarum* and other important species include *V. ordalii*, *V. damsella*, *V. carchariae* and *V. salmonicida* (Egidius, 1987).

Behavioural and clinical symptoms of 'vibriosis'

General behavioural changes such as reduced (lethargic) swimming, disorientation while swimming, swimming on one side, motionless lying at the bottom or pond edges without any escape reflexes (Lightner, 1975; Lightner and Lewis, 1975; Anderson *et al.*, 1988; Nash, 1990; Vera *et al.*, 1992) and loss of appetite in shrimps (Boonyaratpalin, 1990) were observed in vibrio infections. Shrimps may alternate between periods of frantic and disoriented swimming and back flipping to periods of lethargy (Lightner, 1977).

Clinical symptoms of the disease in shrimps include white opaque discolouration of body musculature, melanisation of gill filament, cuticular lesions, darkening of dorsal portion of integument (due to the expansion of melanophores), reddish appendages (due to expansion of integumental erythrophores), pronounced dorsal flexure of abdomen and turbidity and delayed clotting of haemolymph. Reduced number of haemocytes in the haemolymph also was observed in some cases (Lightner, 1975, 1977; Lightner and Lewis 1975; Vera *et al.*, 1992; de la Pena *et al*, 1993). In addition to this, necrosis of appendages and antennae, empty stomach (Anderson *et al.*, 1988) red gills and some times abdomen, erosion of appendages (Delves-Broughton and Poupard, 1976) brown spots on body surface, in gill and lymphoid organ (Lakshmanperumalsamy *et al.*, 1982; Takahashi *et al.*,1985; Soni, 1986; Nash, 1990) were also reported.

Clinical symptoms such as dark localised lesions necrosis of appendages, empty stomach and lack of feacal strands were reported in *P. monodon* larvae (Boonyaratpalin, 1990). Takahashi *et al.* (1984) had observed cloudiness of mid gut gland in postlarvae of *P. monodon* affected with vibriosis. Luminescent bacterial disease in penaeid larvae caused by some vibrios show luminescence in darkness (Pitago, 1988) due to the presence of high number of motile luminous bacteria in the tissue. But asymptomatic vibrionic infection of *P. monodon* in the nursery pond also had been reported in Thailand (Ruangpan, 1982).

A scrutiny of literature has revealed that pathogenic *Vibrio* species could be isolated from haemolymph, hepatopancreas, abdominal muscle, gill and other infected body parts of the affected shrimp (Lightner, 1975, 1977; Lightner and Lewis,1975; Delves-Broughton, 1976; Anderson *et al.*, 1988; Nash, 1990).

Mode of entry of pathogen

The entry of the pathogen into the body systems of the host resulting in'vibriosis'is suggested differently by different workers. Nash (1990) suggested the possible infection *via* gill haemolymph, damaged cuticle haemolymph or oral route. And he further emphasised the importance of oral route particularly when there is prior damage to hepatopancreas. Davis and Sizemore (1982) also have suggested the infection of *Vibrio* spp. which form part of the normal flora of the host, through gut wall. Lightner and Lewis (1975), Sizemore and Davis (1985) and Anderson *et al.* (1988) suggested the infection during ecdysis and through cuticular injuries.

Karunasagar *et al.* (1994) suggested that passage through animals can increase the virulence of bacteria. With their experiments, Prayitno and Latchford (1995) have demonstrated the important role of the environmental parameters such as pH and salinity in the virulence of luminous bacteria related to to *V. harveyi* causing disease in *P. monodon* larvae.

Lightner (1977) has found that moribund shrimp affected with vibrios when cannibalised by other shrimps in the system was suggested the apparent spreading of the disease. The primary mode of transmission of vibriosis in Ayu *Plecoglossus altevelis* from one individual to another was found through water and the direct contact between individual fish was reported accelerating the spread of disease (Kanno *et al.*, 1989).

Experimental pathogenicity studies

Pathogenicity of *Vibrio* spp. were experimentally proved in penaeid shrimps by many workers following different infectivity methods such as bath challenge (immersion), oral administration and intramuscular injections (Vanderzant and Nickelson, 1973; Lightner and Lewis, 1975; Delves-Broughton and Poupard, 1976; Takahashi *et al.*, 1984, 1985b; Baticados *et al.*, 1987; Nash, 1990; de la Pena *et al.*., 1992, 1993; Vera *et al.*, 1992; Karunasagar *et al.*, 1994) and the experimentally infected shrimpsshowed similar behavioural and clinical symptoms which observed in the naturally infected shrimps. The pathogenic role of vibrios were experimentally proved in other crustaceans such as Rock crab *Cancer irroratus* (Newman and Feng, 1982) Swimming crab *Portunus trituberculatus* (Muroga *et al.*, 1994) and American lobster *Homerus americanus* (Bowser *et al.*, 1981).

Prophylaxis and control of 'vibriosis'

Disease prevention through limiting stress, avoiding the introduction of disease causing agents to the system, careful site selection, water preparation, supply of adequate amount of good quality food, careful hatchery maintenance and management are suggested by Lightner (1977) and Boonyaratpalin (1990).

Various water filtration and sterilisation procedures used in hatchery and grow-out systems such as water filtration and chlorination, sterilization with Ultra-Violet radiation and ozonisation were found effective in disease prevention through avoidance of pathogens in the system (Lightner, 1977; Blogoslawski *et al.*, 1978; Brown, 1981; Baticados and Pitago, 1990; Karunasagar *et al.*, 1994).

Vaccination of the cultured population is another prophylactic measure in disease management. Vaccination against 'vibriosis' in penaeid shrimp has been developed recently by Lewis and Lawrence (1985) and Itami *et al.* (1989) and its commercial production with high efficacy and advantages also is claimed (Anon., 1993).

Itami *et al.* (1991) have shown that micro-encapsulated test diet supplemented with formalin killed *Vibrio* cells was reported marked increase in the number of survivors of *P. monodon* larvae. Evaluation of reproductive performance of female *P. stylirostris* injected with heat killed *V. alginolyticus* have been studied by Pizzaro and Alfaro (1994) as a preliminary step in brood stock vaccination.

Even though 'High health shrimps' have claimed for its better growth, disease resistance and survival rate, it can not be considered as a panacea (Pruder, 1994).

Various antimicrobial agents including antibiotics were tested against vibrionic infections in shrimps. Particularly some antimicrobial agents like Oxytetracycline (OTC) (Corliss, 1979; Takahashi *et al.*, 1985a) Chloramphenicol (de la Pena *etal.*, 1993; Hameed and Rao, 1994) and Furanas (Delves-Broughton, 1974; Lightner, 1977) were found effective against this disease. Antibiotics such as Terramycin, Furacin, Tylosin, Tetracycline, Erythromycin, Novobiocin, Sulfamethoxazole-trimethoprime and Amino-benzo-penicillin were also observed effective against vibriosis (Lightner, 1977; de la Pena *et al.*, 1993).

The abuse of antibiotics in the aquaculture systems have caused the generation of drug resistant vibrios and other bacteria. Bacteria resistant to Oxytetracycline, Oxolinic acid and Furazolidone have been reported from marine sediment (Nyagaard, 1992). Rosily *et al.*, have reported a drug resistant *Vibrio* sp. associated with larvae of *P. indicus*. Karunasagar *et al.* (1994) have isolated vibrios from *P. monodon* hatchery which were resistant to vibriostat (0/129) compound, Streptomycin, Chloramphenicol and Co-trimoxasole. Resistance of vibrios to widely used antibiotic Oxytetracycline also has been observed in various shrimp culture regions (Anon., 1993). Development of drug resistance has been and continue to be a significant problem in antibiotic therapy and it involves ecotoxicological and human health effects. So a reduction of use and discharge of

antibacterial substances is suggested (Juwana, 1990; Hektoen *et al.*, 1995). As the routine use of antibiotic result in drug resistance in bacterial strains and accumulation of antibiotic residues in the shrimp and the environment, most of the shrimp pathologist are reluctant to advocate for antibiotic therapy in shrimp culture operations (Nash, 1990; Boonyaratpalin, 1990; Karunasagar *et al.*, 1994).

Removal of stress, avoidance of pathogens from the system and the vaccination are the prophylactic measures considered effective in reducing the possibility of disease out-breaks (Lightner, 1983; de la Pena *et al.*, 1992; Anon., 1993; Karunasagar *et al.*, 1994). The use of drugs and chemicals to prevent disease is suggested as a last resort only (Boonyaratpalin, 1990).

ADVANCEMENTS IN SHRIMP PATHOLOGICAL INVESTIGATIONS

Various advanced procedures and methodologies have been developed for the disease diagnosis in aquaculture as well as for other pathological investigations. Adam (1991) had developed an amplified enzyme linked immunosorbent assay (ELISA) for the detection of *Vibrio parahaemolyticus* biotype *alginolyticus* and it is reported as a specific and sensitive method for detection of bacteria. Karunasagar and Karunasagar (1994) have developed a rapid DNA based technique for the detection of marine *Vibrio* spp., which is a ctx gene based PCR technique that gives very rapid results. Different types of immunological and nucleic acid hybridisation based technologies were described by Mialhe *et al.* (1992). Usefulness of API-20E system for easy identification of pathogenic bacteria has been described by Santos *et al.* (1993). Use of Nucleic acid probes in aquatic bacteriology (Viveres and Guesdon, 1992), Genomic finger-printing technique for characterisation of *Vibrio* spp. (Tsai *et al.*, 1990) are also helpful in pathological investigations in aquatic animals.

The development and application of genomic probes in the detection of shrimp viruses such as IHHNV, HPV, MBV and BP (Lightner and Redman, 1991; Bruce *et al.*, 1994; Lightner *et al.*, 1994), Baculovirus impregnated filter paper method for assessing disinfection protocols in shrimp culture facilities (Lewis *et al.*, 1992), development of simple and rapid diagnostic method to study hepatopancreatic parvo-like virus (HPV) of penaeid shrimps (Lightner *et al.*, 1993), development of *invitro* primary cell culture system for *P. stylirostris* and *P. vennamei* (Leudman and Lightner, 1992) and production of monoclonal antibodies to IHHN virus (Poulose *et al.*, 1994) are the major advancements in the field of shrimp pathology.

MATERIAL AND METHODS

MATERIAL AND METHODS

The study carried out by the candidate at the Central Marine Fisheries Research Institute (CMFRI), Cochin from 1992 to 1995, include shrimp pond ecology with special emphasis on the incidence, isolation and identification of different species of *Vibrio* in the culture system, influence of physico-chemical and environmental parameters such as temperature, salinity and pH on the growth of vibrios, antibiotic sensitivity and pathogenicity of vibrios.

SAMPLING STATIONS

Three stations identified in the vicinity of Cochin Backwater for sampling are Station I Ezhupunna, Station II Chellanum and Station III Thripunithura.

All these culture systems are semi-intensive shrimp culture systems as per the specification given by Fast (1991) with the following characteristics.

Production level	500-2500 kg/ha/year
Stocking rate	3-10/m^2/Crop
Seed source	from wild and hatchery
Feed	natural and supplement
Average water exchange rate	5-10%
Water exchange by	pump
Pond size	>1 ha
Water depth	0.7-1.5 m
Survival	60-80%
Dike construction	earthern
Crop/year	2-3
Management	continuous

SAMPLE COLLECTION

Monthly sampling for one year from September 1992 to September 1993, was conducted for studying the ecology of vibrios in shrimp culture systems.

Sampling for water quality

Water samples were collected from all the four corners and the centre of the ponds using clean glass water bottles for the determination of nitrite, nitrate, ammonia, phosphate, silicate, pH, salinity, alkalinity and hardness. For dissolved oxygen, water was taken in 125 ml clean glass BOD bottles following the Winkler's method and were fixed immediately. The samples thus collected were transported to the laboratory by maintaining below 4°C in an ice box.

ANALYSES OF THE WATER SAMPLE

Temperature (atmospheric, water and sediment) was recorded at the sampling site itself using immersible mercury centigrade thermometer. pH was measured using a digital pH meter (Orion, 420A, USA). Salinity, alkalinity, hardness, nitrite, nitrate and ammonia, phosphate, silicate and dissolved oxygen of the water were determined following Strickland and Parsons (1968), and total hardness following Boyd and Pillai (1985).

STERILISATION

For isolation of bacteria inoculation hood fitted with Ultra-Violet light was used. The hood was first rendered dust free and cleaned with cotton. It was then disinfected with absolute alcohol by swab method, sealed and sterilised by switching on UV light for about 30 minutes.

Glasswares such as petridishes, test-tubes, conical flasks, pipettes, beakers, glass tissue homogenisers, etc. were sterilised in hot air oven at 170°C for 1 hr. All the surgical

instruments such as scissors, blades, needles, scalpels, etc. were either autoclaved or dipped in absolute alcohol and the excess burned off.

Dilution blanks (20 ppt aged sea water), distilled water and mineral oil (for the storage of bacterial culture) were autoclaved (at 121°C and 15 psi) for 20 minutes. Plastic tubes, aerator stones, acrylic tanks, etc. used for pathogenicity experiments were disinfected by immersion for 30 minutes in a 2.6% sodium hypochlorate solution followed by thorough rinsing in autoclaved distilled water (Leong,1983).

SAMPLING FOR BACTERIOLOGY

Juvenile *Penaeus indicus*, pond water and sediment from all the three stations were collected for bacteriological analysis. Juvenile shrimps were collected using a cast net and the catch was emptied to a tub with same pond water. The shrimps were individually collected with a scoop net and packed water-tight in thick sterile polythene bags. During the monthly sampling 15 shrimps were collected from each station (5 each for bacteriology of body surface, gill and digestive system) covering four corners and the centre of the culture pond. Sterile glass water sampler was used for water sample collection and Peterson's grab for bottom sediment. Sediment was transferred asceptically into a thick polythene bag and packed water-tight using rubber bands. Five water samples and five sediment samples were collected from each station in every month. Samples were transported to the laboratory below 4°C in an ice box with in 1 hour.

Preparation of samples for bacteriological analysis

a. Juvenile shrimp

Body surface, gill and digestive system of a composite sample of 5 juvenile *Penaeus indicus* were used for isolation of *Vibrio* and 20 ppt sterile aged sea water was used as diluent.

The surface of juvenile shrimps were gently washed with distilled water and the animals from same station were placed together and rotated clock wise and anti-clock wise vigorously for 2-3 minutes in 100 ml diluent in order to dislodge the bacterial flora from the body surface.

For isolation of vibrios from the gills, the juvenile shrimps were gently washed with sterile distilled water and were asceptically removed. The gill was homogenised using sterile glass tissue homogeniser and serially diluted.

Prior to bacterial sampling from the digestive system, shrimps were dipped in 1% sodium hypochlorate solution for 10 minutes and were washed repeatedly with sterile distilled water. The intestine along with hepatopancreas was separated asceptically. The digestive system of juvenile shrimp sampled at five different spots from same station was pooled, homogenised and serially diluted.

b. Pond water and sediment

Pond water sampled at five different spots from the same station was mixed together and 1 ml from the mix was serially diluted. Similarly for bottom sediment, samples from each station were mixed together and from this composite sample 1 g was serially diluted.

ISOLATION OF VIBRIOS

Vibrios were isolated using ZoBell's agar 2216E (peptone-0.5%, yeast extract-0.1%, ferric phosphate-0.01%, bacto agar-2% (Himedia) pH 7.5 $\pm$ 0.2) prepared in 20 ppt aged sea water and Thiosulphate citrate bile salt sucrose (TCBS) agar (Himedia) with 2% NaCl.

ZoBell's agar was autoclaved (121°C, 15 psi) for 20 minutes and samples were isolated by standard pour plate method. The plates were incubated at room temperature

(28 ± 2°C) for 48 hrs and those plates which had shown 30-300 colonies were used for the isolation. Ten colonies from each sample were isolated at random and were streaked on ZoBell's agar plates to check their purity and transferred subsequently to agar slants.

TCBS agar was brought to boiling, converted into plates and the samples were inoculated by the standard spread plate method. From each sample 10 colonies were isolated at random and the purity was checked on ZoBell's agar plates and transferred to agar slants.

ZoBell's agar was used as a general medium for the isolation of vibrios and TCBS agar as the selective one. ZoBell's agar isolates were considered for all statistical analysis.

Isolated colonies were stocked on ZoBell's agar vials overlaid with sterile mineral oil (Furnis *et al.*, 1979; Baumann and Schubert, 1984). Whenever needed these cultures were restreaked on ZoBell's agar plates and transferred to agar slants.

IDENTIFICATION OF *VIBRIO* SPP.

Identification of *Vibrio* species was carried out following West and Colwell (1984). Tests carried out are given below.

Tests	
Cytochrome oxidase	growth at 42°C
Nitrate reduction	Growth at % Nacl
0/129 sensitivity	0 %
10 µg	3 %
150 µg	6 %
Swarming	8 %
Luminescence	10 %

Thornley's Argenine dihydrolase	Voges-Proskauer reaction
Lysine decarboxylase	Gas from glucose fermentation
Ornithine decarboxylase	

Fermentation to acid

L-arabinose	D-Mannose
M-inositol	Sucrose

Enzyme production

Alginase	Gelatinase
Amylase	Lipase
Chitinase	

Utilization as sole source of carbon

r-aminobuterate	D-glucuronate
Cellobiose	L-leucine
L-citrulin	Putrescine
Ethanol	Sucrose
D-gluconate	D-xylose

Cell morphology was examined using 18 hr old bacterial culture on ZoBell's agar by Gram's staining technique as described by Hucker and Conn (1923,1927). The stained slides were examined under microscope with oil immersion objectives.

OPTIMUM GROWTH REQUIREMENT TEST

Growth of each *Vibrio* isolate at different levels of temperature, pH and salinity were examined.

Temperature

Preference of the isolates of *Vibrio* to grow at different temperatures from 0 to 50°C with 5° intervals was tested by inoculating 0.1 ml of 18 hours old broth (containing 10^4 cfu/ml) into 10 ml tryptone water (1.5% tryptone in sterile distilled water with 2% NaCl and pH 7.5). The inoculated tubes were incubated at the above temperatures in a BOD incubator for 38 hrs and the growth was measured by Turbidometry using a spectrophotometer (Spectronic 1001, USA) at 530 nm. Uninoculated tryptone broth was treated as control. The optical density (OD) of the culture fluid measured by the spectrophotometer was taken as the measure of bacterial growth.

pH

The isolates of *Vibrio* were challenged with different pH levels from 4.0 to 11.0 with 0.5 intervals in 1.5% tryptone water (with 2% NaCl) at 28 ± 0.2°C. pH was adjusted with HCl or NaOH. Inoculation and growth measurements were carried out as mentioned above.

Salinity

The ability of the *Vibrio* isolates to grow at different concentration of NaCl was studied in 1.5% tryptone water. Different NaCl concentrations tested were from 0 to 50 ppt with an interval of 5 ppt. pH was maintained at 7.5 and temperature at 28°C.

ANTIBIOTIC SENSITIVITY TEST

Sensitivity of the *Vibrio* isolates to 40 antibiotics were examined using sensitivity test discs (Himedia). The following are the tested antibiotics.

Amikacin	(10 mcg)	Furazolidone	(50 mcg)
Amoxycillin	(10 mcg)	Gentamycin	(10 mcg)
Ampicillin	(10 mcg)	Kanamycin	(30 mcg)
Augmentin	(10 mcg)	Lincomycin	(2 mcg)
Bacitracin	(10 units)	Methanamine Mandalate	(3 mcg)
Carbenicillin	(100 units)	Methicillin	(3 mcg)
Cefazolin	(30 mcg)	Nalidixic acid	(30 mcg)
Cephalexin	(30 mcg)	Neomycin	(30 mcg)
Cephaloridine	(30 mcg)	Nitrofurantoin	(300 mcg)
Cephalothin	(30 mcg)	Norfloxacin	(10 mcg)
Cephotaxime	(30 mcg)	Novobiocin	(30 mcg)
Chloramphenicol	(30 mcg)	Oleondomycin	(15 mcg)
Chlortetracyclin	(30 mcg)	Oxacillin	(30 mcg)
Ciprofloxacin	(10 mcg)	Oxytetracycline	(30 mcg)
Clindamycin	(2 mcg)	Penicillin	(10 units)
Cloxacillin	(1 mcg)	Polymyxin-B	(300 units)
Colistin	(10 mcg)	Streptomycin	(10 mcg)
Co-trimazine	(25 mcg)	Tetracycline	(30 mcg)
Co-trimoxazole	(25 mcg)	Tobramycin	(10 mcg)
Erythromycin	(15 mcg)	Vancomycin	(30 mcg).

A loop full 18 hours old culture from ZoBell's agar slant was streaked on agar plates and sensitivity test discs were placed over it using sterile forceps. Plates were incubated at room temperature (28 $\pm$ 2°C) for 38 hours. An isolate was considered sensitive to an antibiotic at a particular concentration, if a clear zone (hallow) was observed around the corresponding sensitivity test disc.

A clear zone of above 25 mm diameter was considered highly sensitive (H+++), 16-25 mm diameter was considered sensitive (+++), 8-15 mm diameter moderately sensitive (++) and upto 8 mm low level sensitivity (+).

MINIMAL INHIBITORY CONCENTRATION

Minimal inhibitory concentration of perfuran and furazolidone was determined by using 0, 5, 10, 20, 30, 40, 50, 70, 100, 300, 500 and 1000 μg of these antibiotic per ml of the ZoBell's broth. 1 ml bacterial suspension (with 10^4 cfu/ml) was inoculated into the broth with antibiotic and incubated at room temperature ($28 \pm 2^{o}C$) for 38 hrs. The survival of the bacteria was detected by testing the inoculated broth by simple pour plate method.

PATHOGENICITY EXPERIMENTS

Healthy juveniles of *P. indicus* reared under uniform culture condition with 6-7 cm size were used for these experiments.

Test animals used for these experiments were collected during monsoon months when the pond salinity was below 5 ppt and the occurrence of vibrios in the culture system was below detectable level.

Test animals were transported to laboratory washed with sterile sea water and gradually acclimatised to 20 ppt sea water atleast 7 days before the experiment began. The sea water collected from the adjacent sea was allowed to settle, filtered through bolting silk (50 μ size) and sterile cotton and was finally sterilized by chlorination following the procedures given by Baticados and Pitago (1990). The animals were maintained in one tonne fibre glass tanks connected with air-lift biological filter and fed with sterilized pieces of shrimp meat. The average temperature, pH and salinity maintained in the rearing water were $28 \pm 2^{o}C$, 7.5 ± 0.2, and 20 ± 0.5ppt respectively. Bacteriology of water and juvenile shrimps were tested every three days inorder to check the presence of any vibrio.

Experimental set up for testing pathogenicity

Ten juvenile shrimps sexually undifferentiated for each experiment were kept in 10 litre round acrylic tanks (2 animals in each tank) with aeration. Water was sterilized as mentioned above and in addition with UV light at the end. Continuous flow of the sterilised sea water was maintained in all experimental tanks. The experiments on shrimps were carried for 7 days.

For the experiments NaCl concentration was adjusted with sterilsed concentrated sea water (which was concentrated to 100 ppt salinity by evaporation in sun light) or distilled water. pH was adjusted with sodium hydroxide or hydrochloric acid (Leong, 1983). Low aeration was given in the experimental tanks inorder to keep dissolved oxygen at 5 $\pm$ 0.5 ml/l. Deviation from desired pH levels in tanks due to aeration and shrimp excretion were compensated by continuous sea water flow with desired pH. pH, salinity and temperature of the water were monitored every four hours with digital pH meter, refracto-salinometer and thermometer respectively.

INOCULATION METHODS

Two methods of inoculation were followed for testing pathogenicity : (1) intramuscular injection and (2) oral administration.

Intramuscular injection

The challenging organism was mass cultured on ZoBell's agar plates and harvested with 2% sterile saline. This was diluted further by ten fold serial dilution using the same strength saline. The shrimps were collected from the tank and the site of injection was blotted dry with a cotton swab followed by rubbing with 70% ethyl alcohol for

surface disinfection, 0.05 ml bacterial suspension from each dilution was injected intramuscularly between the fourth and fifth abdominal segment at an angle through the intersegmental membrane using 1 cc tuberculin syringe. The number of bacterial cells in 0.05 ml of bacterial suspension used in the injection are given below.

Viable count of *Vibrio* spp. in 0.5 ml suspension used for the intramuscular injections in juveniles of *Penaeus indicus*

V. anguillarum	*V. parahaemolyticus*
22.5×10^9	30.5×10^9
22.5×10^8	30.5×10^8
22.5×10^7	30.5×10^7
22.5×10^6	30.5×10^6
22.5×10^5	30.5×10^5
22.5×10^4	30.5×10^4
22.5×10^3	30.5×10^3
22.5×10^2	30.5×10^2
22.5×10^1	30.5×10^1

The control animals were provided with 0.05 ml sterile (2%) saline injections. Animals were examined every 2 hours for clinical signs of disease and mortality. The inoculated animals were fed with sterile shrimp meat 5% to their body weight daily and the uneaten food was removed to prevent fouling of the water (Leong, 1983). The experiments were carried out at pH 7.5 ± 0.2, temperature $28 \pm 2^{o}C$ and salinity 20 ± 0.5ppt with continuous flow of sterile sea water.

Oral administration

Administration of vibrios along with feed through the mouth was followed in the experiment to reveal the role of physico-chemical parameter such as pH and salinity in 'vibriosis'.

Fresh shrimp meat was diced (approximately 5 x 5 x 5 mm) and the pieces placed without touching each other on a wet Whatman filter paper in a set of glass petridishes and steam autoclaved (121°C, 15 psi, 15 minutes). After cooling,0.1 ml of suspension of the organism which contained 10^6 cfu/ml was placed on the upper surface of each piece of meat (Leong and Fontain, 1979) and incubated at 28°C for 10-12 hours.

Ten juvenile shrimps were individually isolated for the rearing tank and not fed for 24 hours. After 24 hours each of them was fed with shrimp meat inoculated with bacteria as mentioned above and transferred to experimental tanks which contained sterilised sea water with altered salinity or pH. Levels of pH tested were 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0. Similarly salinity test levels such as 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 ppt where employed for the experiments. Those animals which have not taken the infected feed were excluded. Test animals which were fed once with the inoculated diet were then fed with sterile shrimp meat on successive days at a rate of 5% to their body weight. The left over feed was removed inorder to prevent fouling of the water.

CONFIRMATION TEST FOR PATHOGENICITY

Inorder to confirm the pathogenicity of the candidate bacteria, shrimp muscle and haemolymph were assayed.

Shrimp muscle

The moribund and dead shrimps from the experimental tanks were collected, washed with 1% sodium hypochlorate solution followed by repeated washing with sterile

distilled water. Intestine was removed through a dorsal split on the body using sterile scissors. Care was taken to avoid possible contamination of the muscle. Cephalothorax and legs were also removed using sterile scissors and forceps without contaminating the abdominal region. The shell covering 1st, 2nd and 3rd abdominal segments were then removed carefully (without contaminating the muscle with the outer surface of the shell) and the shrimp was cut into two at the junction of 2nd and 3rd segments by a transverse cut, using a sterile stainless steel blade. The cut surface was pressed gently on ZoBell's and TCBS agar plates and the plates were incubated at room temperature (28 $\pm$ 2°C) for 38 hours. Bacterial colonies developed were transferred to agar slants and later identified following West and Colwell (1985). Apparently healthy shrimps and controls also were assayed for the pathogens in the similar way.

Haemolymph

Juvenile shrimps (apparently healthy, moribund and dead) from the experimental tanks were collected, washed with sterile distilled water and haemolymph was collected from cardiac sinus using a 1 cc tuberculin syringe. Before drawing haemolymph the muscle surface above heart was rubbed gently with 70% ethyl alcohol and allowed to dry. Half of the content was emptied on to a clean and sterile glass slide to observe the time taken to clot and the same slide with haemolymph was Grams' stained and observed under microscope for bacteria. The rest of the haemolymph in the syringe was inoculated equally on to ZoBells' and TCBS agar plates, streaked and incubated. The colonies developed were isolated and identified as given above.

STATISTICAL ANALYSIS

Inorder to find out the relationship between the incidence of vibrios in the culture system and the water quality parameters, their values were subjected to statistical analysis in a computer for the estimation of correlation coefficient 'r'.

The significance 'p' of correlation coefficient 'r' of different parameters such as temperature (Atmoshperic, water and sediment), pH, salinity, alkalinity, total hardness, nitrite, nitrate, ammonia, phosphate, silicate, dissolved oxygen were tested at 1% and 5% levels.

For calculations, data from all the three stations were clubbed together and a few observations which reported abnormal fluctuation in values of the water quality parameters due to the sudden rains during Transition season were side layered and exempted from correlation tests.

CHAPTER I

Chapter 1

ECOLOGY AND INCIDENCE OF VIBRIOS IN SHRIMP CULTURE PONDS AROUND COCHIN

Ecology has a very significant role in aquaculture systems and on the growth and survival of aquatic organisms. Variability in growth and survival of shrimps during culture, has frequently been attributed to the occurrence of pathogenic micro-organisms and their relationship to the pond ecology.

Vibrio spp. are common in the microflora of pond and raceway reared shrimps (Vanderzant *et al.*, 1971; Lightner, 1975; Christopher *et al.*, 1978; Yasuda and Kitao, 1980) and hence they are opportunistic pathogens that causes disease in severely stressed shrimps (Lightner, 1977; Anderson,1990). Delves-Broughton and Poupard (1976) suggested that the 'vibriosis' is most probably closely linked to environmental parameters.

The ability of pathogen to survive and remain infective in the external environment is considered as a major determinant of disease. The incidence of *Vibrio* spp. in the culture environment is greatly influenced by the prevailing environmental parameters. Colwell *et al.* (1985) stated *"It is clear that unless the ecology of the vibrios is fully understood, complete control of disease caused by these micro-organisms will not be possible"*. Hence a deeper study on the occurrence, abundance and seasonal fluctuation of different species of vibrios in relation to environmental parameters in the culture systems in and around Cochin has been undertaken and the results are presented in this chapter.

ENVIRONMENTAL PARAMETERS

Environmental parameters such as temperature (atmospheric, water and soil), dissolved oxygen, pH, salinity, alkalinity, hardness, nitrite, nitrate, ammonia, phosphate and silicate of pond water were studied in order to find out their role in the incidence of *Vibrio* spp. in the culture ponds. Samples were collected from three

TABLE 1.1. *Correlation chart*

Environmental parameters	r	p
Temperature		
Atmospheric	+0.706	0.01 **
Water	+0.689	< 0.01 **
Soil	+0.688	< 0.01 **
pH	+0.141	0.415
Salinity	+0.936	< 0.01 **
Dissolved oxygen	+0.276	0.109
NO_2	+0.095	0.579
NO_3	-0.035	0.839
NH_3	+0.143	0.405
PO_4	-0.222	0.192
Si	-0.023	0.894
Alkalinity	+0.379	< 0.05 *
Total Hardness	+0.876	< 0.01 **

* Significant positive correlation
** Highly significant positive correlation

stations for one year and the data collected were together considered for the correlation tests (Table 1.1). Average values of the environmental parameters during each season at different stations are given in Table 1.2.

Temperature

Atmospheric, water and soil temperature were observed minimum during Monsoon months whereas Transition and Summer months reported higher values.

TABLE 1.2. *Environmental parameters (mean) and incidence of* vibrios *during different seasons*

Seasons / Stations	Temperature (^{0}C)			pH	Salinity	DO	NO_2	NO_3	NH_3	PO_4	Si	Alkalinity	Hardness	No. of vibrios
Station I	Atmopheric	Water	Soil		ppt	ml/l	µg at / l					mg/l	ppm	
Monsoon	25.76	27.38	26.42	7.96	2.50	4.622	0.74	0.80	13.72	3.99	9.49	67.262	336.40	3
Postmonsoon	28.30	29.72	28.95	8.23	5.10	4.373	0.09	-	0.11	5.90	41.56	86.532	1245.00	12
Transition	29.19	30.47	29.33	7.90	15.13	4.116	1.21	4.71	14.10	2.91	23.26	139.487	3151.23	25
Summer	29.10	30.53	29.70	7.86	18.18	3.121	0.88	0.71	53.17	2.71	24.74	122.450	3560.17	20
Station II														
Monsoon	26.56	27.94	27.04	8.11	2.91	2.931	0.36	0.06	21.41	7.86	38.45	102.571	277.48	3
Postmonsoon	29.02	30.77	29.57	8.51	4.09	4.381	0.44	-	-	2.55	11.36	99.572	1693.17	7
Transition	29.97	30.97	30.40	8.16	11.44	4.319	0.82	0.45	-	2.51	3.96	181.598	3285.40	22
Summer	29.67	32.07	30.73	8.30	20.89	4.352	1.10	0.84	10.13	5.33	14.73	182.939	4028.52	40
Station III														
Monsoon	26.54	28.28	27.16	7.07	0.55	2.541	1.08	10.05	18.59	28.77	44.82	28.877	90.67	UD
Postmonsoon	28.90	30.27	29.50	6.62	1.45	3.197	0.19	1.25	-	4.64	62.08	20.010	-	4
Transition	29.37	30.47	29.93	7.49	12.56	3.600	0.30	2.44	17.50	6.17	31.04	47.383	2578.43	18
Summer	28.67	30.50	29.03	7.42	14.55	2.765	2.48	4.87	34.46	8.13	63.96	52.717	2861.50	14

UD = undetectable

Atmospheric temperature

At all the three stations atmospheric temperature was observed lowest during monsoon season, whereas the highest were reported during Summer. Higher number of vibrio isolates were encountered when the atmospheric temperature was higher *i.e.* around 30°C which was observed during Transition and Summer seasons.

The impact of atmospheric temperature on *Vibrio* spp. has proved to be statistically significant ($r = +0.706$; $p = <0.01$).

Water temperature

From the analysis of data, it was observed that water temperature played a vital role in the incidence of *Vibrio* spp. in shrimp culture systems. The water temperature observed was low during Monsoon season and the highest was during summer.

Statistical analysis of data revealed significant positive correlation between the occurrence of *Vibrio* spp. and the water temperature ($r = +0.689$; $p = <0.01$).

Soil temperature

Soil temperature also was found to be important in the incidence of *Vibrio* spp. in culture systems. The lowest soil temperature was reported during Monsoon months invariably at all stations. But the highest was during Summer at station I and II and during Transition season at station III. Incidence of *Vibrio* spp. in the culture system also showed a positive significant correlation with soil temperature ($r = +0.688$; $p = <0.01$).

pH

pH of water fluctuated between 6.62 and 8.51 with which *Vibrio* spp. was not exhibiting any significant correlation ($r = +0.141$; $p = 0.415$).

Salinity

Salinity was found to be the most significant environmental parameter which played an important role in the incidence and distribution of *Vibrio* spp. in shrimp culture systems. At all the three stations, salinity was at the lowest during the Monsoon season with a gradual increase through Postmonsoon and Transition seasons. The highest values were observed during Summer. There existed a positive significant correlation between salinity and the incidence of vibrio ($r = +0.936$; $p = <0.01$).

Dissolved oxygen

The lowest DO at station I was observed during Summer, whereas at station II and III it was observed during Monsoon. The highest values were observed during Monsoon, Postmonsoon and Transition seasons respectively at stations I, II and III. Incidence of *Vibrio* spp. in the culture system did not show any significant correlation with these fluctuation in DO ($r = +0.276$; $p = 0.109$).

Nitrite, nitrate and ammonia

Incidence of *Vibrio* spp. in the culture system did not show any significant statistical relationship with the Nitrite nitrogen, Nitrate nitrogen and Ammonia nitrogen content of the pond water (NO_2 - $r = +0.095$, $p = 0.579$; NO_3 - $r = - 0.035$, $p = 0.839$; NH_3 - $r = + 0.143$, $p = 0.405$).

Phosphate

Similarly the incidence of *Vibrio* spp. in the culture system also did not show any significant statistical correlation with the phosphate phosphorus content of pond water ($r = - 0.222$; $p = 0.192$).

Silicate

Occurrence of *Vibrio* spp. did not show any significant correlation with the silicate-silicon content of pond water ($r = -0.023$; $p = 0.894$). At station I silicate content was the lowest during Monsoon, but at station II and III it was reported highest during Transition season and the higher values at station I, II and III were reported during Postmonsoon, Monsoon and Summer respectively.

Alkalinity

Incidence of *Vibrio* spp. showed significant positive correlation with the alkalinity of the pond water ($r = 0.569$; $p = < 0.05$). Alkalinity of pond water was found to be low during Monsoon and Postmonsoon months and higher during Transition and Summer seasons.

Hardness

Significant statistical correlation was observed between the total hardness of pond water and the incidence and distribution of *Vibrio* spp. in the culture systems ($r = + 0.876$; $p = < 0.01$). The low values of total hardness were observed during Monsoon and Postmonsoon in all the stations and the highest values were reported during Summer months.

SEASONAL ABUNDANCE OF *VIBRIO* SPP. IN CULTURE SYSTEMS

The occurrence and abundance of *Vibrio* spp. show significant variation in culture systems during four different seasons (Table 1.3, Fig. 1.1).

TABLE 1.3. *Occurrence of* Vibrio *spp. during different seasons (isolates from all stations pooled together)*

	Monsoon		Post monsoon		Transition		Summer		Total	
Species	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Va	-	-	-	-	12	18.46	29	39.19	41	24.40
Vc	2	33.33	-	-	5	7.69	2	2.70	9	5.36
Vf	4	66.67	15	65.22	8	12.31	5	6.76	32	19.05
Vfb	-	-	3	13.04	3	4.62	8	10.81	14	8.33
Vn	-	-	-	-	13	20.00	7	9.46	20	11.91
Vo	-	-	4	17.39	6	9.23	6	8.11	16	9.52
Vpa	-	-	-	-	13	20.00	16	21.62	29	17.26
Vpr	-	-	1	4.35	5	7.69	1	1.35	7	4.17
Total	6	100.00	23	100.00	65	100.00	74	100.00	168	100.00
	3.57%		13.69%		38.69%		44.05%		100%	

Va - V. anguillarum, Vc - V. cholerae non-O1, *Vf - V. fischeri* like bacteria, *Vfb - V. fluvialis* biovar II, *Vn - V. neries, Vo - V. ordalii, Vpa - V. parahaemolyticus Vpr - V. proteolyticus.*

Monsoon (June - August)

The number of vibrio isolates encountered during the Monsoon were very less when compared to the other seasons and below detectable levels during late Monsoon (August). The vibrio isolates encountered during Monsoon months formed only 3.57% of the total isolates and the species diversity also was less compared to other seasons (Table 1.3, Fig. 1.2).

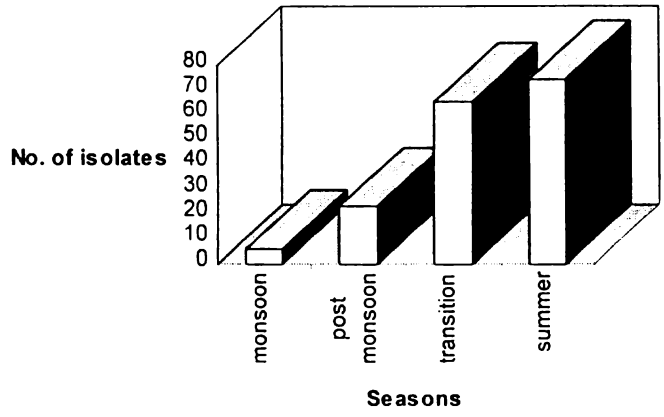


Fig. 1.1. Incidence of Vibrios during different seasons.

Among the isolates of vibrios during this season, 66.67% was formed by *V. fischeri* like bacteria and the rest (33.33%) by *V. cholerae* non-O1. All other *Vibrio* spp. were below detectable levels during this season (Table 1.3).

At station I, *V. fischeri* like bacteria alone (three isolates) was encountered during Monsoon. Whereas at station II, 66.67% of the isolates was formed by *V. cholerae* non-O1 and 33.33% by *V. fischeri* like bacteria and at station III no vibrios were encountered (Table 1.4, 1.5 and 1.6).

All the isolates from juvenile shrimps encountered during Monsoon were from digestive system, whereas in the culture pond 66.67% were isolated from water and 33.33% were from sediment (Table 1.8).

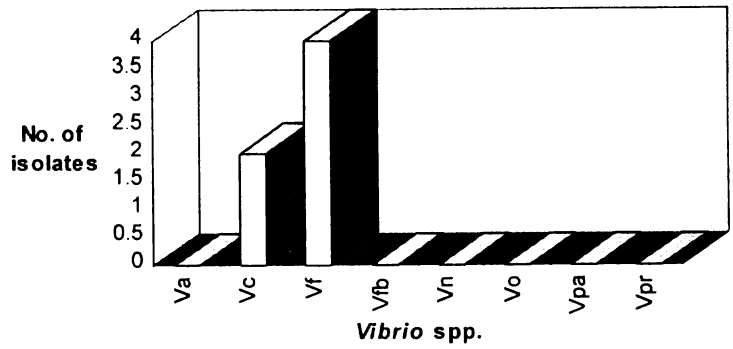


Fig.1.2. Incidence of vibrios during Monsoon.

Postmonsoon (September to November)

During this season the total number of vibrio isolates increased to 23 forming 13.69% of the total isolates and a corresponding higher species diversity was observed. *Vibrio* spp. such as *V. ordalii* (which was formerly known as *V. anguillarum* biovar II), *V. fischeri* like bacteria, *V. fluvialis* biovar II and *V. proteolyticus* were reported during this season (Table 1.3, Fig 1.3).

V. fischeri like bacteria was predominant with 65.22% and the incidence of this species was found to be maximum during this season. *V. ordalii* formed 17.39%, *V. fluvialis* biovar II 13.04% and *V. proteolyticus* 4.35%. Other species such as *V. anguillarum*, *V. cholerae* non-O1, *V. neries* and *V. parahaemolyticus* were below detectable levels.

TABLE 1. 4 . *Occurrence of vibrios at station I during different seasons*

	Monsoon		Postmonsoon		Transition		Summer		Total	
Species	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Va	-	-	-	-	1	4.00	11	55.00	12	20.00
V c	-	-	-	-	4	16.00	-	-	4	6.66
V f	3	100.00	7	58.34	3	12.00	-	-	13	21.67
V fb	-	-	1	8.33	-	-	2	10.00	3	5.00
V n	-	-	-	-	6	24.00	-	-	6	10.00
V o	-	-	4	33.33	4	16.00	1	5.00	9	15.00
V pa	-	-	-	-	6	24.00	6	30.00	12	20.00
V pr	-	-	-	-	1	4.00	-	-	1	1.67
Total	3	100.00	12	100.00	25	100.00	20	100.00	60	100.00

TABLE 1. 5 *Occurrence of vibrios at station II during different seasons*

	Monsoon		Postmonsoon		Transition		Summer		Total	
Species	No.of isolates	%	No.of isolates	%	No.of isolates	%	No.of isolates	%	No.of isolates	%
Va	-	-	-	-	6	27.27	12	30.00	18	25.00
V c	2	66.67	-	-	-	-	2	5.00	4	5.56
V f	1	33.33	5	71.43	2	9.09	-	-	8	11.11
V fb	-	-	1	14.29	-	-	6	15.00	7	9.72
V n	-	-	-	-	5	22.73	4	10.00	9	12.50
V o	-	-	-	-	-	-	5	12.50	5	6.94
V pa	-	-	-	-	7	31.82	10	25.00	17	23.61
V pr	-	-	1	14.29	2	9.09	1	2.50	4	5.56
Total	3	100.00	7	100.00	22	100.00	40	100.00	72	100.00

TABLE 1.6. *Occurrence of vibrios at station III during different seasons*

	Mansoon		Postmansoon		Transition		Summer		Total	
Species0	No.of isolates	%	No.of isolates	%	No.of isolates	%	No.of isolates	%	No.of isolates	%
Va	-	-	-	-	5	27.78	6	42.86	11	30.55
V c	-	-	-	-	1	5.55	-	-	-	2.78
V f	-	-	3	75.00	3	16.67	5	35.71	11	30.55
V fb	-	-	1	25.00	3	16.67	-	-	4	11.11
V n	-	-	-	-	2	11.11	3	21.43	5	13.89
V o	-	-	-	-	2	11.11	-	-	2	5.56
V pa	-	-	-	-	-	-	-	-	-	-
V pr	-	-	-	-	2	11.11	-	-	2	5.56
Total	-	-	4	100.00	18	100.00	14	100.00	36	100.00

During the Postmonsoon season at station 1, 58.34% of vibrios were formed by *V. fischeri* like bacteria followed by *V. ordalii* (33.33%) and *V. fluvialis* biovar II (8.33%) (Table 1.4).

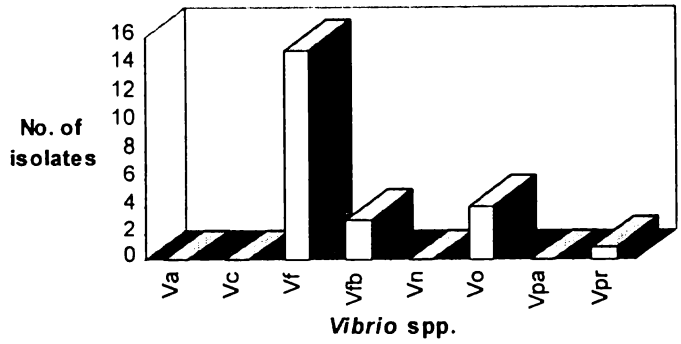


Fig. 1.3. Incidence of vibrios during Postmonsoon.

At station II also *V. fischeri* like bacteria was predominant. It formed 71.43% and other species such as *V. fluvialis* biovar II and *V. proteolyticus* formed 14.29% each. Whereas at station III, *V. fischeri* like bacteria formed 75% and *V. fluvialis* biovar II, 25% (Tables 1.5 and 1.6).

In juvenile shrimp, body surface accounted for 16.66%, gill 41.67% and digestive system 41.67% of the total vibrios from juvenile shrimp. In culture pond, water accounted for 72.73% and sediment 27.27% (Table 1.8).

Transition (December - February)

During Transition months a higher incidence of vibrios with greater species diversity was observed in the culture system which formed 38.69% of the total vibrio isolates (Table 1.3, Fig. 1.4).

Among the vibrio isolates, *V. neries* and *V. parahaemolyticus* formed 20% each, *V. anguillarum* 18.46%, *V. fischeri* like bacteria 12.31%, *V. ordalii* 9.23%, *V. cholerae* non-O1 7.69%, *V. proteolyticus* 7.69% and *V. fluvialis* biovar II formed 4.62%. The incidence of *V. proteolyticus*, *V.neries*, *V. cholerae* non-O1 and *V. ordalii* were found in good numbers during this season (*V. ordalii* showed similar occurrence in Summer also) (Table 1.3).

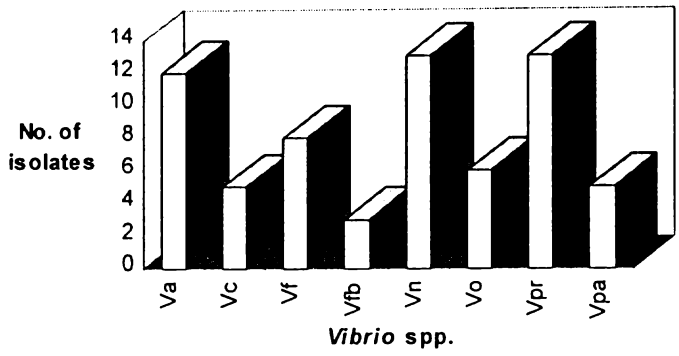


Fig.1.4. Incidence of vibrios during Transition.

At station I, species such as *V. neries* (24%), *V. parahaemolyticus* (24%), *V. ordalii* (16%), *V. cholerae* non-O1(16%), *V. fischeri* like bacteria (12%), *V. anguillarum* (4%) and *V. proteolyticus* (4%) were isolated during this season (Table 1.4).

At station II during Transition months, 31.82% was formed by *V. parahaemolyticus*, 27.27% by *V. anguillarum*, 22.73% by *V. neries* and 9.09% each by *V. fischeri* like bacteria and *V. proteolyticus* (Table 1.5).

At station III, during Transition months, *V. anguillarum* formed 27.78%, *V. fischeri* like bacteria and *V. fluvialis* biovar II formed 16.67% each, *V. ordalii*,

V. neries and *V. proteolyticus* formed 11.11% each and *V. cholerae* non-O1 5.55% (Table 1.6).

In juvenile shrimp 17.39% was isolated from body surface, 28.26% from gill and 54.35% from digestive system. At the same time in culture pond, water accounted for 47.37% and sediment 52.63% (Table 1.8).

Summer (March - May)

Summer months were found to be the most ideal season for the growth and distribution of *Vibrio* spp. in culture systems and a higher number of isolates (74) forming 44.05% was observed during this season. All the eight species of *Vibrio* encountered during the Transition period were occurred in Summer also (Fig. 1.5).

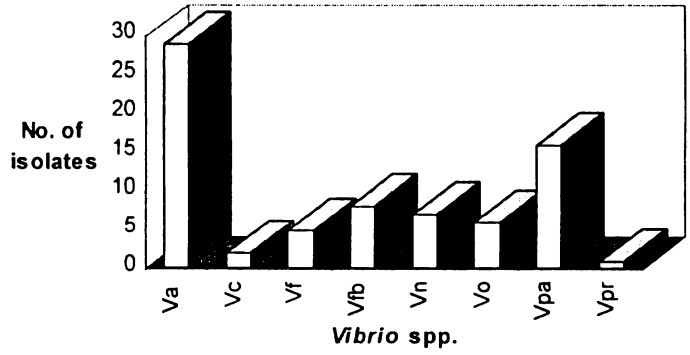


Fig. 1.5. Incidence of vibrios during Summer.

Out of the 74 isolates encountered *V. anguillarum* formed the highest component (39.19%), followed by *V. parahaemolyticus* (21.52%), *V. fluvialis* biovar II (10.81%), *V. neries* (9.46%), *V. ordalii* (8.11%), *V. fischeri* like bacteria (6.76%), *V. cholerae* non-O1 (2.70%) and *V. proteolyticus* (1.35%) (Table 1.3). The highest number of *V. anguillarum* and *V. parahaemolyticus* were encountered during Summer season.

20 vibrio isolates were encountered from station I during the Summer, out of which *V. anguillarum* formed 55%, *V. parahaemolyticus* 30%, *V. fluvialis* biovar II 10% and *V. ordalii* 5% (Table 1.4).

The highest number of isolates at station II was encountered during Summer. 40 vibrio isolates were encountered with the following species composition; *V. anguillarum* 30%, *V. parahaemolyticus* 25%, *V. fluvialis* biovar II 15%, *V. ordalii* 12.5%, *V. neries* 10%, *V. cholerae* non-O1 5% and *V. proteolyticus* 2.5% (Table 1.5).

TABLE 1.7. *Seasonal abundance of vibrios*

Juvenile shrimp		
Season	No. of isolates	%
Monsoon	3	2.52
Postmonsoon	12	10.08
Transition	46	38.66
Summer	58	48.74
Total	119 (70.83%)	100.00
Culture pond		
Monsoon	3	6.10
Postmonsoon	11	22.48
Transition	19	38.77
Summer	16	32.65
Total	49 (29.17%)	100.00

At station III 14 vibrio isolates were encountered with *V. anguillarum* (42.86%), *V. fischeri* like organism (35.71%), *V. neries* (21.43%) during this season (Table 1.6). This is a very poor representation of the isolates compared to the earlier two stations in Summer.

Out of the total vibrios isolated from juvenile shrimp body surface, gill and digestive system accounted for 20.69%, 24.14% and 55.17% respectively. In culture pond, water accounted for 68.75% and sediment 31.25%.

HABITAT OF THE ISOLATED VIBRIOS

The incidence and seasonal abundance of *Vibrio* spp. in the shrimps and their culture system showed marked differences with respect to the habitat. Out of 168 isolates of vibrios, 119 (70.83%) were isolated from juvenile shrimp and the rest 49 (29.17%) from culture pond (Table 1.7).

Juvenile shrimp

Among the isolated vibrios, 119 were obtained from juvenile shrimp, of which digestive system accounted for 54.62%, gill 26.89% and the body surface 18.49% (Table 1.8).

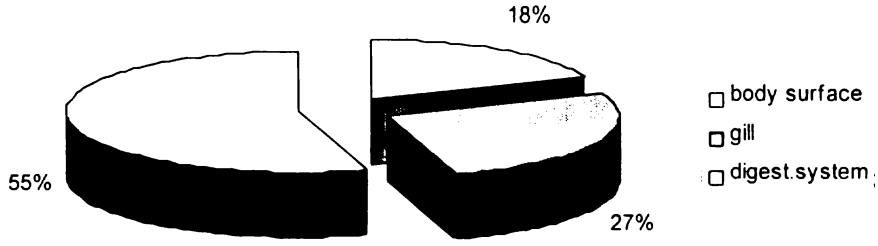


Fig. 1.6. Incidence of vibrios in different body parts of juvenile shrimp.

Body surface

Body surface accounted for 18.49 % of the vibrio isolates from the juvenile shrimp (Table 1.8). Body surface of the juvenile shrimp harboured species such as *V. parahaemolyticus* (22.73%), *V. anguillarum* (18.18%), *V. fischeri* like bacteria (18.18%) *V. ordalii* (13.64%), *V. cholerae* non-O1 (9.09%), *V. proteolyticus* (9.09%), *V. fluvialis* biovar II (4.54%) and *V. neries* (4.54%) (Table 1.11).

TABLE 1.8. *Occurrence of* Vibrio *spp. in juveniles of* P. indicus *and in culture ponds during different seasons*

	Monsoon		Postmonsoon		Transition		Summer		Total	
	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Juvenile shrimp										
Body surface	-	-	2	16.66	8	17.39	12	20.69	22	18.49
Gill	-	-	5	41.67	13	28.26	14	24.14	32	26.89
Digestive system	3	100.00	5	41.67	25	54.35	32	55.17	65	54.62
Total	3	100.00	12	100.00	46	100.00	58	100.00	119	100.00
Culture pond										
Water	2	66.67	8	72.73	9	47.37	11	68.75	30	61.22
Bottom sediment	1	33.33	3	27.27	10	52.63	5	31.25	19	38.78
Total	3	100.00	12	100.00	19	100.00	16	100.00	49	100.00

TABLE 1.9. *Distribution pattern of* Vibrio *spp. in juveniles of* P. indicus *and in culture ponds during different seasons*

	Monsoon		Postmonsoon		Transition		Summer		Total	
	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Juvenile shrimp										
Body surface	-	-	2	9.09	8	36.36	12	54.55	22	100.00
Gill	-	-	5	15.63	13	40.62	14	43.75	32	100.00
Digestive system	3	4.62	5	7.69	25	38.46	32	49.23	65	100.00
Culture pond										
Water	2	6.67	8	26.67	9	30.00	11	36.66	30	100.00
Bottom sediment	1	5.26	3	15.79	10	52.63	5	26.32	19	100.00

During Monsoon no vibrio was encountered from juvenile body surface and during Postmonsoon months the incidence of vibrios was observed very low. Only 2 isolates were encountered, which formed 9.09% of the total vibrios isolated from the body surface. *V. fischeri* like bacteria and and *V. proteolyticus* were the two species reported during this season (Table 1.11).

From the body surface 36.36% of the vibrio isolates were encountered during Transition months, where *V. ordalii*, *V. fischeri* like bacteria and *V. parahaemolyticus* formed 25% each and *V. cholerae* non-O1 and *V. proteolyticus* formed 12.5% each.

Summer reported maximum vibrio population on body surface. Among the vibrios isolated during Summer, *V. anguillarum* formed 33.33% and *V. parahaemolyticus* 25%. Other species such as *V. ordalii*, *V. cholerae* non-O1, *V. fischeri* like bacteria, *V. neries* and *V. fluvialis* biovar II formed 8.33% each (Table 1.9, 1.11).

TABLE 1.10. *Composition of vibrios in body parts of juvenile* P. indicus

	Body surface		Gill		Digst. system		Total	
Species	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Va	4	18.18	5	15.62	22	33.85	31	26.05
Vc	2	9.09	3	9.38	1	1.54	6	5.04
Vf	4	18.18	7	21.87	10	15.38	21	17.65
Vfb	1	4.54	5	15.62	3	4.62	9	7.56
Vn	1	4.55	4	12.50	10	15.38	15	12.61
Vo	3	13.64	3	9.38	5	7.69	11	9.24
Vpa	5	22.73	3	9.38	11	16.92	19	15.97
Vpr	2	9.09	2	6.25	3	4.62	7	5.88
Total	22	100.00	32	100.00	65	100.00	119	100.00

Gill

Gill accounted for 26.89% of the vibrios isolated from juvenile shrimp. *V. fischeri* like bacteria was the predominant species which formed 21.88% and

V. anguillarum and *V. fluvialis* biovar II formed 15.63% each. *V. neries* formed 12.5%, *V. ordalii V. cholerae* non-O1 and *V. parahaemolyticus* formed 9.38% each and *V. proteolyticus* formed 6.25% (Table 1.8, 1.12).

Bacteriological analysis revealed that the occurrence of *Vibrio* spp. was below detectable numbers in gill during Monsoon months. Whereas during Postmonsoon season 5 isolates (15.63% of the total vibrios isolated from gill) belonging to *V. fischeri* like bacteria (80%) and *V. fluvialis* biovar II (20%) were reported (Table 1.9, 1.12).

During Transition months 13 isolates were reported from gill (40.62% of the total vibrio isolates from gill) and the species observed were *V. anguillarum* (23.08%), *V. cholerae* non-O1, *V. fischeri* like bacteria and *V. neries* (15.38% each), *V. ordalii*, *V. fluvialis* biovar II, *V. parahaemolyticus* and *V. proteolyticus* (7.69% each).

Summer season registered highest incidence of *Vibrio* species in gills with 14 isolates (43.75% of the total vibrios from gill) with *V. fluvialis* biovar II as the predominant species with 21.43%. Other species encountered during Summer months were *V. anguillarum* (14.29%), *V. ordalii* (14.29%), *V. neries* (14.29%), *V. parahaemolyticus* (14.29%), *V. cholerae* non-O1 (7.14%), *V. fischeri* like bacteria (7.14%) and *V. proteolyticus* (7.14%) (Table 1.9, 1.12).

Digestive system

The highest occurrence of vibrio isolates in juvenile *P. indicus* was reported from the digestive system, which formed 54.62% of the vibrio isolates from juvenile shrimp (Table 1.8).

V. anguillarum was the predominant species in the digestive system of juvenile shrimp with 33.85%. Whereas *V. parahaemolyticus* formed 16.92 %, *V. fischeri* like bacteria and *V. neries* 15.38% each. Other species reported from digestive system were

TABLE 1.11. *Composition of* Vibrio *spp. on the body surface of juvenile* P. indicus *during different seasons*

	Monsoon		Postmonsoon		Transition		Summer		Total	
Species	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Va	-	-	-	-	-	-	4	33.33	4	18.18
Vc	-	-	-	-	1	12.50	1	8.33	2	9.09
Vf	-	-	1	50.00	2	25.00	1	8.33	4	18.18
Vfb	-	-	-	-	-	-	1	8.33	1	4.54
Vn	-	-	-	-	-	-	1	8.33	1	4.54
Vo	-	-	-	-	2	25.00	1	8.33	3	13.64
Vpa	-	-	-	-	2	25.00	3	25.00	5	22.73
Vpr	-	-	1	50.00	1	12.50	-	-	2	9.09
Total	-	-	2	100.00	8	100.00	12	100.00	22	100.00

TABLE 1.12. *Composition of* Vibrio *spp. in the gill of juvenile* P. indicus *during different seasons*

	Monsoon		Postmonsoon		Transition		Summer		Total	
Species	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Va	-	-	-	-	3	23.08	2	14.29	5	15.63
Vc	-	-	-	-	2	15.38	1	7.14	3	9.38
Vf	-	-	4	80.00	2	15.38	1	7.14	7	21.88
Vfb	-	-	1	20.00	1	7.69	3	21.43	5	15.63
Vn	-	-	-	-	2	15.38	2	14.29	4	12.50
Vo	-	-	-	-	1	7.69	2	14.29	3	9.38
Vpa	-	-	-	-	1	7.69	2	14.29	3	9.38
Vpr	-	-	-	-	1	7.69	1	7.14	2	6.25
Total	-	-	5	100.00	13	100.00	14	100.00	32	100.00

TABLE 1.13. *Composition of* Vibrio *spp. in the digestive system of juvenile* P. indicus *during different seasons*

	Monsoon		Postmonsoon		Transition		Summer		Total	
Species	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Va	-	-	-	-	8	32.00	14	43.75	22	33.85
Vc	1	33.33	-	-	-	-	-	-	1	1.54
Vf	2	66.67	3	60.00	3	12.00	2	6.25	10	15.38
Vfb	-	-	-	-	-	-	3	9.38	3	4.62
Vn	-	-	-	-	7	28.00	3	9.38	10	15.38
Vo	-	-	2	40.00	-	-	3	9.38	5	7.69
Vpa	-	-	-	-	4	16.00	7	21.87	11	16.92
Vpr	-	-	-	-	3	12.00	-	-	3	4.62
Total	3	100.00	5	100.00	25	100.00	32	100.00	65	100.00

V. ordalii (7.69%), *V. fluvialis* biovar II (4.62%) *V. proteolyticus* (4.62%) and *V. cholerae* non-O1 (1.54%) (Table 1.10).

Only three isolates were encountered during Monsoon months of which *V. fischeri* like bacteria constituted 66.67% and 33.33% by *V. cholerae* non-O1. The incidence of these isolates were observed during early Monsoon season (first fortnight of June) and their incidence was below detectable levels during the later part of Monsoon.

During Postmonsoon season also the incidence of *Vibrio* spp. was low which formed only 7.69% of the total vibrios from digestive system. Out of the 5 isolates encountered *V. fischeri* like bacteria formed 60% and *V. ordalii* 40% during this period (Tables 1.9, 1.13).

Vibrio population from digestive system encountered during Transition months formed 38.46% of the total vibrios. Out of the 25 isolates, *V. anguillarum* was the predominant (32%) species followed by *V. neries* (28%). Other species isolated from the digestive system of the juvenile shrimp were *V. parahaemolyticus* (16%), *V. fischeri* like bacteria (12%) and *V. proteolyticus* (12%).

Summer season has found to be the ideal season for growth and multiplication of vibrios in juvenile shrimp digestive system which formed 49.23% of the total vibrios isolated from digestive system (Table 1.9). Among the 32 vibrio isolates of Summer, *V. anguillarum* was the predominant species with 43.75%, whereas the incidence of other species exhibited the following pattern. *V. parahaemolyticus* (21.87%), *V. ordalii* (9.38%), *V. fluvialis* biovar II (9.38%), *V. neries* (9.38%) and *V. fischeri* like bacteria (6.25%).

Culture pond

49 isolates were encountered from culture *pond i.e.* from pond water and bottom sediment. Of which 61.22% (30 isolates) was isolated from pond water and 38.78% (19 isolates) from bottom sediment (Table 1.8, Table 1.14).

Pond water

Out of the 30 isolates 36.66% was obtained during Summer, 30% during Transition months, 26.67% during Postmonsoon and 6.67% during Monsoon (Table 1.9).

Fig. 1.7. Incidence of vibrios in culture pond.

In the pond water *V. fischeri* like bacteria predominated with 23.33%, other species encountered were *V. parahaemolyticus* (20.00%), *V. anguillarum* and *V. neries* 13.33% each and *V. ordalii*, *V. cholerae* non-O1 and *V. fluvialis* biovar II 10.00% each. The occurrence of *V. proteolyticus* was not observed in pond water at all (Table 1.14).

During Monsoon , only two isolates were obtained and it together formed 6.67% of the total vibrios isolated from water. *V. cholerae* non-O1and *V. fischeri* like bacteria were those species which were observed during early days of Monsoon. 26.67% of the

vibrio isolates from water were encountered during Postmonsoon represented by *V. fischeri* like bacteria (62.5%), *V. ordalii* (25%) and *V. fluvialis* biovar II (12.5%).

During Transition period 9 vibrio isolates were from pond water with 30.00% of the total vibrios isolated from pond water. Out of the 9 isolates, *V. neries* formed 33.33%, *V. cholerae* non-O1 and *V. parahaemolyticus* 22.22% each and *V. fluvialis* biovar II and *V. ordalii* 11.11% each (Table 1.9, 1.15).

The highest incidence of *Vibrio* spp. in the pond water was observed during Summer, with 36.66% of the total vibrio isolates from water. During this season *V.anguillarum* and *V. parahaemolyticus* were the predominant species with 36.36% each and other species encountered were *V. fischeri* like bacteria, *V. fluvialis* biovar II and *V. neries* with a composition of 9.09% each (Table 1.15).

TABLE 1.14. *Composition of* Vibrio *spp. in the pond water and sediment*

	Water		Sediment		Total	
Species	No. of isolates	%	No. of isolates	%	No. of isolates	%
Va	4	13.33	6	13.58	10	20.41
Vc	3	10.00	-	-	3	6.12
Vf	7	23.33	4	21.05	11	22.45
Vfb	3	10.00	2	10.53	5	10.20
Vn	4	13.33	1	5.26	5	10.20
Vo	3	10.00	2	10.53	5	10.20
Vpa	6	20.00	4	21.05	10	20.41
Vpr	-	-	-	-	-	-
Total	30	100.00	19	100.00	49	100.00

Pond sediment

Bacteriological analysis revealed that the incidence of vibrio was found least in bottom sediment. Only 19 isolates were obtained from sediment which constituted 38.78% of the total vibrio isolated from the culture pond (water and sediment) (Table 1.8). *Vibrio* spp. such as *V. anguillarum*, *V. ordalii*, *V. fischeri* like bacteria,

TABLE 1.15. *Composition of* Vibrio *spp. in pond water during different seasons*

	Monsoon		Postmonsoon		Transition		Summer		Total	
Species	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Va	-	-	-	-	-	-	4	36.36	4	13.33
V c	1	50.00	-	-	2	22.22	-	-	3	10.00
V f	1	50.00	5	62.50	-	-	1	9.09	7	23.33
V fb	-	-	1	12.50	1	11.11	1	9.09	3	10.00
V n	-	-	-	-	3	33.33	1	9.09	4	13.33
V o	-	-	2	25.00	1	11.11	-	-	3	10.00
V pa	-	-	-	-	2	22.22	4	36.36	6	20.00
V pr	-	-	-	-	-	-	-	-	-	-
Total	2	100.00	8	100.00	9	100.00	11	100.00	30	100.00

TABLE 1.16. *Composition of* Vibrio *spp. in the pond sediment during different seasons*

	Monsoon		Postmonsoon		Transition		Summer		Total	
Species	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Va	-	-	-	-	1	10.00	5	100.00	6	31.58
V c	-	-	-	-	-	-	-	-	-	-
V f	1	100.00	2	66.67	1	10.00	-	-	4	21.05
V fb	-	-	1	33.33	1	10.00	-	-	2	10.53
V n	-	-	-	-	1	10.00	-	-	1	5.26
V o	-	-	-	-	2	20.00	-	-	2	10.53
V pa	-	-	-	-	4	40.00	-	-	4	21.05
V pr	-	-	-	-	-	-	-	-	-	-
Total	1	100.00	3	100.00	10	100.00	5	100.00	19	100.00

V. fluvialis biovar II, *V. neries* and *V. parahaemolyticus* were represented from pond bottom sediment. But species such as *V. cholerae* non-O1 and *V.proteolyticus* were below detectable numbers (Table 1.14).

V. anguillarum was the predominant species (31.58%) in the sediment, followed by *V. fischeri* like bacteria and *V. parahaemolyticus* (21.05% each). Other species such as *V. ordalii* and *V. fluvialis* biovar II formed 10.53% each and *V. neries* 5.26% (Table 1.16).

During Monsoon, only *V. fischeri* like bacteria was reported from sediment and formed only 5.26% of the total vibrios encoutered from sediment. 3 isolates were encoutered during Postmonsoon, out of which *V. fischeri* like bacteria formed 66.67% and *V. fluvialis* 33.33% (Table 1.9, 1.16).

The highest incidence of vibrios in sediment was observed during Transition months. Out of the 10 isolates (52.63%) encountered *V. parahaemolyticus* formed 40% and other species such as *V. ordalii* formed 20%, *V. anguillarum*, *V. fischeri* like bacteria, *V. fluvialis* biovar II and *V. neries* formed 10% each. During Summer months the incidence of vibrios in the sediment was low when compared to Transition months. 5 isolates belonged to a single species *V. anguillarum* were encountered during this period which formed 26.32% of the total vibrios isolated from sediment (Table 1.16, 1.9).

VIBRIO SPECIES ENCOUNTERED FROM SHRIMP CULTURE SYSTEMS

The following eight *Vibrio* spp. were seen in the shrimp culture system.

V. anguillarum
V. cholerae non-O1
V. fischeri like bacteria
V. fluvialis biovar II
V. neries
V. ordalii
V. parahaemolyticus
V. proteolyticus

Vibrio anguillarum

V. anguillarum formed 24.4% and was found to be predominant in shrimp culture systems. Occurrence of *V. anguillarum* was below detectable levels during Monsoon and Postmonsoon. However during Transition and Summer seasons incidence of this species was reported in higher numbers. Out of the 41 isolates of *V. anguillarum* 29.27% were reported during Transition months and 70.73% during Summer, which was found to be suitable for their growth and multiplication (Table 1.17).

TABLE 1.17. *Occurrence (%) of* Vibrio *spp. during different seasons*

Species	Monsoon	Postmonsoon	Transition	Summer	Total	
					No. of isolates	%
Va	-	-	29.27	70.73	41	24.40
Vc	22.22	-	55.56	22.22	9	5.36
Vf	12.50	46.87	25.00	15.63	32	19.05
Vfb	-	21.43	21.43	57.14	14	8.33
Vn	-	-	65.00	35.00	20	11.90
Vo	-	25.00	37.50	37.50	16	9.52
Vpa	-	-	44.83	55.17	29	17.26
Vpr	-	14.29	71.43	14.29	7	4.17
Total					168	100.00

Out of the 60 isolates from station I, *V. anguillarum* formed 20% (12 isolates). At station II,18 isolates were encountered, which formed 25% of the total (72) isolates from that station. Out of 36 isolates encountered from station III, this species formed 30.56% (Table 1.18).

Out of the 41 isolates of *V. anguillarum* 75.61% (31 isolates) was from juvenile shrimp (Table 1.19), of which digestive system harboured a maximum of 70.97% followed by gill (16.13%) and body surface (12.9%) (Table 1.20). 24.39% isolates were encountered from culture pond (Table 1.19), of which pond water accounted for 40% and bottom sediment 60% (Table 1.21).

From shrimp body surface *V. anguillarum* could be isolated only during Summer (Table 1.22). Whereas its incidence in gill was higher during Transition (60%) and Summer months (40%) (Table 1.23). Out of the 22 isolates of *V. anguillarum* from digestive system of juvenile shrimp, majority were observed during Summer (63.64 %) indicating that Summer months are suitable for its growth and multiplication followed by Transition months (36.36%) (Table 1.24).

In pond water, incidence of *V. anguillarum* was observed only during Summer (Table 1.25). Analyses revealed that the occurrence of *V. anguillarum* was highest in sediment during Summer season (83.33%) followed by Transition months (16.67%), whereas during Monsoon and Postmonsoon it was below detectable numbers (Table 1.26).

***Vibrio cholerae* non-O1**

The incidence of *V. cholerae* non-O1 in the culture system was low when compared to other species. Only 9 isolates were encountered from the culture system (including both juvenile shrimp and culture pond), which formed 5.36% of the total vibrio isolates (Table 1.17).

55.56% of the total *V. cholerae* non-O1 isolates were encountered during Transition months. Summer and Monsoon seasons showed uniform distribution of 22.22% each and it was not observed during Postmonsoon season (Table 1.17).

At station I, only 4 *V. cholerae* non-O1 isolates were encountered and it formed only 6.67% of the total vibrios isolated from this station. At all other stations their incidence was still lower (5.56% and 2.78% at station II and III respectively) (Table 1.18).

Out of the 9 isolates, 66.67% were encountered from juvenile shrimp and 33.33% from culture pond (Table 1.19). The highest incidence (50%) was in gill followed by body surface (33.33%) and digestive system (16.67%) (Table 1.20). In culture pond, isolates were encountered only from water and in sediment it was below detectable level (Table 1.21).

TABLE 1.18. *Occurrence of* Vibrio *spp. in three different stations*

	Station I		Station II		Station III		Total	
Species	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Va	12	20.00	18	25.00	11	30.56	41	24.40
Vc	4	6.67	4	5.56	1	2.78	9	5.36
Vf	13	21.67	8	11.11	11	30.56	32	19.05
Vfb	3	5.00	7	9.72	4	11.11	14	8.33
Vn	6	10.00	9	12.50	5	13.89	20	11.90
Vo	9	15.00	5	6.94	2	5.56	16	9.52
Vpa	12	20.00	17	23.61	-	-	29	17.26
Vpr	1	1.67	4	5.56	2	5.56	7	4.17
Total	60	100.00	72	100.00	36	100.00	168	100.00

In the gills of juvenile shrimp, incidence of *V. cholerae* non-O1 was higher during Summer (66.66%) followed by Transition months (33.33%) (Table 1.23). During other seasons it was not reported. From body surface, isolates were obtained only during Transition and Summer seasons (50% each) (Table 1.22). From digestive system only one isolate was encountered during Monsoon (Table 1.24).

In pond water the incidence of *V. cholerae* non-O1 was observed during Transition months (66.67%) and Monsoon season (33.33%) and no isolates was encountered during Postmonsoon and Summer months (Table 1.25).

***Vibrio fischeri* like bacteria**

V. fischeri like bacteria formed 19.05% of the total vibrio isolates during all seasons. Out of the 32 isolates 46.87% were during Postmonsoon season, followed by Transition (25%), Summer (15.63%) and Monsoon(12.5%) seasons (Table 1.17).

TABLE 1.19. *Composition (%)* of Vibrio *spp. in culture system*

	Juvenile shrimp		Culture pond		Total	
Species	No. of isoates	%	No. of isoates	%	No. of isolates	%
Va	31	75.61	10	24.39	41	100.00
Vc	6	66.67	3	33.33	9	100.00
Vf	21	65.63	11	34.37	32	100.00
Vfb	9	64.29	5	35.71	14	100.00
Vn	15	75.00	5	25.00	20	100.00
Vo	11	68.75	5	31.25	16	100.00
Vpa	19	65.52	10	34.48	29	100.00
Vpr	7	100.00	-	-	7	100.00
Total	119		49		168	

At station I, *V. fischeri* like bacteria formed 21.67% of the total vibrios isolates, whereas at station II and III it accounted for 11.11% and 30.56% respectively (Table 1.18). 65.63% was reported from juvenile shrimp and the rest (34.37%) from culture pond. In the juvenile shrimp highest occurrence of *V. fischeri* like bacteria was observed in digestive system (47.62%), 33.33% in gill and 19.05% in body surface respectively. (Tables 1.19, 1.20).

During Postmonsoon and Transition months the incidence of this species was higher in digestive system (30% each), whereas during Summer and Monsoon it was low (20% each) (Table 1.24).

In gill 57.14% were observed during Postmonsoon and 28.57% during Transition period, but during Summer season it formed only 14.29%. They were absent in the gill during Monsoon (Table 1.23).

In body surface highest occurrence (50%) was observed during Transition period, whereas during Postmonsoon and Summer it formed 25% each. As in the case of gill their occurrence was not reported during Monsoon (Table 1.22).

TABLE 1.20. Vibrio *isolates in different body parts of juvenile* P. indicus

	Body surface		Gill		Digest. system		Total	
Species	No. of isoates	%	No. of isoates	%	No. of isolates	%	No. of isolates	%
Va	2	12.90	5	16.13	22	70.97	31	26.05
Vc	2	33.33	3	50.00	1	16.67	6	5.04
Vf	4	19.05	7	33.33	10	47.62	21	17.65
Vfb	1	11.11	5	55.56	3	33.33	9	7.56
Vn	1	6.67	4	26.67	10	66.67	15	12.60
Vo	3	27.27	3	27.27	5	45.45	11	9.24
Vpa	5	26.32	3	15.79	1	57.89	19	15.97
Vpr	2	28.57	2	28.57	3	42.86	7	5.88
Total	22	18.49	32	26.89	65	54.62	119	100.00

The culture pond showed the higher occurrence of *V.fischeri* like bacteria in pond water (63.64%) than in the bottom sediment (36.36%) (Table 1.21). This species

TABLE 1.21. Vibrio *isolates from pond water and bottom sediment*

Species	Water		Sediment		Total	
	No. of isolates	%	No. of isolates	%	No. of isolates	%
Va	4	40.00	6	60.00	10	20.41
Vc	3	100.00	-	-	3	6.12
Vf	7	63.64	4	36.36	11	22.45
Vfb	3	60.00	2	40.00	5	10.20
Vn	4	80.00	1	20.00	5	10.20
Vo	3	60.00	2	40.00	5	10.20
Vpa	6	60.00	4	40.00	10	20.41
Vpr	-	-	-	-	-	-
Total	30	61.22	19	38.78	49	100.00

from pond water was observed maximum (71.43%) during Postmonsoon season followed by Summer and Monsoon seasons (14.29% each). During Transition

months it was not encountered (Table 1.25). The highest occurrence of this species in sediment also was observed during Postmonsoon (50%) and in Transition and Monsoon seasons it accounted for 25% each. During Summer the occurrence of *V. fischerii* like bacteria was below detectable numbers (Table 1.26).

***Vibrio fluvialis* biovar II**

V. fluvialis biovar II was encountered in low numbers in culture system with 8.33% of the total vibrios. Summer season was observed with highest occurrence (57.14%) of this species and during Transition and Postmonsoon months it formed 21.43% each. *V. fluvialis* biovar II was not reported during Monsoon (Table 1.17).

When the total vibrio isolates of each station is considered, *V. fluvialis* biovar II formed 5%, 9.72% and 11.11% at station I, II and III respectively (Table 1.18).

Out of the 14 isolates of *V. fluvialis* biovar II, 64.29% was obtained from juvenile shrimp and the rest (35.71%) from the culture pond (Table 1.19). In juvenile shrimp, gill harboured the highest number of *V. fluvialis* biovar II (55.56%), and in digestive system and body surface it formed 33.33% and 11.11% respectively (Table 1.20). In culture pond, water harboured 60% of *V. fluvialis* biovar II and the bottom sediment 40% (Table 1.21).

In gill, the incidence of *V. fluvialis* biovar II was highest during Summer (60%) whereas during Postmonsoon and Transition period it formed 20% each and was not encountered during monsoon (Table 1.23). In the digestive system and body surface of juvenile shrimp this species was encountered during Summer only (Table 1.22, 1.24). The incidence of *V. fluvialis* biovar II in pond water was observed during Postmonsoon, Transition and Summer with uniform pattern (33.33% each) (Table 1.25)

and in the sediment it was observed during Postmonsoon and Transition months in equal numbers. During the other two seasons it was not observed in sediment (Table 1.26).

TABLE 1.22. *Occurrence (%)* Vibrio *spp. on the body surface of juvenile* P.indicus *during different seasons*

	Monsoon	Postmonsoon	Transition	Summer	Total No.	%
Va	-	-	-	100.00	4	18.18
V c	-	-	50.00	50.00	2	9.09
V f	-	25.00	50.00	25.00	4	18.18
V fb	-	-	-	100.00	1	4.54
V n	-	-	-	100.00	1	4.54
V o	-	-	66.67	33..33	3	13.64
V pa	-	-	40.00	60.00	5	22.73
V pr	-	50.00	50.00	-	2	9.09
Total					22	100.00

Vibrio neries

Incidence of *V. neries* in culture systems has reported only during Transition and Summer months with 65% and 35% respectively. This species constituted 11.9% of the total vibrio isolates (Table 1.17).

In the percentage composition of total vibrios isolated, *V. neries* formed 10% at station I, 12.50% at station II and 13.89% at station III (Table 1.18).

Out of the 20 isolates 75% were isolated from juvenile shrimp and the rest (25%) from culture pond (Table 1.19). In juvenile shrimp, 66.67% of the isolates were reported from digestive system, 26.67% from gills and 6.67% from body surface (Table 1.20). In culture pond 80.00% of the isolates were obtained from pond water and 20.00% from sediment (Table 1.21).

Incidence of *V. neries* in digestive system was highest (70.00%) during Transition period followed by Summer (30.00%) (Table 1.24). In gill this species

TABLE 1.23. *Occurrence (%) of* Vibrio *spp. in the gill of juvenile* P. indicus *during different seasons*

	Monsoon	Postmonsoon	Transition	Summer	Total No.	%
Va	-	-	60.00	40.00	5	15.63
Vc	-	-	66.66	33.33	3	9.38
Vf	-	57.14	28.57	14.29	7	21.88
Vfb	-	20.00	20.00	60.00	5	15.63
Vn	-	-	50.00	50.00	4	12.50
Vo	-	-	33.33	66.67	3	9.38
Vpa	-	-	33.33	66.67	3	9.38
Vpr	-	-	50.00	50.00	2	6.25
Total					32	100.00

was found equally distributed (50% each) during Transition and Summer months (Table 1.23). In the body surface it was observed only during Monsoon (Table 1.22).

TABLE 1.24.*Occurrence (%) of* Vibrio *spp. in the digestive system of juvenile* P. indicus *during different seasons*

Species	Monsoon	Postmonsoon	Transition	Summer	Total No.	%
Va	-	-	36.36	63.64	22	33.85
Vc	100.00	-	-	-	1	1.54
Vf	20.00	30.00	30.00	20.00	10	15.38
Vfb	-	-	-	100.00	3	4.62
Vn	-	-	70.00	30.00	10	15.38
Vo	-	40.00	-	60.00	5	7.69
Vpa	-	-	36.36	63.64	11	16.92
Vpr	-	-	100.00	-	3	4.62
Total					65	100.00

75% of the isolates from water were reported during Transition months and the rest (25%) during Summer months (Table 1.25). In the bottom sediment the incidence of *V. neries* was observed only during Transition months (Table 1.26).

Vibrio ordalii

V. ordalii (formerly known as *V. anguillarum* biovar II) accounted for 9.52% of the total isolates. The highest occurrence of *V. ordalii* was during Transition and Summer months (37.5% each) followed by Postmonsoon season (25%). Whereas during Monsoon the incidence of *V. ordalii* could not be detected in the culture systems (Table 1.17).

TABLE 1.25. *Occurrence (%) of* Vibrio *spp. in pond water during different seasons*

	Monsoon	Postmonsoon	Transition	Summer	Total No.	%
Va	-	-	-	100.00	4	13.33
Vc	33.33	-	66.67	-	3	10.00
Vf	14.29	71.43	-	14.29	7	23.33
Vfb	-	33.33	33.33	33.33	3	10.00
Vn	-	-	75.00	25.00	4	13.33
Vo	-	66.67	33.33	-	3	10.00
Vpa	-	-	33.33	66.67	6	20.00
Vpr	-	-	-	-	-	-
Total					30	100.00

Out of the 60 isolates encountered at station I, *V. ordalii* accounted for 15.00%, but at station II and III their incidence was comparatively low (6.94% and 5.56% respectively) (Table 1.18).

Out of the total 16 isolates, 68.75% were isolated from juvenile shrimp and the rest 31.25% from culture pond (Table 1.19). In juvenile shrimp digestive system harboured 45.45% and gill and body surface formed 27.27% each (Table 1.20). In Culture pond, water accounted for 60% and bottom sediment for 40% (Table 1.21).

In shrimp digestive system, *V. ordalii* was highest incidence during Summer (60%), followed by Postmonsoon (40%). During Monsoon and Transition periods it was below detectable numbers (Table 1.24). In gill 66.67% was reported during Summer and the rest during Transition (33.33%) (Table 1.23). And from body surface

66.67% were encountered during Transition months and 33.33% during Summer (Table 1.22).

The highest incidence (66.67%) of *V. ordalii* in pond water was reported during Postmonsoon (66.67%) and the rest (33.33%) during Transition months (Table 1.25). In sediment it was isolated only during Transition months (Table 1.26).

Vibrio parahaemolyticus

One of the major shrimp pathogens *V. parahaemolyticus* formed 17.26% of the total vibrio isolates from culture system (Table 1.17).

The incidence of *V. parahaemolyticus* was reported only during Summer and Transition seasons. Out of 29 isolates of this species, 55.17% was observed during Summer and 44.83% during Transition season (Table 1.17).

At station I and II *V. parahaemolyticus* was 20% and 23.61% among the total isolated vibrios, but at station III it was not encountered at all (Table 1.18).

Juvenile shrimp harboured 65.52% of the total *V. parahaemolyticus* and the remaining 34.48% from culture pond (Table 1.19). In the juvenile shrimp 57.89% were isolated from shrimp digestive system, 26.32% from body surface and 15.79% from gill (Table 1.20). In culture pond 60% of the *V. parahaemolyticus* isolates was found from pond water and the remaining 40% from sediment (Table 1.21).

The highest incidence of *V. parahaemolyticus* in digestive system was reported during Summer season (63.64%) and during Transition months it was 36.36% (Table 1.24). Similarly in gills maximum number of *V. parahaemolyticus* isolates were encountered during Summer (66.67%) followed by Transition season (33.33%)

(Table 1.23). In the body surface also the highest occurrence was reported during Summer (60.00%) and Transition (40.00%) (Table 1.22).

In culture ponds, Summer reported the maximum occurrence (66.67%) of *V. parahaemolyticus* and Transition months showed 33.33% (Table 1.25). In the sediment their presence (100%) was observed only during Transition months (Table 1.26).

TABLE 1.26. *Occurrence (%) of* Vibrio *spp. in sediment during different seasons*

Species	Monsoon	Postmonsoon	Transition	Summer	Total No.	%
Va	-	-	16.67	83.33	6	31.57
Vc	-	-	-	-	-	-
Vf	25.00	50.00	25.00	-	4	21.05
Vfb	-	50.00	50.00	-	2	10.53
Vn	-	-	100.00	-	1	5.26
Vo	-	-	100.00	-	2	10.53
Vpa	-	-	100.00	-	4	21.05
Vpr	-	-	-	-	2	-
Total					19	100.00

Vibrio proteolyticus

V. proteolyticus was found poorer (4.17%) among the isolates from the culture system and their occurrence was highest during Transition months (71.43%). During Postmonsoon and Summer it formed 14.29% each, but during Monsoon it did not report (Table 1.17).

At station I *V. proteolyticus* formed only 1.67% of the total vibrio isolates. However, at station II and III it accounted for 5.56% each (Table 1.18).

V. proteolyticus was encountered only from juvenile shrimp and their occurrence in the culture pond, (both water and sediment) was not reported (Table 1.19).

In juvenile shrimp, digestive system harboured 42.86% and gill and body surface 28.57% each (Table 1.20). This species was encountered from the digestive system only during Transition months (Table 1.24). In gills 50% was observed during Transition months and the rest (50%) during Summer (Table 1.23). Occurrence of *V. proteolyticus* on body surface reported during Postmonsoon (50%) and Transition (50%) seasons (Table 1.22).

EFFECT OF SUDDEN RAINFALL ON THE INCIDENCE OF VIBRIOS

According to Vanderzant *et al.* (1971) the difference in microbial flora of marine environment can be attributed to the effect of environmental changes caused by rain fall. During this study an important observation was made during Transition season. It so happened that the sample collection in the month of February for bacteriological assay

TABLE 1.27. *Effect of unprecedented rain on environmental parameters at three different stations*

	Station I		Station II		StationIII	
Parameter	Before rain	After rain	Before rain	After rain	Before rain	After rain
Temperature Atmospheric(°c)	31.50	27.00	31.60	27.50	30.20	27.80
Water (°c)	31.50	29.00	33.90	26.50	31.30	28.90
Soil (°c)	30.00	27.50	32.00	28.00	31.00	28.00
pH	7.70	7.70	7.60	8.35	7.10	7.40
Salinity (ppt)	19.90	14.00	14.40	11.00	15.96	12.81
D.O ml/l	3.643	3.043	3.593	3.265	2.865	3.265
No_2 µ g at/l	1.851	1.625	1.208	1.020	1.118	0.452
No_3 "	7.392	6.750	0.821	0.535	-	-
NH_3 "	29.19	10.44	-	-	12.31	16.38
PO_4 "	2.507	4.640	2.333	3.000	3.707	8.173
Si "	18.88	46.55	1.444	3.777	50.22	30.88
Alkalinity (mg/l)	193.95	102.05	252.51	180.79	45.20	56.13
Hardness (ppm)	3455	3640	3102	3813	2472	3574

as well as for water quality parameters were conducted in the morning hours of a day which was preceded by a rainy night. The bacteriological analysis revealed that the

incidence of *Vibrio* spp. in the culture system had come down to a significantly low level and their occurrence was observed only in the digestive system of the juvenile shrimp. Zobell's agar plates showed the presence of branching and highly spreading bacterial colonies which were not observed during other samplings except for early Monsoon sampling. These bacteria were later identified as *Bacillus* spp. Few more rains had occurred during the following weeks and the samples collected during the first half of the March also exhibited low incidence of vibrio in the culture system.

TABLE 1.28. vibrio *isolates encountered before and after the sudden rain*

	Station I		Station II		Station III	
	Before rain	After rain	Before rain	After rain	Before rain	After rain
Juvenile shrimp						
Body surface	3	0	0	0	2	0
Gill	3	0	0	0	1	0
Digestive system	2	1	5	3	6	0
Culture pond						
Water	1	0	1	0	3	0
Sediment	3	0	3	0	1	0
Total	12	1	9	3	13	0

Total isolated vibrios in the sampling before rain - 34 and after rain - 4

There was a marginal fluctuation observed in environmental parameters especially with temperature salinity and alkalinity during this period (Table 1.27). In order to confirm this observation, investigations were made during Transition months in the subsequent year and samplings were made in the morning hours (6-8 hours after the rain). This could confirm the existence of a relationship between unprecedented rainfall and the incidence of the vibrios (Table 1.27, 28).

DISCUSSION

ECOLOGY OF VIBRIOS

Environment plays an important role in the incidence, abundance and distribution of any organism including bacteria particularly vibrios. During the present study, the incidence and abundance of vibrios were observed varying from station to station, season to season and between different body parts within the animal. Pond water and sediment also showed variation in the distribution of vibrios. Among the environmental parameters studied, temperature (atmospheric, water and soil), salinity, alkalinity and hardness were showed significant positive correlation with the incidence of vibrios.

Salinity showed highly significant positive statistical correlation ($r = +0.936$; $p = < 0.01$) indicating the profound role of salinity in the incidence of vibrios in the culture system.

Sodium ions stimulate the growth of all *Vibrio* spp. and are absolute requirement of most of them. They are found in aquatic habitats with a wide range of salinities (Baumann *et al.*, 1984). Colwell *et al.* (1984) in their study, mentioned the fascinating role of salinity in the incidence of vibrios in Chesapeake Bay and concluded that the majority of vibrios, both pathogenic and nonpathogenic, either require or enhance in growth by the presence of salt. Difference in the microbial flora of shrimps due to the fluctuation in salinity was reported by Vanderzant *et al.* (1971) also.

Salinity was lowest during Monsoon at all stations and a gradual increase was observed through Postmonsoon and Transition periods. The highest level was observed during Summer. In accordance with the increasing salinity, the incidence and abundance of vibrios were also increased showing a positive correlation. Vibrios

encountered during Monsoon accounting only 3.57% of the total, gradually increased during Postmonsoon season (13.69%). A still higher incidence (38.69%) was found during Transition and the highest incidence (44.05%) during Summer.

Since the vibrios in the culture systems were halophilic, their incidence in the culture system was greatly influenced by salinity. Salinity has been found to be the major environmental factor affecting the physiological mechanism of estuarine organisms as suggested by Kinne (1966).

Generally during Monsoon salinity goes down and the pond water becomes almost freshwater due to dilution by rain water and the flood water discharge of rivers into the Vembanad Lake resulting in the distortion of the existing population structure of the micro-organism. Vibrio population declined and became undetectable as Monsoon advanced. Their reappearance in the culture system accompanied with increase in the water salinity during Postmonsoon was noticeable. Since the culture media prepared in aged sea water (with a salinity of 20 ppt) has not shown the presence of any vibrio colonies during late Monsoon sampling. The possibility of bacterial dormancy in the culture system as hypothesised by Stevenson (1978) has not been observed in the present study. But the chance of survival of the bacteria in "unplatable forms" in the culture system is yet to be investigated. Probably the coastal water which retains vibrio population in low numbers during monsoon (Alavandi, 1989), enriches after the Monsoon and carries to the culture system through the tidal flow.

Alavandi (1989) reported low count of heterotrophic bacteria in the coastal waters of Cochin during Monsoon due to the discharge of flood waters from Vembanad Lake, which obviously caused reduction in salinity. He also has observed a peak in the

coastal water bacterial count during early Summer. Hood *et al.* (1984), while studying *Vibrio cholerae* in two Florida estuaries, reported the relevance of a range of salinities in the distribution of these organism.

Straub and Dixon (1993) have reported the direct correlation between the changes in salinity and diversity of bacterial flora of brine shrimp from San Francisco Bay, California. The present study also agrees with the earlier reports and the incidence of different species of vibrios, their distribution and abundance were found greatly influenced by the prevailing salinity of the shrimp culture system.

The relationship between the incidence of vibrios and total hardness of the pond water in the culture system has shown a significantly positive correlation ($r = +0.876$; $p = < 0.01$). The hardness was the lowest during Monsoon as in the case of salinity and increased during Postmonsoon and Transition periods. When the total hardness of the water was highest during Summer, a corresponding increase in the incidence and abundance of vibrios was also noticed.

No earlier reports are available regarding the correlation between total hardness of the water and the incidence of vibrios. It is obvious that the dissolved solutes in the water increase with the salinity resulting in the increase of total hardness of the water and thereby the influence of the incursion of salt water into the culture system could be demonstrated as the decisive factor for the migration, incidence, abundance and distribution of vibrios in the culture environment.

Temperature (atmospheric, water and soil) was the second important parameter in the incidence of vibrios. Highly significant positive correlation between temperature and incidence of vibrios was observed [$r = +0.706$ (atmospheric temp.), +0.689 (water temp.), +0.688 (soil temp.) $p = < 0.01$].

Alavandi (1989) related the major peak in the bacterial population of coastal waters of Cochin during early Summer to the prevailed suitable temperature of the season. Some other earlier reports also suggests the significance of temperature in the incidence, distribution and abundance of *Vibrio* spp. In their study of ecology of pathogenic vibrios in Chesapeake Bay, Colwell *et al.* (1984) reported reduced growth of vibrios at low temperature and higher growth at higher temperature. Hood *et al.* (1984) also confirmed the relevance of temperature in the distribution of *Vibrio cholerae* in Florida estuaries.

van't Hoff's rule says that increase in temperature will result in an increase in metabolism of the organism. External temperature therefore will profoundly influences all vital activities of the organism *i.e.* development, growth, reproduction, etc. (Nicol, 1967).

The higher incidence, distribution and abundance of the *Vibrio* spp. in the culture system during Transition and Summer seasons can also be related to the higher metabolic activity including multiplication (reproduction) of the vibrios due to the elevated temperature observed during those seasons along with other important environmental factors.

Alkalinity of the water showed significant correlation with the incidence of vibrios ($r = +0.379$; $p = < 0.05$) indicating that the alkalinity of the pond water plays a significant role in the occurrence, distribution and abundance of *Vibrio* spp. in the juvenile shrimp and in culture pond along with other environmental parameters.

Other environmental parameters such as pH, dissolved oxygen, nitrate, nitrite, ammonia, phosphate and silicate did not show any significant statistical relationship with the incidence of vibrios.

pH and oxygen are reported as important parameters that determines the incidence and distribution of micro-organism (Vanderzant *et al.*, 1971; Vanderzant and Nichelson, 1973). The accelerated multiplication of vibrios by the influence of alkaline pH in hatchery has also been reported by Singh *et al.* (1989). But in the present study pH, DO, nitrogen (nitrate, nitrite or ammonia), phosphate or silicate were not observed to influence the incidence and abundance of vibrios in culture system.

This does not mean that *Vibrio* spp. are capable to survive and multiply irrespective of extreme levels of these parameters. Instead, it is inferred that the range of these factors observed in the culture system could not influence the incidence, distribution or abundance of the vibrios significantly.

Christopher *et al.* (1978) could not establish any relationship between the changes in number and type of micro-organisms in pond reared shrimps or pond water and changes in water characteristics. However in view of the present investigation, it is concluded that a profound relationship between some of the prevailing environmental parameters such as temperature, salinity, alkalinity and hardness and the incidence, distribution and abundance of vibrios in the system is existed. This observation is well coincides with the earlier reports of Vanderzant *et al.* (1971), Vanderzant and Nichelson (1973), Varga and Hirtle (1975), Colwell (1984), West and Lee (1984), Singh *et al.* (1989), de la Pena *et al.* (1992), and Straub and Dixon (1993).

DISTRIBUTION OF VIBRIOS WITHIN THE STATIONS

There were also fluctuations in incidence of vibrios within the three localities. The highest incidence (72 isolates) of vibrios was reported at station II followed by station I (60 isolates), which are near to the bar mouth and the lowest incidence of vibrios (36 isolates) was observed at station III (Thripunithura) which is situated away from bar

mouth and near to freshwater river discharge and this infers that the incidence of vibrios is closely connected with the salinity and other related environmental factors of the culture system.

SEASONAL FLUCTUATION IN THE ABUNDANCE OF VIBRIOS

The low incidence of vibrios at different stations during Monsoon is related to the low levels of temperature (atmospheric, water and soil), salinity and hardness reported during this season. These observations coincide with the studies made by Alavandi (1989) who reported lowest count of heterotrophic bacterial population in the coastal waters of Cochin during rainy seasons (Monsoon) and Vanderzant *et al.* (1971) who noticed the influence of rainfall as the cause for differences in microbial flora of the Gulf Coast, Pacific and Pond reared shrimps.

The incidence of vibrios during Postmonsoon season which was observed to be higher (accounted for 13.69% of the total isolated vibrios) than that reported during Monsoon, is directly related to the increase observed in the levels of important environmental parameters such as temperature, salinity, alkalinity and hardness. The influence of these environmental parameters in the incidence and abundance of microbial population has been reported by several workers (Vanderzant *et al.*, 1971; Baumann *et al.*, 1984; Straub and Dixon, 1993; Hood *et al.*, 1984).

Vibrio spp. during Transition period formed 38.69% of the total isolates and included all the eight species. When compared to Postmonsoon season the number of vibrio isolates was nearly 3 times higher and the species diversity also doubled. At station I and III the incidence and abundance of vibrios were observed highest during this season. Alavandi (1989) reported a major peak in the count of heterotrophic

bacteria in the coastal waters off Cochin during January-February and related this to factors such as temperature, evaporation of surface water and low variation in salinity.

Out of the isolates of vibrio, Summer accounted 44.05%. All the eight species encountered during Transition season were reported during Summer also.

Highest incidence of vibrios during Summer has been reported by Colwell *et al.* (1984) and Hood *et al.* (1984) in prawns and Davis and Sizemore (1982) in Blue crab *Callinectes sapidus*. Philip and Lakshmanaperumalsamy (1995) also have reported the highest population of total heterotrophic and proteolytic bacteria (including vibrios) in *P. indicus* and *Metapenaeus dobsoni* from the coastal waters off Cochin during Summer.

The important environmental parameters such as salinity and hardness which maintained a highly significant positive correlation with the incidence of vibrios in the culture systems showed the highest values during Summer. The synergistic effect of these important parameters which prevailed during the period can be suggested as the reason for the high abundance of vibrios.

The increase in the abundance of vibrios in the culture system with the progress from Monsoon to Summer through Postmonsoon and Transition can be related to the enhancement of growth due to the corresponding increase in salinity, as suggested by Baumann *et al.* (1984) and also due to the increase in the metabolic rate with the increase in temperature (Nicol, 1967). The role of other environmental parameters especially hardness and alkalinity along with other known factors are also likely to be important in the incidence of vibrios.

Since the required levels of salinity, temperature and other factors for the optimum growth of vibrios varies from species to species, their incidence pattern, population structure and abundance over seasons also show variation.

VIBRIOS IN DIFFERENT BODY PARTS OF JUVENILE SHRIMP

Out of the 168 isolates of vibrio, 70.83% was encountered in different parts of the body (body surface, gill and digestive system) of juvenile shrimps. The shrimp body seems to provide suitable microenvironment for the highly competent vibrios to attach and multiply. As suggested by Stevenson (1978), ability of the microbes to attach to and colonize the particulate material affords them with the microenvironment higher in nutritional concentration than the surrounding water.

The lowest incidence (2.52%) of vibrios was observed during Monsoon and showed subsequent increase through Postmonsoon (10.08%) and Transition (38.66%). The highest incidence was reported during Summer (48.78%). This pattern of vibrios over different season in the juvenile shrimps can be related to the environmental parameters particularly salinity and temperature of the culture system as discussed earlier.

Vibrios are the predominant bacteria in the natural microbial flora of the penaeid shrimps and several workers have demonstrated the association of *Vibrio* spp. with penaeids (Vanderzant *et al.*, 1971; Lightner, 1975; Christopher *et al.*, 1978; Singh, 1986; Singh *et al.*, 1989; Hameed, 1993; Philip and Lakshmanaperumalsamy, 1995).

Incidence of vibrios in the body surface of marine shrimps was reported by Bose and Chandrasekharan (1976) and similarly the occurrence of vibrios in the body surface of other crustaceans such as copepods, also has been demonstrated (Sochard *et al.*,

1979). In the present investigation on juvenile shrimps also, incidence (18.49%) of vibrios was observed from the body surface of the shrimps. Among the body parts the lowest incidence of vibrios was reported in the body surface and this observation well coincides with the report of Philip and Lakshmanaperumalsamy (1995) in *P. indicus* and *M. dobsoni* from coastal waters off Cochin. Vibrios are chitinoclastic which find the exoskeleton of shrimp as a suitable environment for their attachment and growth. In case of rupture or damage of the exoskeleton the bacteria may cause invasion and multiplication resulting in brown spot on the body surface or other shell diseases. Infection through damaged cuticle which result in 'vibriosis' was suggested by Anderson *et al.* (1988) and Nash (1990).

Gill of juvenile shrimps also was observed harbouring vibrios. The incidence of vibrios in gill was higher than that of body surface. This observation also coincides with the earlier report of Philip and Lakshmanaperumalsamy (1995). Among the isolates of vibrios from juvenile shrimps, 26.89% was encountered in gill. Accumulation of organic matter in the gill may possibly provide a suitable environment for the attachment and colonisation of these organisms. The possibility of these bacteria to serve as pathogens as a result of their entry through the gill during adverse environmental conditions can not be ruled out as suggested by Nash (1990). The abundance of vibrios in gill which formed 30% of the total gill flora has been reported in Blue crab *Callinectes sapidus* by Davis and Sizemore (1982). The fluctuation in the seasonal abundance of vibrio in gill observed during different season can be related to the changes in the culture pond ecology over the seasons.

Among the body parts assayed, digestive system of the shrimp was observed to harbour highest number of vibrios (54.62%). The digestive system of juvenile shrimp

along with the food appears to provide a very suitable nutrient rich environment for higher incidence and abundance of vibrios.

Chitinolytic bacteria dominated with *Vibrio* have been reported from digestive system (including hepatopancreas, stomach and intestine) of *P. indicus* by Ninawe *et al.* (1987) and Singh *et al.* (1991). Similar observations in other penaeids were also reported (Yasuda and Kitao, 1980; Dempsey and Kitting, 1987; Dempsey *et al.*, 1989). Since *Vibrio* spp. are capable of producing other hydrolytic enzymes, their high incidence in the digestive system of penaeid shrimp juveniles suggest the profound role in the process of digestion. Report of earlier workers also justifies this observation (Dempsey and Kitting, 1987; Dempsey *et al.*, 1989; Singh *et al.*, 1989, 1991). Philip and Lakshmanaperumalsamy (1995), in their study on occurrence of total heterotrophic and proteolytic bacteria in prawn from coastal waters off Cochin, have reported vibrios as the predominant organism in the intestine of *P. indicus* and *M. dobsoni*.

Seasonal fluctuation was observed in the incidence and abundance of vibrios in the digestive system. Summer was found ideal period for its growth and multiplication followed by Transition. But during Monsoon their incidence was the lowest. As discussed earlier the favourable environmental parameters prevailed during Summer and Transition could be suggested as the reason for their higher abundance.

Cruz (1991) reported that the rate of feeding of shrimp was governed by the temperature and maximal feeding was observed during warm Summer months. Since the profound role of vibrios in digestion has been suggested by several workers, the high rate of feeding and increased metabolic rate can also be related to the higher abundance of vibrios in the gut during the Summer and a hypothesis has been propounded as follows: "The increase in feeding, metabolic activities and growth in juvenile shrimps

during warmer months (Transition and Summer) are due to the abundance of vibrios in the digestive system which have a profound role in the process of digestion".

INCIDENCE OF VIBRIOS IN THE CULTURE POND

Culture pond (water and sediment) accounted for 29.17% vibrios with a higher incidence of vibrios during Transition and Summer than in Monsoon and Postmonsoon.

Vibrios were found to be more in water (61.22%) than in sediment (38.78%). The water temperature was invariably higher than the sediment temperature at all the three stations over the four seasons. Since temperature is an important environmental factor in the occurrence and abundance of vibrios and as has been reported by some workers, their higher abundance in water (than in sediment) is found to be influenced significantly by the higher temperature. Since the increase in temperature results in increased metabolism as per van't Hoff's rule, the vibrios which are motile will possibly prefer the water than sediment for its growth and multiplication and this may most probably a reason for their higher abundance in water. In their study on the bacterial flora of certain marine fishes and prawns in Cochin waters in relation to their environs, Karthiani and Iyer (1975) reported absence of vibrios in the bottom mud.

The fluctuation in the seasonal abundance of vibrios in water was similar to their abundance in body parts during different seasons. Highest number of isolates were encountered during Summer followed by Transition and Postmonsoon and the lowest during Monsoon season. This pattern of fluctuation can be related to the changes in the pond ecology during different seasons.

In the case of sediment, a difference in the abundance of vibrios during Transition and Summer season was observed. Transition season was reported with highest number

of isolates followed by Summer. No reason for this observation could be explained on the basis of the environmental parameters noted during the study. The role of certain unidentified factors other than temperature, zooplankton and nutrients was reported by Kaneko and Colwell (1973) in the abundance of *V. parahaemolyticus*.Similarly the highest number of vibrios during Transition season might be related to some other factors.

SPECIES DIVERSITY OF VIBRIOS IN THE CULTURE SYSTEM

Wide distribution of the pathogenic vibrios in the shrimp culture system was observed and reported by de la Pena *et al.* (1992). Among the species *V. anguillarum* was the most abundant encountered in the culture systems of *P. indicus* followed by *V. fischeri* like bacteria and *V. parahaemolyticus*. Among the three species *V. anguillarum* and *V. parahaemolyticus* were found as major pathogenic vibrios by several workers and *V. fischeri* also has been reported as a shrimp pathogen by Pillai (1984). But since the group of isolates that showed close resemblance to *V. fischeri* except for the luminescence are specified as *V. fischeri* like bacteria.

Other species of vibrios are *V. ordalii, V. cholerae* non-O1, *V. fluvialis* biovar II, *V. neries* and *V. proteolyticus* among which Lightner (1993) has reported *V. fluvialis* as a disease causing organism in penaeid shrimps. Eventhough species other than *V. anguillarum*, *V. parahaemolyticus*, *V. fischeri* like bacteria and *V. fluvialis* are not reported as shrimp pathogens , the chances of an opportunistic infection can not be ruled out. Since vibrios are considered as opportunistic pathogens, an adverse environment that causes stress to the shrimps and at the same time conducive for vibrios for infection can cause disease.

All the isolated species including pathogenic vibrios showed higher abundance in body parts than in the culture pond. More than 60% of all species were encountered from juvenile shrimps and *V. proteolyticus* was reported only from juvenile shrimps. This indicates that most suitable microenvironment is provided by the host for the attachment, growth and multiplication of vibrios and among the body parts analysed digestive system was found to harbour more vibrios.

The abundance of vibrios in the digestive system and other body parts does not cause any abnormality or disease in shrimps when the shrimps are healthy. Instead it helps in the process of digestion as suggested by Dempsey and Kitting (1987), Dempsey *et al.* (1989) and Sigh *et al.* (1989, 1991). The shrimp's defense mechanism is capable of preventing the possible infections. But prolonged physiological stress due to an unfavourable environmental condition or any other diseases make shrimps susceptible to vibrio infection.

Both the major shrimp pathogens *V. anguillarum* and *V. parahaemolyticus* were encountered in highest number during Summer season (followed by Transition) where the water temperature was highest. So the chances of vibrio infections in shrimp in culture system are high during Summer and Transition months. Higher incidence of vibrios in the environment and "vibriosis" in the shrimp farm coincides with increase in water temperature and it has been reported by Sano *et al.* (1987) and de la Pena *et al.* (1992, 1993).

Since the shrimp culture system selected for the sampling in the present study were semi-intensive type, with proper continuous management practices, the incidence of disease was not reported as a problem. But, as pathogenic vibrios are already distributed widely in these culture systems, intensification of culture practice with

increased stocking rate (which result in environmental degradation and stress on the shrimps) will definitely cause 'vibriosis'.

Avoidance of these pathogens from the culture system to eliminate the chances of disease out-breaks is practically difficult. Especially in semi-intensive culture system, where incoming water is not chlorinated or treated before flowing into the farms, vibrios remain as a part of natural microbial flora. Even in the case of water treatment (by chlorination or by any other method) the total and absolute removal of the bacteria is not practicable and the low number of vibrios entering the system will multiply in the digestive system and other body parts of the shrimp as discussed earlier and also the faecal pellets will add up its population in the pond water and sediment.

EFFECT OF UNPRECEDENTED RAINFALL ON VIBRIOS IN THE CULTURE SYSTEM

The interesting phenomena observed in the vibrio population in shrimp culture system by the effect of sudden rain during Transition season can not be related alone to the marginal fluctuation observed in the the environmental parameters in culture system. Reduction in environmental parameters such as temperature and salinity was observed due to the rain at all the three stations and the hardness of the water was found increased. Other parameters also showed minor fluctuations. But, these levels were not detrimental to the *Vibrio* spp., as similar levels were observed in the culture system during other periods. But the vibrio showed tremendous decline in their population soon after the rain during Transition period. No vibrio was isolated from culture pond (water and sediment) or from body parts, but they were observed in the digestive system. This again indicates that the gut is providing an excellent micro-environment

for the growth and multiplication of vibrios even in adverse environmental conditions. But if such conditions continue, they may not be able to survive long.

Bacillus sp. generally found in the dust, encountered immediately after the unprecedented and sudden rain, was an interesting observation during the present investigation. The probable reason for the occurrence of *Bacillus* sp. is to be investigated. The transport of this alien microbe which does not form part of the natural flora of the culture system (juvenile shrimp, pond water or sediment) might have entered possibly through the rain water into the system. Since this bacteria were reported also during early Monsoon, their occurrence in the culture system is closely related to the rainfall. Though the antagonistic effect of these bacteria on vibrio is not fully known, the circumstantial evidence indicates a probable inhibition of vibrios by the extra-metabolites produced by them.

The separate or combined effect of environmental changes in the system and the presence of *Bacillus* sp. might possibly be the causative factors for this unusual phenomenon.

CHAPTER II

Chapter II

CHARACTERS OF VIBRIOS ISOLATED

Identification of the vibrios isolated (Table 2.1) were carried out according to West and Colwell (1984). *Vibrio anguillarum* biovar II is renamed as *V. ordalii* following Schiewe *et al.* (1981) and the group of bacterial isolates which showed close resemblance to *V. fischeri* except for the character luminescence is specified as *V. fischeri* like bacteria. *V. cholerae* isolates which did not agglutinate in *V. cholerae* O group 1 antiserum are referred to as non-O1 (Colwell, 1984).

OPTIMUM REQUIREMENT FOR GROWTH

Extent of growth of isolates of vibrios in varying temperature, salinity and pH were examined, according to the procedures described under the section "Material and methods".

V. anguillarum

Temperature

V. anguillarum did not exhibit growth below 15°C, but reported high growth above 20°C. The optimum temperature was 30°C and beyond 35°C the growth was below detectable level (Table 2.2, Fig. 2.1).

Salinity

V. anguillarum exhibited growth over a wide range of salinity ranging from 0 to 50 ppt. Higher growth was observed between 20 to 30 ppt with the peak at 20 ppt (Table 2.3, Fig. 2.1).

TABLE 2.1. *Characters of* Vibrio *spp. isolated*

Test	*Va*	*Vc*	*Vf*	*Vfb*	*Vn*	*Vo*	*Vpa*	*Vpr* *
Cytochrome oxidase	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	-	+	+
0/129 sensitivity 10 µg	+	+	+	-	-	+	-	-
150 µg	+	+	+	+	+	+	+	+
Swarming	-	-	-	-	-	-	v	-
Luminescence	-	-	-	-	-	-	-	-
Thornley's Argenine dihydrolase	+	-	-	+	+	-	-	+
Lysine decarboxylase	-	+	+	-	-	-	+	+
Ornithine decarboxylase	-	+	-	-	-	-	+	-
Growth at 42°C	-	+	-	-	-	-	+	-
Growth at % NaCl 0 %	+	+	-	+	-	-	-	+
3 %	+	+	+	+	+	+	+	+
6 %	+	-	+	+	+	+	+	+
8 %	-	-	+	-	-	-	+	+
10 %	-	-	-	-	-	-	-	-
Voges-Proskauer reaction	+	+	-	-	-	-	-	+
Gas from glucose fermentation	-	-	-	+	-	-	-	-
Fermentation to acid								
L-arabinose	-	-	-	+	-	-	+	-
m-inositol	+	-	-	-	-	-	-	-
D-mannose	+	v	+	+	-	-	+	+
Sucrose	+	+	-	+	+	+	-	-
Enzyme production								
Alginase	-	-	-	-	-	-	-	-
Amylase	+	+	-	v	-	-	+	+
Chitinase	+	+	-	+	+	v	+	+
Gelatinase	+	+	-	+	+	+	+	+
Lipase	+	+	+	+	-	-	+	+
Utilization as sole source of carbon								
r-aminobuterate	-	-	-	+	+	-	-	-
Cellobiose	+	-	+	-	-	-	-	-
L-citruline	-	-	-	-	+	-	-	-
Ethanol	-	-	-	+	+	-	+	-
D-gluconate	+	+	-	+	+	-	+	+
D-glucuronate	-	-	-	-	-	-	-	-
L-leucine	-	-	-	-	+	-	+	-
Putrescine	-	-	-	+	+	-	+	+
Sucrose	+	+	-	+	+	+	-	-
D-xylose	-	-	-	-	-	-	-	-

**Va* - *V. anguillarum*, *Vn* - *V. neries*, *Vc* - *V. cholerae* non-O1 *Vo* - *V. ordalii*, *Vf* - *V. fischeri* like bacteria, *Vpa* - *V. parahaemolyticus*, *Vfb* - *V. fluvialis* biovar II, *Vpr* - *V. proteolyticus*.

+ : positive trait for atleast 90% of strains;

- : negative trait for atleast 90% of strains, and

v : differs for strains within the species.

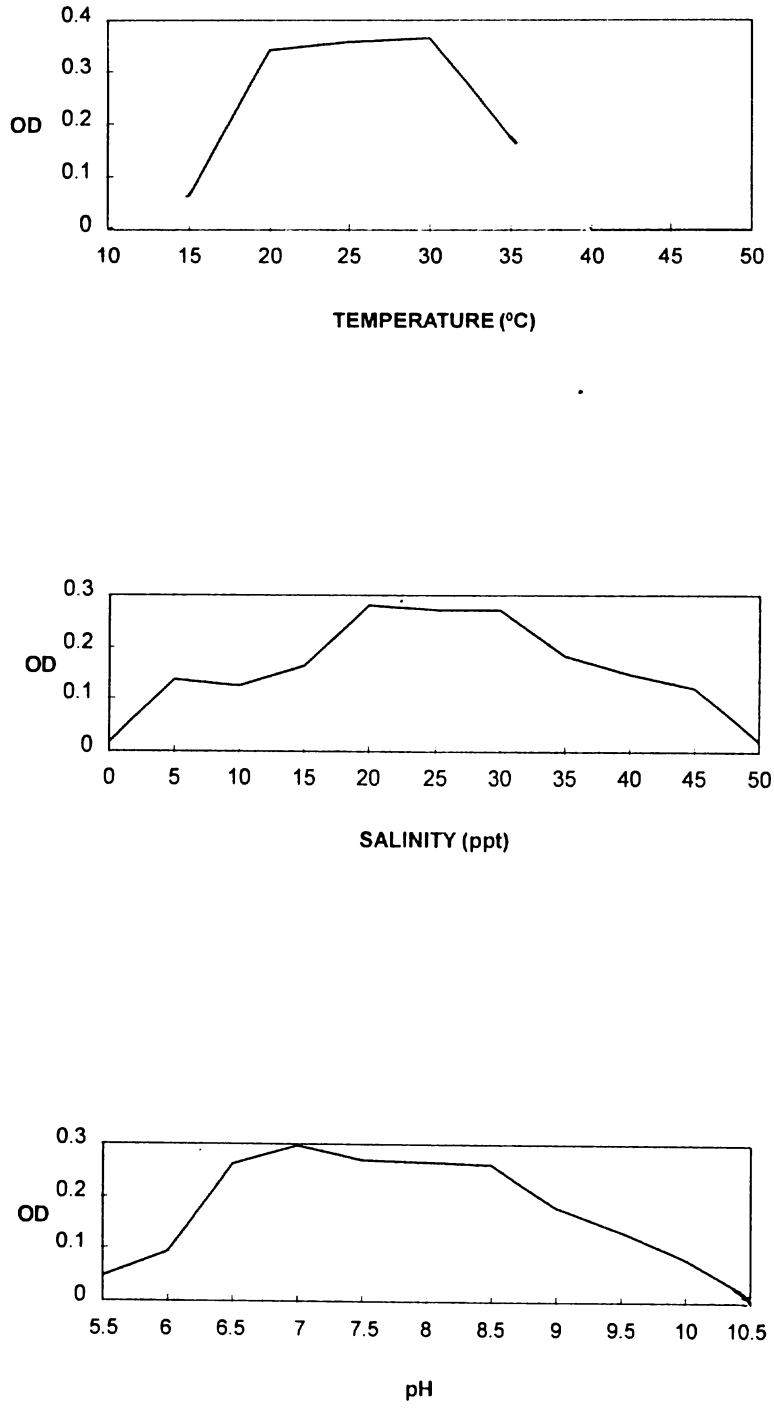


Fig. 2.1. Growth pattern of *V. anguillarum*.

pH

pH range between 6.5 and 8.5 was found favourable for the growth of *V. anguillarum* and the maximum growth was observed at pH 7.0. Below pH 5.5 and above 10.5 the growth was not noticed (Table 2.4, Fig.2.1).

TABLE 2.2. *Growth of isolated vibrios at different temperatures (None of the isolates showed growth below 15°C)*

Species		15°C	20°C	25°C	30°C	35°C	40°C	45°C	50°C
	OD	0.063	0.341	0.358	0.366	0.174	0.000	0.000	0.000
V. anguillarum	SD ±	0.022	0.012	0.031	0.012	0.021			
	OD	0.042	0.168	0.257	0.233	0.161	0.121	0.019	0.010
V. cholerae non-O1	SD ±	0.002	0.012	0.015	0.011	0.009	0.015	0.004	0.001
	OD	0.062	0.107	0.113	0.145	0.095	0.010	0.000	0.000
V. fischeri like bacteria	SD ±	0.005	0.008	0.011	0.010	0.008	0.002		
	OD	0.024	0.113	0.136	0.147	0.090	0.038	0.000	0.000
V. fluvialis biovar II	SD ±	0.002	0.012	0.015	0.014	0.008	0.002		
	OD	0.064	0.271	0.257	0.287	0.155	0.023	0.000	0.000
V. neries	SD ±	0.005	0.020	0.022	0.025	0.014	0.002		
	OD	0.056	0.179	0.327	0.335	0.251	0.012	0.000	0.000
V. ordalii	SD ±	0.004	0.009	0.028	0.035	0.024	0.002		
	OD	0.059	0.293	0.296	0.304	0.181	0.058	0.010	0.000
V. parahaemolyticus	SD ±	0.006	0.031	0.032	0.035	0.009	0.005	0.002	
	OD	0.014	0.059	0.060	0.038	0.026	0.015	0.000	0.000
V. proteolyticus	SD ±	0.002	0.006	0.007	0.005	0.003	0.002		

* OD - Optical density, SD - Standard deviation.

V. cholerae non-O1

Temperature

The minimum temperature required for detectable growth was 15°C and growths observed even at 50°C. A higher growth was observed over 25° to 30°C with a peak at 25° C (Table 2.2, Fig. 2.2).

TABLE 2.3. *Growth of isolated vibrios at different salinities*

Species		0ppt	5ppt	10ppt	15ppt	20ppt	25ppt	30ppt	35ppt	40ppt	45ppt	50ppt
	OD	0.014	0.138	0.124	0.163	0.281	0.271	0.271	0.182	0.150	0.120	0.020
V. anguillarum	SD±	0.002	0.008	0.015	0.017	0.025	0.024	0.025	0.016	0.012	0.008	0.002
	OD	0.000	0.178	0.193	0.294	0.290	0.246	0.200	0.101	0.100	0.050	0.000
V. Cholerae non-01	SD±		0.015	0.020	0.025	0.031	0.022	0.018	0.009	0.008	0.002	
	OD	0.000	0.122	0.123	0.152	0.099	0.099	0.105	0.069	0.065	0.052	0.040
V. fischeri like bacteria	SD±		0.010	0.008	0.012	0.006	0.007	0.009	0.004	0.005	0.004	0.002
	OD	0.022	0.032	0.036	0.112	0.138	0.165	0.174	0.164	0.155	0.102	0.060
V. fluvialis biovar II	SD±	0.003	0.003	0.002	0.009	0.012	0.017	0.019	0.018	0.014	0.009	0.003
	OD	0.000	0.114	0.109	0.158	0.217	0.202	0.177	0.133	0.097	0.080	0.010
V. neries	SD±		0.012	0.013	0.016	0.019	0.018	0.010	0.012	0.005	0.005	0.002
	OD	0.000	0.115	0.122	0.195	0.239	0.280	0.212	0.179	0.103	0.082	0.010
V. ordalii	SD±		0.009	0.010	0.020	0.021	0.030	0.019	0.017	0.008	0.005	0.002
	OD	0.000	0.152	0.144	0.168	0.265	0.344	0.492	0.362	0.210	0.192	0.180
V. parahaemolyticus	SD±		0.008	0.010	0.014	0.021	0.032	0.038	0.029	0.023	0.021	0.012
	OD	0.060	0.070	0.058	0.058	0.052	0.057	0.041	0.024	0.022	0.025	0.020
V. proteolyticus	SD±	0.003	0.002	0.004	0.006	0.004	0.005	0.003	0.003	0.002	0.003	0.003

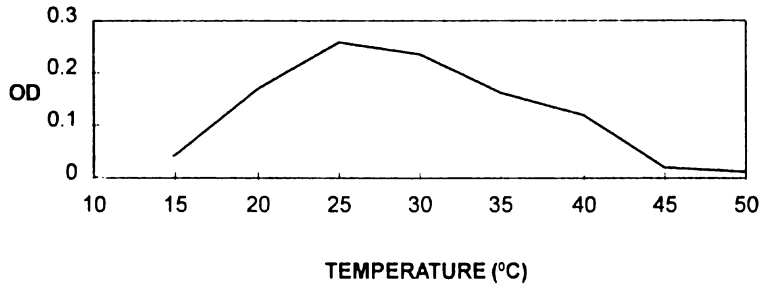
*OD - Optical dencity, SD - Standard deviation.

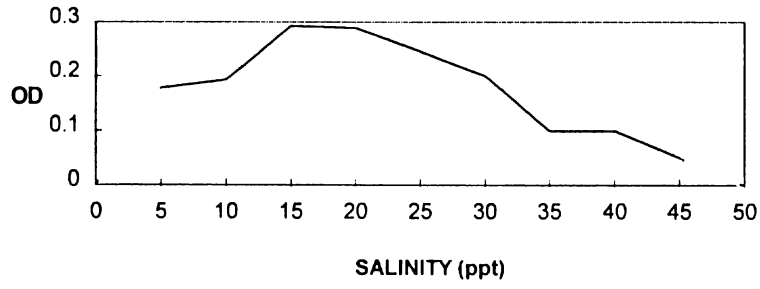
TABLE 2.4. *Growth of isolated vibriosat different pH*

(None of the isolates showed growth below pH 5.5 and above 10.5)

Species		5.5	6	6.5	7	7.5	8	8.5	9	9.5	10	10.5
	OD	0.046	0.093	0.260	0.298	0.269	0.267	0.260	0.182	0.133	0.082	0.010
V. anguillarum	SD ±	0.003	0.005	0.019	0.022	0.024	0.023	0.022	0.015	0.015	0.009	0.002
	OD	0.033	0.064	0.081	0.102	0.223	0.252	0.206	0.101	0.056	0.000	0.000
V. cholerae non - 01	SD ±	0.002	0.005	0.009	0.009	0.018	0.021	0.019	0.008	0.006		
	OD	0.027	0.056	0.068	0.071	0.095	0.119	0.098	0.013	0.000	0.000	0.000
V. fischeri like bacteria	SD ±	0.003	0.004	0.007	0.007	0.010	0.012	0.008	0.002			
	OD	0.000	0.018	0.088	0.165	0.199	0.236	0.171	0.075	0.043	0.000	0.000
V. fluvialis biovar II	SD ±		0.002	0.009	0.018	0.020	0.022	0.019	0.010	0.002		
	OD	0.000	0.012	0.130	0.160	0.204	0.181	0.153	0.062	0.042	0.022	0.000
V. neries	SD ±		0.002	0.011	0.013	0.019	0.016	0.015	0.004	0.003	0.002	
	OD	0.000	0.000	0.013	0.025	0.160	0.300	0.266	0.158	0.141	0.115	0.024
V. ordalii	SD ±			0.002	0.001	0.020	0.027	0.025	0.014	0.013	0.012	0.002
	OD	0.047	0.104	0.149	0.210	0.251	0.282	0.241	0.125	0.054	0.000	0.000
V. parahaemolyticus	SD ±	0.003	0.010	0.013	0.021	0.026	0.029	0.025	0.013	0.002		
	OD	0.000	0.035	0.046	0.065	0.062	0.057	0.054	0.000	0.000	0.000	0.000
V. proteo lyticus	SD ±		0.003	0.005	0.007	0.005	0.005	0.002				

* OD-Optical density, SD-Standard deviation.





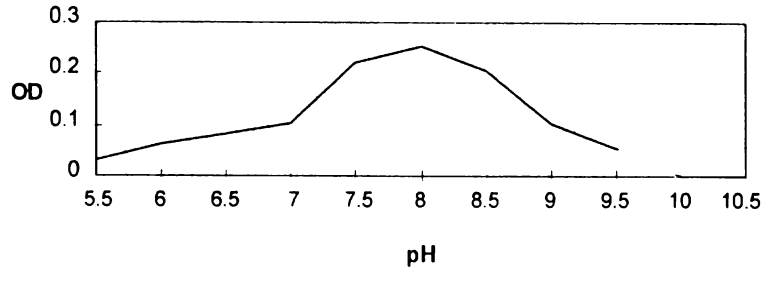


Fig. 2.2. Growth pattern of *V. cholerae non-01.*

Salinity

A wide range (5-45 ppt) of salinity was favouring the growth of *V. cholerae* non-O1 and a higher growth level was observed between 15 and 25 ppt salinity with a peak at 15 ppt (Table 2.3, Fig. 2.2).

pH

The growth of *V. cholerae* non-O1 isolates was observed good between pH 5.5 and 9.5 and a more favourable and better growth was reported between pH 7.5 and 8.5 with peak at pH 8.0 (Table 2.4, Fig. 2.2).

V. fischeri like bacteria

Temperature

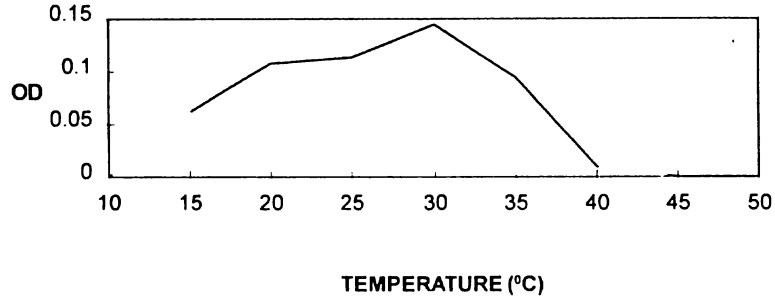
The minimum temperature required for the detectable growth of *V. fischeri* like bacteria was observed 15°C. Higher growth was observed between 20° and 30°C with the maximum growth at 30°C. Above 40°C the growth was undetectable (Table 2.2, Fig. 2.3).

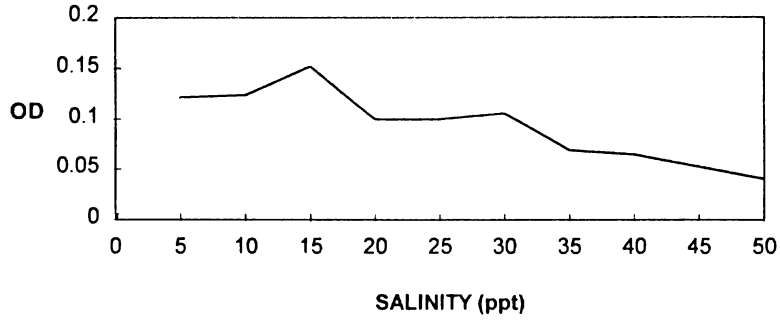
Salinity

5 ppt was observed to be the minimum salinity for recordable growth and the growth was observed even at a higher level of 50 ppt. Maximum growth was observed at 15 ppt and the range that registered comparatively higher growth was between 5-15 ppt (Table 2.3, Fig. 2.3).

pH

V. fischeri like bacteria was flourishing in the pH between 7.5 and 8.5 with a maximum at pH 8.0. The lower and higher levels of pH for detectable growth were 5.5 and 9.0 respectively (Table 2.4, Fig. 2.3).





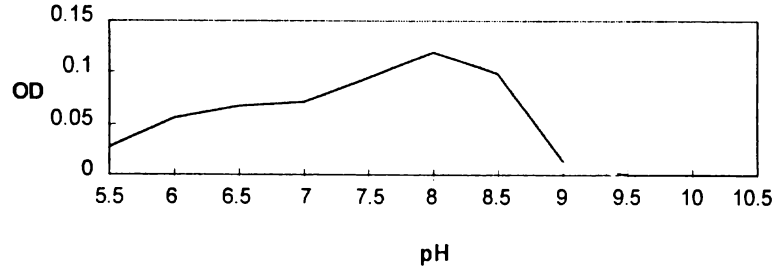


Fig. 2.3. Growth pattern of *V. fischeri* like bacteria.

V. fluvialis biovar II

Temperature

V. fluvialis biovar II showed a high growth level at 20°, 25° and 30°C, with the maximum growth at 30°C. The experiment revealed that the minimum temperature required for detectable growth as 15°C and a maximum 40°C (Table 2.2, Fig. 2.4).

Salinity

V. fluvialis biovar II showed a wide range of salinity tolerance. The growth was observed from O ppt to 50 ppt. However, the growth was recorded high, in the range of 25-35ppt with a peak at 30 ppt (Table 2.3, Fig. 2.4).

pH

The survival of this species was observed between pH 6.0 and 9.5 and the higher level of growth was reported between 7.0-8.5. The maximum growth was observed at pH 8.0 (Table 2.4, Fig. 2.4).

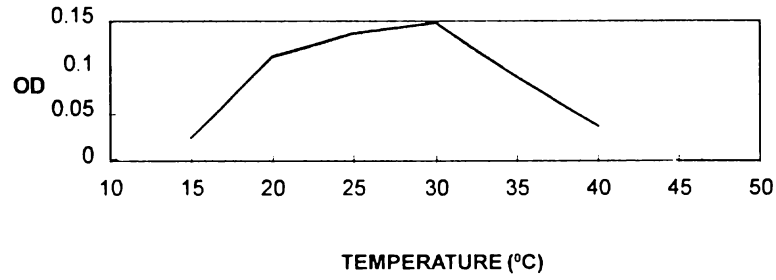
V. neries

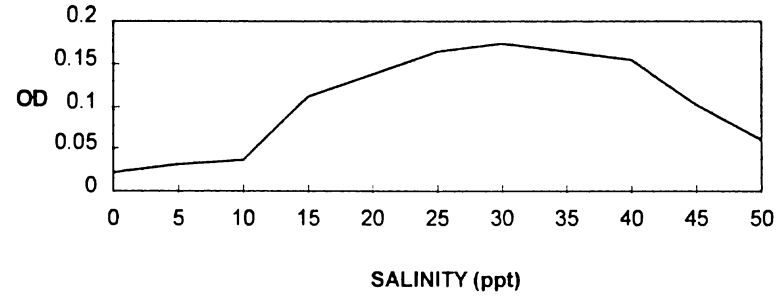
Temperature

30°C has given the maximum growth and the range of temperature where higher level of growth registered was between 20 and 30°C. The upper and lower limits for the recordable growth was 40°C and 15° respectively (Table 2.2, Fig.2.5).

Salinity

V. neries has grown in a a minimum salinity level of 5 ppt and in the range of 15-30 ppt in a higher growth level with a peak at 20 ppt. The survival of this species was detected even at 50 ppt (Table 2.3, Fig. 2.5).





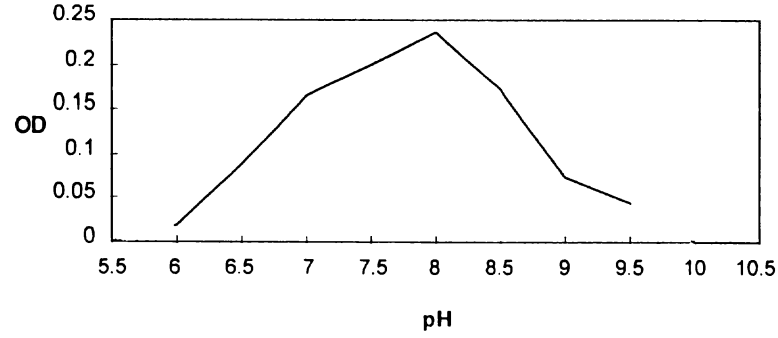


Fig. 2.4. Growth pattern of *V. fuvialis* biovar II.

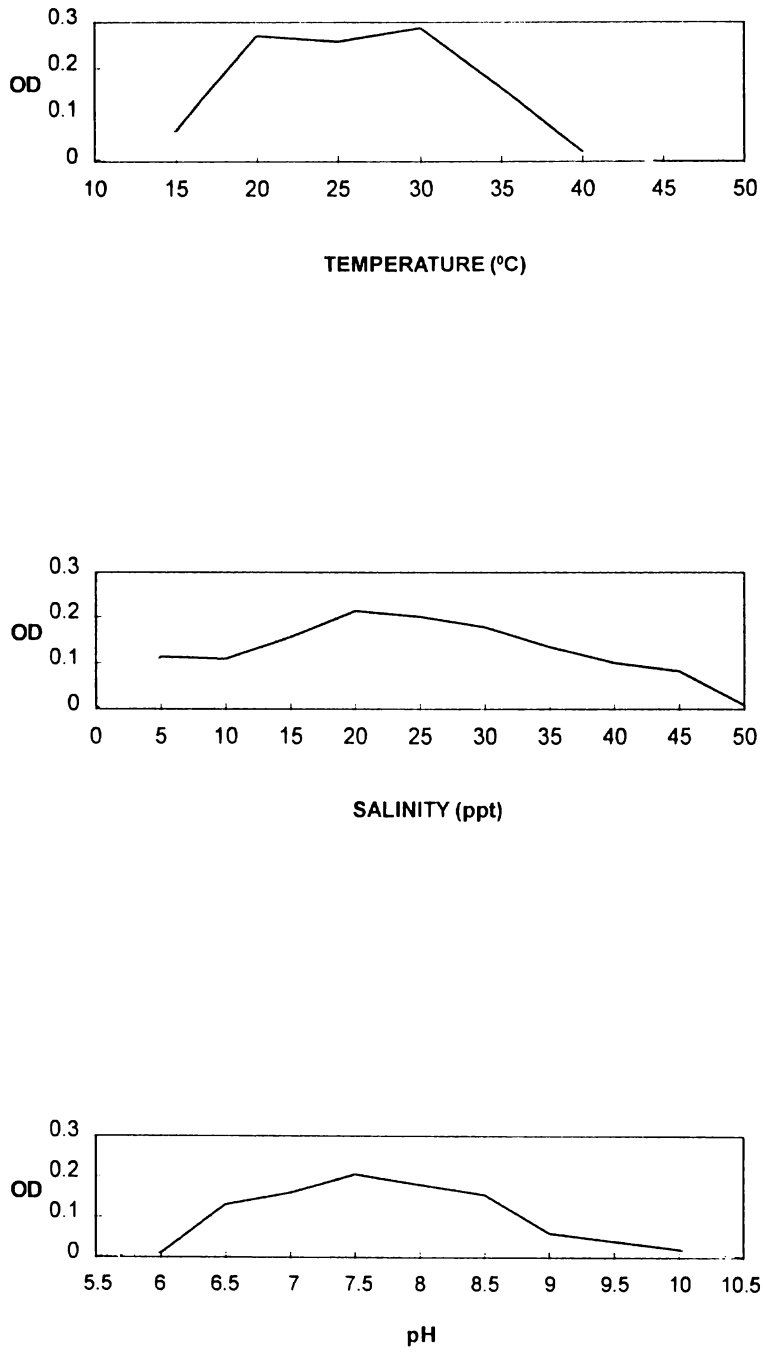


Fig. 2.5. Growth pattern of *V. neries.*

pH

The ideal pH for the maximum growth and multiplication of *V. neries* was observed at 7.5. The favourable pH range for growth was 6.0-10.0 with a higher growth between 7.5 and 8.0 (Table 2.4).

V. ordalii

Temperature

Growth of *V. ordalii* was not detectable upto 10°C. Over a range of 15° to 40°C they exhibited higher growth between 25° to 35°C and the maximum was observed at 30°C (Table 2.2, Fig. 2.6).

Salinity

Growth was observed between the range 5 ppt and 50 ppt, and the maximum growth was found at 25 ppt. Below 5 ppt growth was not detectable (Table 2.3,Fig.2.6).

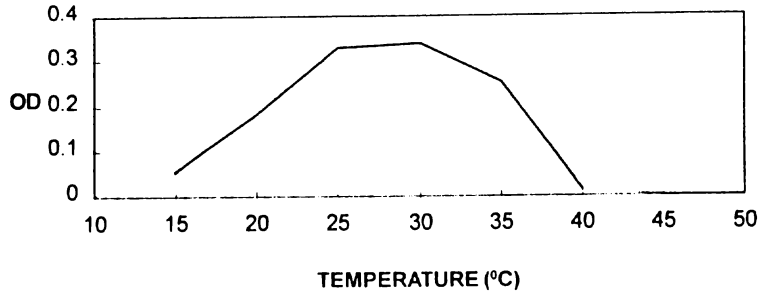
pH

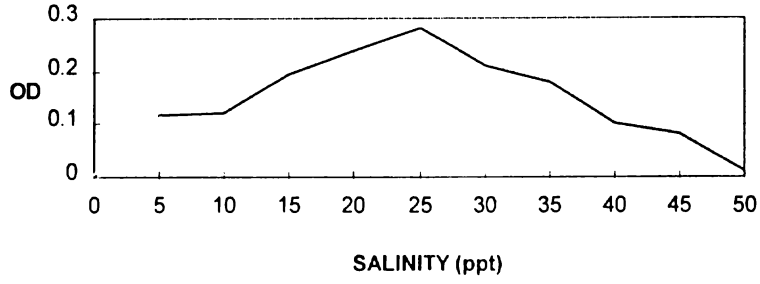
The minimum pH required for detectable growth of *V. ordalii* was 6.5 and upper limit observed was pH 10.5. Maximum growth was observed at pH 8.0 (Table 2.4, Fig. 2.6).

V. parahaemolyticus

Temperature

The temperature range within which the survival of *V. parahaemolyticus* was found between 15° and 45°C. However a higher growth was observed between 20°-30°C with a peak at 30°C (Table 2.2, Fig. 2.7).





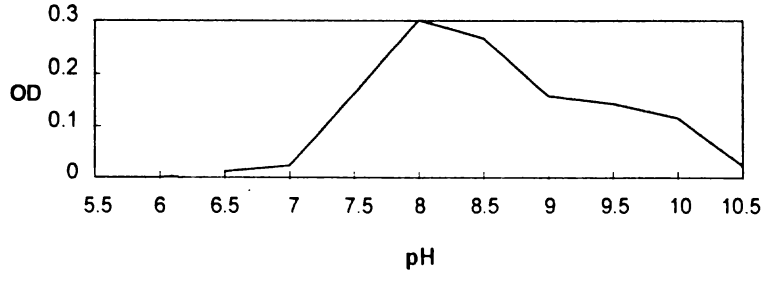
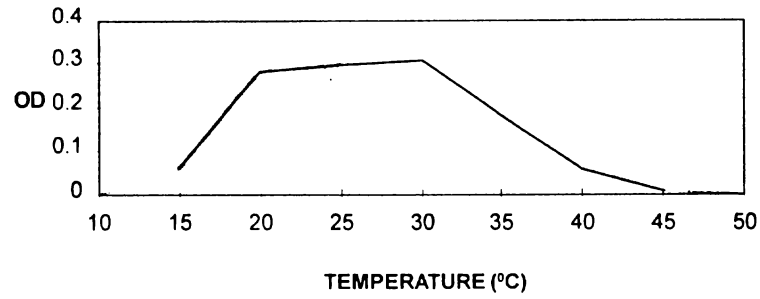
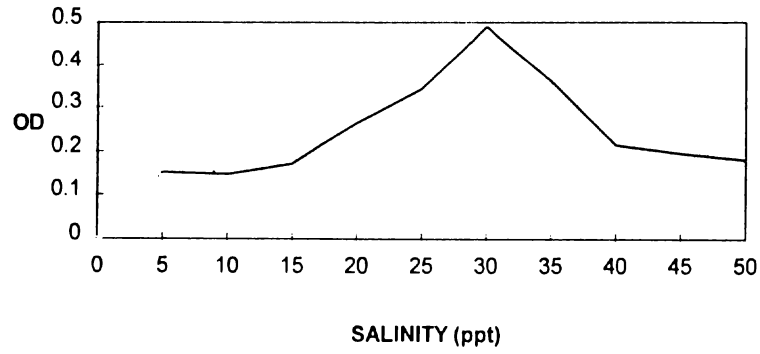


Fig. 2.6. Growth pattern of *V. ordalii.*





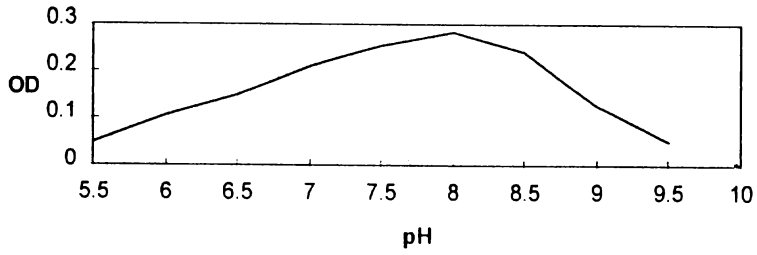


Fig. 2.7. Growth pattern of *V. parahaemolyticus.*

Salinity

The minimum salinity required for recordable growth of *V. parahaemolyticus* was 5 ppt and it showed survival even at 50 ppt. A higher growth was observed between 20 and 35 ppt with a peak at 30 ppt (Table 2.3, Fig. 2.7).

pH

Growth was observed within the pH 5.5 to 9.5. Significantly higher growth was recorded at a pH 7.0, 7.5 and 8.5 with the highest peak at pH 8.0 (Table 2.4).

V. proteolyticus

Temperature

The temperature range which favoured the survival of *V. proteolyticus* was observed between 15° and 40°C. However, a better growth was recorded within 20°-30°C with the highest at 25°C (Table 2.2, Fig. 2.8).

Salinity

This species was found tolerating a wider salinity range between 0 ppt and 50 ppt. Unlike other species, *V. proteolyticus* reported maximum growth at a salinity of 5 ppt and beyond which it showed a very slow decline in growth upto 50 ppt (Table 2.3Fig. 2.8).

pH

A pH range of 6.0-8.0 was observed favouring the growth of *V. proteolyticus*. Higher growth was observed between pH 6.5 and 8.0 and the maximum growth was recorded at pH 7.

In general, the isolates of different species of vibrios showed a temperature range (15° to 40°C) for detectable growth and higher growth was observed at 20°, 25° and 30°C. Species other than *V. cholerae* non-O1 and *V. parahaemolyticus* could not register growth

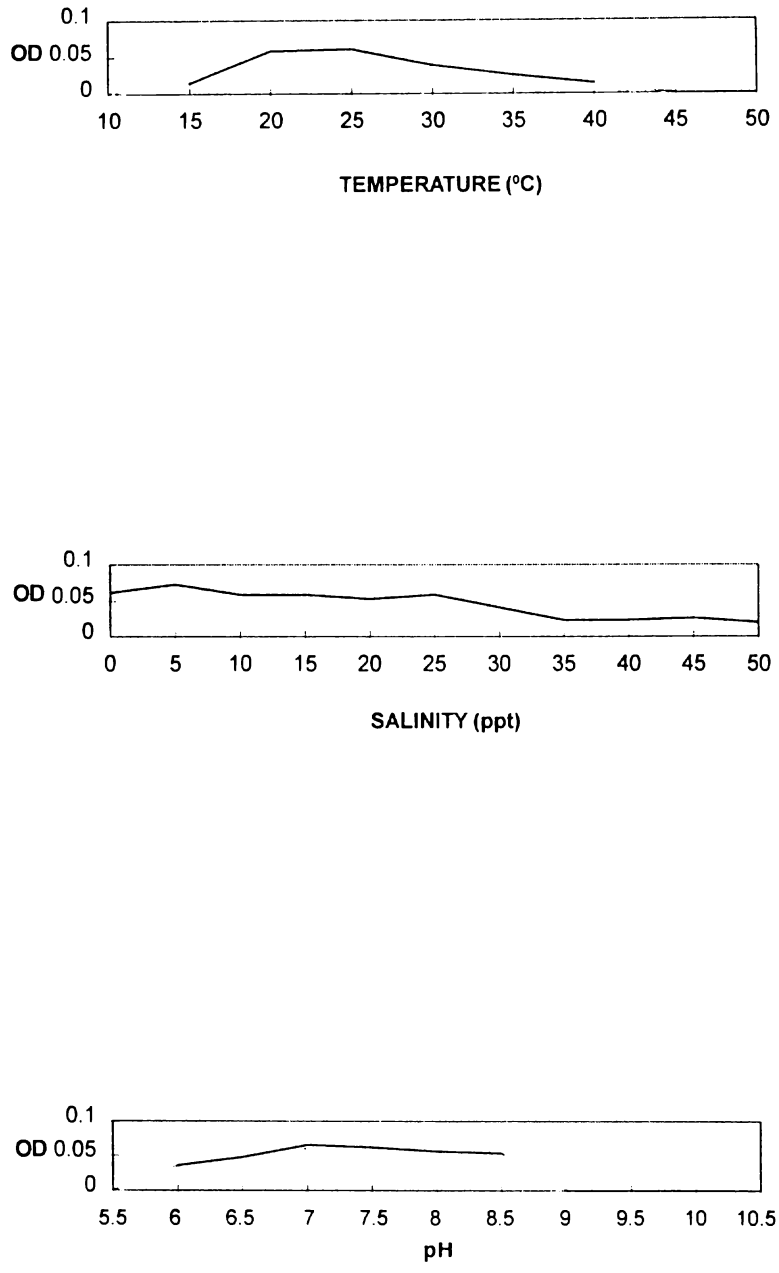


Fig. 2.8. Growth pattern of *V. proteolyticus*.

at 42°C. At low temperature (< 20°C) the growth was found to decline and became undetectable at 10°C for all species. Isolates of *Vibrio* spp. such as *V. anguillarum*, *V. fischeri* like bacteria, *V. fluvialis* biovar II, *V. ordalii* and *V. parahaemolyticus* recorded highest growth at 30°C, whereas for the isolates of other species (*V. Cholerae* non-O1 and *V. proteolyticus*) 25°C was the optimum.

Growth response of vibrios to different levels of salinity were observed differing from species to species. For *V. anguillarum* the high growth salinity range observed was 20-30 ppt with an optimum level at 20 ppt Similarly for *V. cholerae* non-O1 it was 15-20 ppt (optimum 15 ppt), *V. fischeri* like bacteria 5-15 ppt (optimum 15 ppt), *V. fluvialis* biovar II 20-35 ppt (optimum 30 ppt), *V. neries* 20-30 ppt (optimum 20 ppt), *V. ordalii* 20-30 ppt (optimum 25 ppt) *V. parahaemolyticus* 20-35 ppt (optimum 30 ppt) and for *V. proteolyticus* 0-25 ppt (optimum 5 ppt).

V. anguillarum showed a high growth over a pH range of 6.5-8.5 with an optimum level at 7. For other species it was observed as follows *V. cholerae* non-O1 7.5-8.5 (optimum 8), *V. fischeri* like bacteria 7.5-8.5 (optimum 8), *V. fluvialis* biovar II 7-8.5 (optimum 8), *V. neries* 7-8.5 (optimum 7.5), *V. ordalii* 7.5-8.5 (optimum 8), *V. parahaemolyticus* 7-8.5 (optimum 8), *V. proteolyticus* 7-8 (optimum 7). This indicates that the pH level required for the growth of vibrio from culture system varies from species to species.

ANTIBIOTIC SENSITIVITY TEST

Antibiotic sensitivity of vibrio isolates from the shrimp culture ponds was carried out in the laboratory. In this study 40 antibiotics were tested with antimicrobial

sensitivity test discs. The antibiogram is given in Table 2.5 with the following pattern.

H+++ = Highly sensitive; Clear zone diameter above 25 mm for at least 90% of strains.

+++ = Sensitive; Clear zone diameter 16-25 mm for atleast 90% of strains.

++ = Moderately sensitive; Clear zone diameter 8-15 mm for at least 90% of strains.

+ = Less sensitive; Clear zone diameter below 8 mm for at least 90% of strains.

- = Resistant to more than 90% of strains.

***Amikacin* (10 mcg)**

Isolates of *V. proteolyticus* was sensitive (+++) to Amikacin. A moderate (++) sensitivity pattern was demonstrated by *V. fischeri* like bacteria and *V. parahaemolyticus*, whereas *V. ordalii* and *V. fluvialis* biovar II were less sensitive (+). Isolates of *V. anguillarum, V. cholerae* non-O1 and *V. neries* were resistant (-) to Amikacin.

***Amoxycillin* (10 mcg)**

The only species resistant (-) to Amoxycillin was *V. proteolyticus*. Very high level (H+++) sensitivity was seen from by the isolates of *V. fluvialis* biovar II, and all other species were sensitive (+++).

***Ampicillin* (10 mcg)**

Isolates of *V. fluvialis* biovar II was found sensitive (+++) to Ampicillin. *V. anguillarum* showed a moderate (++) sensitivity and isolates of all other species such as *V. ordalii, V. cholerae* non-O1, *V. fischeri* like bacteria, *V. neries, V. parahaemolyticus* and *V. proteolyticus* were resistant (-) to this antibiotic.

***Augmentin* (10 mcg)**

All isolates of *V. anguillarum, V. ordalii, V. cholerae* non-O1, *V. fischeri* like bacteria, *V. neries, V. parahaemolyticus* and *V. proteolyticus* were found resistant (-), but *V. fluvialis* biovar II was sensitive (+++) to Augmentin.

TABLE 2.5. *Antibiogram*

ANTIBIOTIC	CONC.	*Va*	*V c*	*V f*	*V fb*	*V n*	*V o*	*V pa*	*V pr**
AMIKACIN	10 mcg	-	-	++	+	-	+	++	+++
AMOXYCILLIN	10 mcg	+++	+++	+++	H+++	+++	+++	+++	-
AMPICILLIN	10 mcg	++	-	-	+++	-	-	-	-
AUGMENTIN	10 mcg	-	-	-	+++	-	-	-	-
BACITRACIN	10 units	-	-	-	-	-	-	-	-
CARBENICILLIN	100 mcg	+++	+++	+++	+++	++	+++	+++	-
CEFAZOLIN	30 mcg	+++	+++	+++	H+++	+++	+++	+++	-
CEPHALEXIN	30 mcg	++	-	+	+++	+	++	-	-
CEPHALORIDINE	30 mcg	-	-	-	++	-	-	-	-
CEPHALOTHIN	30 mcg	++	+	++	+++	++	++	++	-
CEPHOTAXIME	30 mcg	+++	+++	+++	+++	++	+++	+++	-
CHLORAMPHENICOL	30 mcg	+++	+++	+++	-	+++	+++	+++	+++
CHLORTETRACYCLINE	30mcg	++	+++	++	+++	++	+++	+++	-
CIPROFLOXACIN	10 mcg	+++	+++	+++	+++	+++	+++	+++	++
CLINDAMYCIN	2 mcg	+++	+++	+++	+++	+++	+++	+++	+
CLOXACILLIN	1 mcg	+++	+++	+++	+++	+++	+++	+++	+
COLISTIN	10 mcg	-	-	-	-	-	-	-	+
CO-TRIMAZINE	25 mcg	+++	+++	+++	-	+++	+++	+++	+++
CO-TRIMOXAZOLE	25 mcg	+++	+++	+++	+++	+++	+++	+++	+++
ERYTHROMYCIN	15 mcg	+++	+++	+++	+++	+++	+++	+++	++
FURAZOLIDONE	50 mcg	+++	+++	+++	+++	-	-	-	-
GENTAMYCIN	10 mcg	++	++	++	++	++	++	+++	+++
KANAMYCIN	30 mcg	++	+	-	++	-	+	+++	+++
LINCOMYCIN	2 mcg	++	-	-	-	-	-	+	-
METH. MANDALATE	3 mcg	+++	+++	+++	+++	+++	+++	++	+++
METHICILLIN	5 mcg	-	-	-	-	-	-	-	-
NALIDIXIC ACID	30 mcg	H+++	++	+++	+	+++	H+++	+	++
NEOMYCIN	30 mcg	+++	+++	+++	+++	++	+++	+++	+++
NITROFURANTOIN	300 mcg	+++	+++	+++	+++	+++	+++	+++	-
NORFLOXACIN	10 mcg	+++	+++	+++	+++	+	+++	+++	+
NOVOBIOCIN	30 mcg	+++	+++	+++	+++	+++	+++	+++	+
OLEANDOMYCIN	15 mcg	+	-	+	+++	-	++	++	-
OXACILLIN	30 mcg	-	-	-	-	-	-	-	-
OXYTETRACYCLIN	30 mcg	+	-	+	++	+	+	++	-
PENICILLIN	10 units	-	-	-	-	-	-	-	-
POLYMYXIN-B	300 units	++	+	+	++	++	++	+	+++
STREPTOMYCIN	10 mcg	++	+	+	++	-	+	++	++
TETRACYCLINE	30 mcg	++	+	++	+++	++	++	++	-
TOBRAMYCIN	10 mcg	++	+	-	+++	+	+	+++	+++
VANCOMYCIN	30 mcg	-	-	-	++	-	-	+	-

* *Va- V. anguillarum*, *V c-V. cholerae* non-01, *V f - V. fischeri* like bacteria, *V fb- V. fluvialis* biovar II, *V n- V. neries*, *V o-V. ordalii*, *V pa- V. parahaemolyticus*, *V pr - V. proteolyticus*.

H+++ -Clear zone diameter above 25 mm for at least 90% of strains (Highly sensitive), +++ - Clear zone diameter 16-25 mm for at least 90% of strains (Sensitive), ++ - Clear zone diameter 8-15 mm for at least 90% of strains (Moderately sensitive), + - Clear zone up to 8 mm for at least 90% of strains (Less sensitive), - Resistant.

***Bacitracin* (10 units)**

All the Vibrio isolates from shrimp culture system were resistant (-) to Bacitracin.

***Carbenicillin* (100 mcg)**

V. anguillarum, V. ordalii, V. cholerae non-O1, *V. fischeri* like bacteria, *V. fluvialis* biovar II and *V. parahaemolyticus* were sensitive (+++) to Carbenicillin and isolates of *V. neries* were moderately (++) sensitive. But *V. proteolyticus* was resistant (-) to this drug.

***Cefazolin* (30 mcg)**

Sensitivity of *V. fluvialis* biovar II to Cefazolin was observed exceptionally high (H+++). Similarly, other species such as *V. anguillarum, V. ordalii, V. cholerae* non-O1, *V. fischeri* like bacteria, *V. neries* and *V. parahaemolyticus* also were sensitive (+++) to this antibiotic. But *V. proteolyticus* was found resistant (-) to Cefazolin.

***Cephalexin* (30 mcg)**

Moderate sensitivity (++) to Cephalexin was demonstrated by *V. anguillarum* and *V. ordalii. V. fischeri* like bacteria and *V. neries* were less sensitive (+). However, *V. fluvialis* biovar II, was sensitive (+++), but isolates of *V. cholerae* non-O1, *V. parahaemolyticus* and *V. proteolyticus* were observed resistant (-) to Cephalexin.

***Cephaloridine* (30 mcg)**

V. fluvialis biovar II was moderately sensitive to Cephaloridin (++) whereas isolates of all other species were resistant (-) to it.

***Cephalothin* (30 mcg)**

Isolates of *V. anguillarum, V. ordalii, V. fischeri* like bacteria, *V. neries* and *V. parahaemolyticus* were moderately sensitive (++) to Cephalothin and *V. fluvialis*

biovar II was sensitive (+++). *V. cholerae* non-O1 showed low level sensitivity (+), whereas V. Proteolyticus was reported resistant (-) to this anibiotic.

***Cephotaxime* (30 mcg)**

All the *Vibrio* isolates except *V. proteolyticus* showed different levels of sensitivity to Cephotaxime. *V. neries* showed moderate (++) sensitivity and isolates of all other species were sensitive (+++).

***Chloramphenicol* (30 mcg)**

V. fluvialis biovar II was found resistant (-) to Chloramphenicol, but all other isolates were sensitive (+++) to it.

***Chlortetracycline* (30 mcg)**

V. proteolyticus has shown resistance (-) to Chlortetracycline. *V. anguillarum* and *V. neries* were moderately sensitive (++) and *V. ordalii, V. cholerae* non-O1, *V. fischeri* like bacteria, *V. fluvialis* biovar II and *V. parahaemolyticus* were sensitive (+++).

***Ciprofloxacin* (10 mcg)**

All the isolates from the culture systems were sensitive (+++) to Ciprofloxacin except *V. proteolyticus* which showed a moderate level (++) of sensitivity.

***Clindamycin* (2 mcg)**

Sensitivity of isolates of *V. proteolyticus* to Clindamycin was low (+), but all other species were sensitive (+++) to it.

***Cloxacillin* (1 mcg)**

Isolates of all *Vibrio* spp. showed different levels of sensitivity to Cloxacillin. *V. proteolyticus* was less sensitive (+) and all others were sensitive (+++).

***Colistin* (10 mcg)**

All isolates of vibrios except *V. proteolyticus* were resistant (-) to the antibiotic Colistin.

***Co-trimazine* (25 mcg)**

Isolates of *V. anguillarum, V. ordalii, V. cholerae* non-O1, *V. fischeri* like bacteria, *V. neries, V. parahaemolyticus, V. proteolyticus* were sensitive (+++) to Co-trimazine, but *V. fluvialis* biovar II was resistant (-) to Co-trimazine.

***Co-trimoxazole* (25 mcg)**

Co-trimoxazole was the most effective drug among the tested antibiotics. All isolates from the shrimp culture ponds were sensitive (+++) to it.

***Erythromycin* (15 mcg)**

Except *V. proteolyticus* which was moderately sensitive (++), all species were sensitive (+++) to Erythromycin.

***Furazolidone* (50 mcg)**

V. anguillarum, V. cholerae non-O1, *V. fischeri* like bacteria and *V. fluvialis* biovar II were sensitive (+++) to Furazolidone. Whereas other species such as *V. ordalii, V. neries, V. parahaemolyticus* and *V. proteolyticus* were found resistant (-) to it.

***Gentamycin* (10 mcg)**

V. parahaemolyticus and *V. proteolyticus* were sensitive (+++) to Gentamycin and all others were moderately (++) sensitive..

***Kanamycin* (30 mcg)**

V. parahaemolyticus and *V. proteolyticus* were sensitive (+++) to Kanamycin. *V. anguillarum* and *V. fluvialis* biovar II showed moderate (++) and *V. ordalii* and *V. cholerae*

non-O1 showed low level (+) sensitivity. But isolates of *V. fischeri* like bacteria and *V. neries* were resistant (-) to Kanamycin.

***Lincomycin* (2 mcg)**

Isolates of *V. anguillarum* were moderately sensitive (++) to Lincomycin and *V. parahaemolyticus* showed a low level (+) of sensitivity. Isolates of other species were resistant (-) to it.

***Methanamine Mandalate* (3 mcg)**

Isolates of all *Vibrio* spp. except *V. parahaemolyticus* were sensitive (+++) to this antibiotic *V. parahaemolyticus* was moderately sensitive (++).

***Methicillin* (5 mcg)**

None of the isolated vibrios were sensitive to Methicillin.

***Nalidixic acid* (30 mcg)**

V. anguillarum and *V. ordalii* were highly sensitive (H+++) to Nalidixic acid. *V. fischeri* like bacteria and *V. neries* were sensitive (+++), whereas isolates of *V. cholerae* non-O1 and *V. proteolyticus* reported moderate sensitivity (++) and *V. fluvialis* biovar II and *V. parahaemolyticus* showed low level (+) sensitivity.

***Neomycin* (30 mcg)**

All *Vibrio* spp. except *V. neries* were sensitive (+++) *V. neries* showed a moderate level (++) of sensitivity.

***Nitrofurantoin* (300 mcg)**

Isolates of *V. proteolyticus* were resistant (-) to Nitrofurantoin, whereas all other species were sensitive (+++) to it.

***Norfloxacin* (10 mcg)**

Low level sensitivity (+) to Norfloxacin was demonstrated by the isolates of *V. neries* and *V. proteolyticus*, whereas isolates of all other species were sensitive (+++) to it.

***Novobiocin* (30 mcg)**

All *Vibrio* spp. except *V. proteolyticus* (which showed a low level (+) sensitivity) were sensitive (+++) to Novobiocin.

***Oleandomycin* (15 mcg)**

Isolates of *V. cholerae* non-O1, *V. neries* and *V. proteolyticus* were resistant (-) to Oleandomycin. *V. anguillarum* and *V. fischeri* like bacteria demonstrated a low level sensitivity (+). *V. ordalii* and *V. parahaemolyticus* were moderately sensitive (++) and *V. fluvialis* biovar II was sensitive (+++) to Oleandomycin.

***Oxacillin* (30 mcg)**

All *Vibrio* isolates obtained from shrimp culture system were observed resistant (-) to Oxacillin.

***Oxytetracycline (OTC)* (30 mcg)**

V. fluvialis biovar II and *V. parahaemolyticus* registered moderate sensitivity (++) and *V. anguillarum, V. ordalii, V. fischeri* like bacteria and *V. neries* have shown low level (+) sensitivity. But isolates of *V. cholerae* non-O1 and *V. proteolyticus* were resistant (-) to Oxytetracycline.

***Penicillin* (10 units)**

Isolates of all *Vibrio* spp. encountered in shrimp culture system were resistant (-) to Penicillin.

***Polymyxin-B* (300 units)**

V. cholerae non-O1, *V. fischeri* like bacteria and *V. parahaemolyticus* showed a low level (+) sensitivity and isolates of other species such as *V. anguillarum, V. ordalii, V. fluvialis* biovar II and *V. neries* showed moderate sensitivity (++). However as *V. proteolyticus* was sensitive (+++) to Polymyxin-B.

***Streptomycin* (10 mcg)**

Moderate (++) sensitivity was showed by *V. anguillarum, V. fluvialis* biovar II, *V. parahaemolyticus* and *V. proteolyticus*. Whereas *V. ordalii, V. cholerae* non-O1 and *V. fischeri* like bacteria showed low level (+) sensitivity. But *V. neries* was resistant (-) to Streptomycin.

***Tetracycline* (30 mcg)**

V. fluvialis biovar II was sensitive to Tetracycline. Isolates of *V. anguillarum, V. fischeri* like bacteria, *V. neries* and *V. parahaemolyticus* have shown moderate (++) sensitivity whereas *V. cholerae* non-O1 low sensitivity (+). But *V. proteolyticus* found resistant (-) to Tetracycline.

Tobramycin (10 mcg)

Isolates of *V. fluvialis* biovar II, *V. parahaemolyticus* and *V. proteolyticus* were sensitive (+++) to Tobramycin. Moderate (++) sensitivity was exhibited by isolates of *V. anguillarum*, whereas isolates of *V. ordalii, V. cholerae* non-O1 and *V. neries* showed low level (+) sensitivity and isolates of *V. fischeri* like bacteria were observed resistant.

***Vancomycin* (30 mcg)**

Isolates of most of the species such as *V. anguillarum, V. ordalii, V. cholerae* non-O1, *V. fischeri* like bacteria, *V. neries* and *V. proteolyticus* were resistant to Vancomycin. However isolates of *V. fluvialis* biovar II were moderately (++) sensitive and

V. parahaemolyticus showed low (+) sensitivity.

In general isolates of 8 species of *Vibrio* demonstrated sensitivity as follows.

TABLE 2.6. *Antibiotic Sensitivity /Resistance of Vibrios*

Species	Antibiotics		
	Total	Sensitive	Resistant
V. anguillarum	40	31	9
V. cholerae non-O1	40	26	14
V. fischeri like bacteria	40	28	12
V. fluvialis b II	40	32	8
V. neries	40	25	15
V. ordalii	40	29	11
V. parahaemolyticus	40	30	10
V. proteolyticus	40	19	21

MINIMAL INHIBITORY CONCENTRATION TEST

Experiments were conducted in order to find out minimal inhibitory concentration of two antibiotics such as Furazolidone and Perfuran which are being used in shrimp culture.

As specified in the "Material and Methods", tolerance tests of *Vibrio* isolates were conducted at 0, 5, 10, 20, 30, 40, 50, 70, 100, 300, 500 and 1000 µg/l levels.

Furazolidone

V. cholerae non-O1 and *V. fluvialis* biovar II showed sensitivity to Furazolidone even at 5 µg/l level. But isolates of all other species such as *V. anguillarum, V. ordalii,*

V. fischeri like bacteria, *V. neries, V. parahaemolyticus* and *V. proteolyticus* were found to be resistant at this level. *V. fischeri* like bacteria showed sensitivity at 20 µg/l level,

TABLE 2.7. *Effect of different concentrations of Furazolidone on Vibrios*

Species	Concentration (µg/l)								
	0	5	10	20	30	40	50	70	100
V. anguillarum	R	R	R	R	R	R	R	S	S
V. cholerae non - 01	R	S	S	S	S	S	S	S	S
V. fischeri like bacteria	R	R	R	S	S	S	S	S	S
V. fluvialis biovar II	R	S	S	S	S	S	S	S	S
V. neries	R	R	R	R	R	R	R	S	S
V. ordalii	R	R	R	R	R	R	R	S	S
V. parahaemolyticus	R ——————> up to 300 µg/l.								
V. proteolyti cus	R ——————> even at 1000 µg/l.								

* R - Resistant trait for at least 90% of strains.

S - Sensitive trait for at least 90% of strains.

whereas *V. anguillarum* revealed sensitivity at 50 µg/l level. Isolates of *V. ordalii* and *V. neries* were observed sensitive at 70 µg/l and *V. parahaemolyticus*at 300 µg/l. But *V. proteolyticus* was resistant to Furazolidone even at 1000 µg/l level (Table 2.7).

Perfuran

Sensitivity to Perfuran was reported at 5 µg/l by the isolates of *V. ordalii, V. fischeri* like bacteria, and *V. fluvialis* biovar II, where as isolates of all other species except *V. proteolyticus* were sensitive to perfuran at 20 µg/l level. But again *V. proteolyticus* was observed resistant even at 1000 µg/l level (Table 2.8).

TABLE 2.8. *Effect of different concentrations of Perfuran on Vibrios*

Species	Concentration (µg/l)								
	0	5	10	20	30	40	50	70	100
V. anguillarum	R	R	R	S	S	S	S	S	S
V. cholerae non-O1	R	R	R	S	S	S	S	S	S
V fischeri like bacteria	R	S	S	S	S	S	S	S	S
V. fluvialis biovar II	R	S	S	S	S	S	S	S	S
V. neries	R	R	R	S	S	S	S	S	S
V. ordalii	R	S	S	S	S	S	S	S	S
V. parahaemolyticus	R	R	S	S	S	S	S	S	S
V. proteolyticus	R	R ——————> even above 1000 µg/l.							

* R - Resistant trait for at least 90% of strains.

S - Sensitive trait for at least 90% of strains.

DISCUSSION

OPTIMUM GROWTH REQUIREMENT OF VIBRIOS

Temperature

The water temperature has emerged as a major environmental parameter controlling the diversity and distribution of vibrios. In general the range of temperature that promoted growth and multiplication of vibrios was between 15°-40°C. As discussed earlier the temperature regulated the metabolism of the organism and controlled all their vital activities such as development, growth and reproduction.

Colwell *et al.* (1984), in their study on ecology of pathogenic vibrios in Chesapeake Bay, have reported reduced growth of vibrios at low temperature and high growth at higher temperature. Similarly reduction or inhibition of growth of *V. parahaemolyticus* at temperature below 10°C also has been reported by them. de la Pena *et al.* (1993) have

reported the optimum growth range of some pathogenic vibrio strains as 13°-31°C with the specific optimum growth at 27°C. Newman and Feng (1982) has reported the optimum temperature for *Vibrio* isolates as 15°-25°C.

Based on the earlier reports and the results of the present study it can be concluded that vibrios isolated from shrimp culture system, prefer a temperature range of 15°-40°C for their metabolic activities and growth and a range of 20°-30°C for higher growth.

Salinity

As suggested by Kinne (1966), salinity is also a master environmental factor affecting the physiological mechanism of estuarine organisms. The significant positive correlation ($r = 0.936$; $p = <0.01$) observed between the salinity and the incidence of vibrios in the shrimp culture systems indicates the profound role of salinity in the occurrence of *Vibrio* spp.

Several workers had reported the absolute requirement of salt (Sodium ions) for growth of *Vibrio* spp. and in some cases the growth enhancement is attributed by specific salt concentrations (Baumann *et al.*, 1984; Colwell *et al.*, 1984).

The optimum concentration of salt for the growth of vibrios were reported as 0.5-4% by Newman and Feng (1992) and 2-4% by de la Pena *et al.* (1993). In the present investigation, isolates of different species of *Vibrio* showed a detectable growth within a range of salinity 5-45 ppt (0.5-4.5% salt concentration) and the widest range (0-50 ppt) of salinity tolerance was showed by *V. anguillarum*, *V. fluvialis* biovar II and *V. proteolyticus*. But the highest growth (peak) of different species was reported at specific salinities as reported by Baumann *et al.* (1984).

pH

Influence of pH in the bacterial multiplication has been reported by several workers (Vanderzant *et al.*,1971; Vanderzant and Nickelson 1973; Singh *et al.*, 1989). A pH range of 6-9 with an optimum of 7-8 for the growth of vibrio strains was reported by de la Pena *et al.* (1993).

In the present study the widest range was observed with the isolates of *V. anguillarum* (5.5-10.5) and the narrowest with *V. fluvialis* biovar II (6- 9.5). Isolates of all species exhibited growth over a pH range of 6-9 coinciding with the report of de la Pena *et al.* (1993). But the highest growth (peak) of different *Vibrio* spp. was reported at different specific pH levels.

Among the species tested for optimum growth requirement *V. proteolyticus* showed very low growth at different levels of temperature, salinity and pH. The incidence of this species in the culture system also was observed very low (Only seven isolates were encountered forming 4.17% of the total vibrio isolates). This indicated the fastidiousness of this species which was observed to be reluctant to grow on artificial culture media or broth. This low number encountered may not be reflecting their actual level of occurrence in the culture system.

The optimum growth requirement tests of vibrios isolated from the shrimp culture system revealed that, though the temperature, pH and salinity requirement for the highest growth (peak) found to vary from species to species, a range of each of these parameters was proved to be very important in the incidence of vibrios. A temperature range of 25°-35°C, pH range of 7.0-8.5 and salinity range of 15-30 ppt were reported to be ideal for the growth of vibrios and this indicate that a natural estuarine or brackishwater system

provides a very suitable environment for the survival and growth of vibrios and this is the reason for the wide distribution of vibrios in the shrimp culture systems.

ANTIBIOTIC SENSITIVITY

Among the tested antibiotics, Co-trimoxasol was the drug that observed most effective against *Vibrio* spp. isolated from the shrimp culture system. Isolates of all the eight species encountered were sensitive (+++) to this antibiotic (with a clear zone of diameter 16-25 mm around the antibiotic test disc). Antibiotics such as Ciprofloxacin, Erythromycin, Methanamine mandalate and Neomycine were also observed to be very effective against vibrios.

Antibiotics such as Clindamycin, Cloxacillin, Gentamycin, Nalidixic acid, Norfloxacin, Novobiocin and Polymyxin-B were the other antibiotics which showed different levels of effectiveness. Sensitivity to Amoxycillin, Carbenicillin, Cefazolin, Cephotaxime, Chloramphenicol, Chlortetracycline, Co-trimazine and Nitrofurantoin has shown by most of the *Vibrio* spp.

Sensitivity of vibrios towards antibiotics such as Chloramphenicol, Erythromycin, Novobiocin, Nalidixic acid and Tetracycline as has reported by earlier workers (Lightner, 1977; de la Pena *et al.*, 1993), was also observed during the present study.

All the tested species were found resistant to Bacitracin, Methicillin, Oxacillin and Penicillin. Similarly some other antibiotics such as Ampicillin, Augmentin, Cephaloridine, Colistin, Lincomycin and Vancomycin also were observed less effective against vibrios.

Though Colriss (1979) and Takahashi *et al.* (1985) had reported oxytetracycline (OTC) as an effective drug against 'vibriosis', the effect of OTC on *Vibrio* spp. in the present investigation was observed low when compared with several other tested antibiotics.

Similarly Penicillin which was reported as moderately sensitive to vibrio strains (de la Pena *et al.*, 1993) was not observed to be effective at all against the *Vibrio* spp. in shrimp culture systems in the present study.

In general the resistance pattern was found to be Bacitracin, Methicillin, Oxacillin, Penicillin > Colistin > Cephaloridine > Augmentin > Lincomycin, Vancomycin > Ampicillin> Furazolidone > Amikacin, Cephalexin, Oleandomycin > Oxytetra cycline > Kanamycin > Streptomycin > Tetracycline, Cephalothin> Tobramycin > Chlortetracycline > Carbenicillin > Cephotaxime> Chloramphenicol, Co-trimazine, Norfloxacin > Amoxycillin, Cephazolin > Polymyxin-B > Gentamycin > Nalidixic acid> Norfloxacin > Ciprofloxacin, Methanamine Mandalate, Neomycin> Clindamycin, Cloxacillin, Erythromycin, Novobiocin> Co-trimoxazole.

Among the isolated Vibrios, *V. anguillarum, V. fischeri* like bacteria, *V fluvialis* biovar II, *V. ordalii* and *V. parahaemolyticus* were sensitive to more than 70 % of the tested antibiotics. Other species such as *V. cholerae* non-O1 and *V. neries* were sensitive to more than 60%. But *V. proteolyticus* was sensitive only to less than 50% of the tested antibiotics which showed resistance to 21 antibiotics (out of 40 antibiotics tested) at their tested concentrations.

MINIMAL INHIBITORY CONCENTRATION

Among the antibiotics tested for minimal inhibitory concentration, Perfuran was reported effective against vibrios than Furazolidone. The minimal inhibitory concentration of Furazolidone required to prevent the growth of vibrios was higher for most of the species. At a concentration of 20 µg/l isolates of all *Vibrio* spp. isolated from the shrimp culture system (except *V. proteolyticus*) was sensitive to the antibiotic Perfuran.

Whereas in the case of Furazolidone even at a concentration of 50 µg/l only 4 species were sensitive to the drug and at 70 µg/l concentration, 6 species showed sensitivity. *V. parahaemolyticus* showed sensitivity to this drug only at a higher concentration of 300 µg/l. These observations indicate the effective inhibitory property of Perfuran on vibrios than of Furazolidone.

V. proteolyticus was observed to be resistant to both of these antibiotics even at a very high concentration of 1000 µg/l. This high resistance of this species toward several antibiotics was observed in antibiotic sensitivity test also.

Eventhough many tested antibiotics were reported to be effective against vibrios encountered in the shrimp culture system, their use during disease-out breaks is suggested as a last resort only, since development of drug resistance has been and continue to be a significant problem in the antibiotic therapy and it involves ecotoxicological and human health problems.

Multiple drug resistance was exhibited by isolates of all *Vibrio* spp. But among the species tested for antibiotic sensitivity and minimal inhibitory concentration, *V. proteolyticus* was the toughest one which demonstrated a wide range of drug resistance against the tested antibiotics.

CHAPTER III

CHAPTER III

ROLE OF PHYSICO-CHEMICAL PARAMETERS IN 'VIBRIOSIS'

V. anguillarum and *V. parahaemolyticus* are identified as the major pathogens involved in 'vibriosis' of crustaceans (Lightner and Lewis, 1975; Delves-Broughton and Poupard, 1976; Lightner, 1977; Bower *et al.*, 1981; Lightner, 1983; Chong and Chao, 1986; Anderson *et al.*, 1988; Boonyaratpalin, 1990; Sindermann, 1990 a) especially shrimps. Eventhough vibrios are part of normal microbial flora of shrimps and culture ponds (Vanderzant *et al.*, 1971; Lightner, 1975; Christopher *et al.*, 1978; Nash, 1990), they are considered as opportunistic pathogens. They cause disease in severely stressed shrimps (Lightner, 1977; Anderson, 1990), when the stress is caused by the influence of environmental parameters (Delves-Broughton and Poupard, 1976).

PATHOGENICITY OF *V. ANGUILLARUM*

Intramuscular injection

100% mortality was observed within 2 hrs after the injection in juvenile shrimps which were administered with high dose of 45 x 10^9 cells/ml (22.5 x 10^8 cells/Juvenile). Higher levels of abnormality in juvenile shrimp behaviour including disoriented erratic swimming and jumping were exhibited by the inoculated animal (Table 3.1). Injection with 45 x 10^8 cell/ml (22.5x10^7 cells/animal) resulted in 100% mortality between 4 and 5 hrs after injection.

An injection dose of 45 x 10^7 cells/ml (22.5x10^6 cells/Juvenile) resutlted only 70% mortality following jumping and occasional lying at the bottom between 48 and 72 hrs after injection. Whereas a dose below 45x10^7 cells/ml (22.5x10^6 cells/Juvenile) has not resulted

in death. The juvenile shrimps were weak for 1 or 2 days, but later recovered to normal condition within 3 to 5 days (Plate I, II).

TABLE 3.1. *Pathogenicity experiment - Intra-muscular injection*
Dose - 0.05 ml of bacterial suspension/animal
Pathogen - Vibrio anguillarum

Injection dose (bacterial cells/animal)	% of mortality	Duration in hrs
22.5×10^9	100	1-2
22.5×10^8	100	1-2
22.5×10^7	100	4-5
22.5×10^6	70	48-72
22.5×10^5	-	168
22.5×10^4	-	168
22.5×10^3	-	168
22.5×10^2	-	168
22.5×10^1	-	168

PATHOGENICITY IN STRESS CONDITIONS

The experimental animals were tested for their behaviours and infections in different levels of pH and salinity.

pH alterations as stress factor

Sudden changes in pH of water were made in the experimental tanks to induce stress to the juvenile shrimps, reared at pH 7.5 + 0.02 in the laboratory before the experiment. They were orally administered with *V. anguillarum* and transferred into experimental tanks with higher and lower pH levels such as 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0 and the sudden change in pH of water was employed as the stress factor to study the pathogenicity.

Plate I. Setup for pathogenicity experiments.

Plate II. Stages of juvenile shrimps between intramuscular injection (with pathogen) and subsequent death.

At pH 5.0 and 5.5, 100% mortality was noticed within 3 to 7 hrs following erratic swimming and jumping. Bacterial assay of the test juvenile shrimps and control juveniles revealed the invasion of body muscle by an *Aeromonas* sp. which was not inoculated. Meanwhile the inoculated *V. anguillarum* could not be isolated from test shrimps.

TABLE 3.2. *Pathogenicity experiments-pH as stress*

Pathogen - V. anguillarum

pH	% of mortality	
	Test juveniles	Control juveniles
5	100	100
5.5	100	100
6	100	0
6.5	60	0
7	0*	0
7.5	0	0
8	0	0
8.5	0	0
9	0	0
9.5	100	100
10	100	100

* Invasion of *V. anguillarum* into the body muscle was reported, but no motality.

At pH 6.5, 60% mortality was reported in test shrimps within 6 hrs since inoculation, whereas control juveniles exhibited initial erratic swimming and recovered within 3 to 4 hrs. *V. anguillarum* was isolated as pure culture from the body muscle. At pH 7.0 the test juveniles exhibited initial erratic swimming which subsided within 1 to 2 hrs, but the control juveniles were normal. Bacterial assay of the body muscle of test juveniles revealed the invasion of *V. anguillarum*.

At pH 7.5, 8.0 and 8.5 both control and test juveniles were found normal and no bacterial invasion was reported in the body muscle or haemolymph.

Abnormal swimming and erratic movements were exhibited by both test and control juveniles animals at pH 9. In control juveniles it subsided within 2 to 3 hrs whereas in the test animals it persisted for 20 to 24 hrs. Bacterial assay of the inoculated shrimp body muscle or haemolymph did not reveal any bacterial occurrence.

At pH 9.5, 100% mortality was noticed in both control and test juveniles following higher behavioural abnormality. Death was reported within 2 to 3 hrs after inoculation in test juveniles and 4 to 5 hrs in control animals. Bacterial assay of the animal did not reveal the occurrence of *V. anguillarum* either in muscle or in haemolymph, but *Aeromonas* sp. which was not inoculated, was observed in both the test and control juvenile muscle.

At pH 10, 100% mortality was noticed in both test and control juveniles within 2 hrs and no bacterial isolation could be achieved from either the body muscle or haemolymph of the juvenile shrimp.

In general, at lower pH (from 5.5-7.0) *V. anguillarum* was observed to cause infections and resulting death in juvenile shrimp (Table 3.2) whereas *Aeromonas* sp. which was a resident bacterial flora of the gut of the juveniles was found to infect juveniles at pH 5.0 and 5.5.

Behavioural abnormalities noticed in the juvenile shrimps included reduced (lethargic) swimming, disorientation in swimming, occasional jumping and motionless lying at the bottom. Similarly clinical signs such as opacity of body musculature, reddening of extremities (especially of telson, uropod and telson) and delay in clotting of haemolymph were also exhibited by the affected juveniles (Plate III, IV).

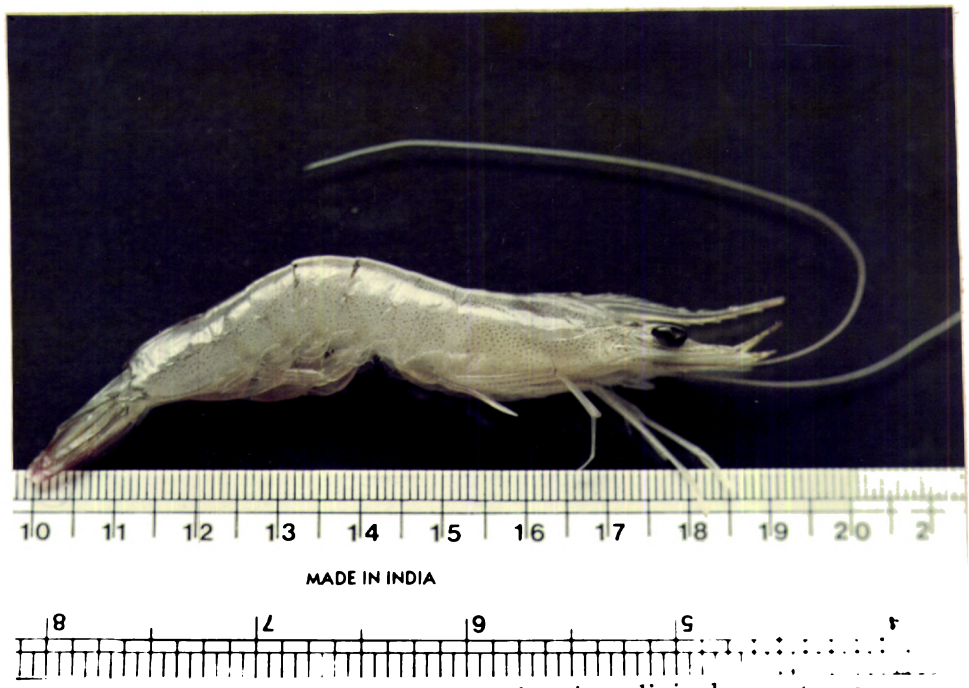


Plate III. Juvenile shrimp showing clinical symptoms of vibrio infection (after oral inoculation).

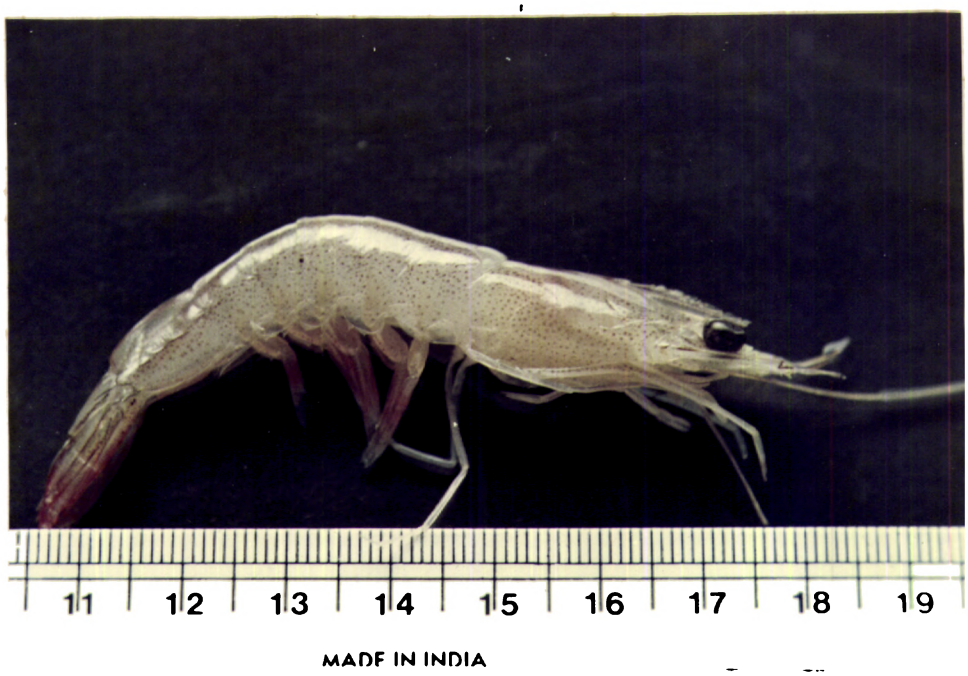


Plate IV. Dead juvenile shrimp due to vibrio infection.

Alterations in salinity as stress factor

As mentioned in "Material and Methods", different levels of salinities were employed to induce stress to the juveniles and to study its role in inducing and enhancing the pathogenicity of *V. anguillarum*. The salinity where the juveniles were reared was 20 ppt and the test animals after oral administration of the pathogen were suddenly transferred to experimental tanks with altered salinities of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 ppt.

Juvenile shrimps with orally administered pathogen, when introduced into 0 ppt showed abnormal erratic swimming and an exposure of 1 to 2 hrs resulted 60% mortality. The mortality was 20% in control juveniles. However, 100% mortality was encountered both in experimental and control animals within 3 to 4 hrs. Bacterial assay of the juveniles did not show any bacterial invasion in body muscle or haemolymph.

At 5, 10 and 15 ppt, abnormal erratic swimming was observed both in test juvenile shrimps and controls, which later subsided within 3 hrs. Bacterial assay of the juveniles did not reveal the occurrence of pathogen in body muscle or haemolymph.

Neither test shrimps or control juveniles exhibited any abnormality or bacterial invasion in body muscle or haemolymph at 20 ppt salinity. But at 25 ppt, test juvenile shrimps exhibited erratic movements, whereas the control animals were observed to be normal. Bacterial assay of the inoculated juveniles revealed the invasion of *V. anguillarum* into the muscle, but the haemolymph was found free of pathogen.

30% mortality was reported in the test juvenile shrimps at 30 ppt salinity within 42 to 72 hrs after the inoculation. Abnormal erratic swimming, opaqueness in body musculature, reddening of telson, uropods and pleopods were exhibited by the affected animals. *V. anguillarum* could be isolated from the body muscle of the infected juveniles and haemolymph was found sterile.

At 35 ppt, both the inoculated juveniles and control animals exhibited abnormal behaviours which subsided within 2 to 3 hours after inoculation and the bacterial assay of the body muscle and haemolymph did not reveal any invasion of the inoculated organism.

TABLE 3.3. *Pathogenicity experiments - Salinity as stress*

Pathogen - V. anguillarum

Salinity (ppt)	% of mortality	
	Test juveniles	Control juveniles
0	100	100
5	0	0
10	0	0
15	0	0
20	0	0
25	0*	0
30	30	0
35	0	0
40	50	0
45	100	0
50	100	100

* Invasion of *V. anguillarum* into the body muscle was reported, but no motality.

50% mortality was noticed in test juvenile shrimps within 42 to 72 hrs at 40 ppt salinity, whereas control animals remained normal. *V. anguillarum* could be isolated in pure culture from the affected animal's body muscle.

At 45 ppt, 100% mortality within 5-6 hrs in test shrimps was observed, but the control animals were found surviving even on the seventh day of inoculation. *V. anguillarum* was isolated in pure culture from the moribund animal muscle.

Behavioural abnormalities and clinical signs observed at 30 ppt stress level were noticed at 40 and 45 ppt also. The inoculated pathogen *V. anguillarum* could be isolated in pure culture from the muscle of moribund animals, but the haemolymph was found sterile. 100% mortality following erratic movements and disoriented swimming was reported both in controls and test juvenile shrimps within 1 to 3 hrs and muscle and haemolymph were found uninfected.

In general, the infection of *V. anguillarum* in inoculated and stressed juvenile shrimp was reported to be maximum during 30, 40 and 45 ppt levels of salinity (Table 3.3). A significant observation noticed was that the salinity stress at 35 ppt had not resulted in the invasion of *V. anguillarum* eventhough the immediate lower (30 ppt) and higher (40 ppt) levels resulted in infection (Plate V).

PATHOGENICITY OF *V. PARAHAEMOLYTICUS*

Intramuscular injection

100% mortality was noticed within 2 hrs when juvenile shrimps were injected with a dose of $61x10^9$ cell/ml ($30.5x10^8$ cells/juvenile) and $61x10^{10}$ cells/ml ($30.5x10^9$ cells/ juvenile) of *V. parahaemolyticus* (Table 3.4). Similarly $61x10^8$ cells/ml ($30.5x10^7$ cells/ juvenile) resulted 100% mortality within 3 to 4 hrs following erratic movements and tendency to jump out of water.

Though intramuscular inoculation below $61x10^8$cells/ml ($30.5x10^7$cell/animal) did not resulted in mortality, juvenile shrimps were weak for 2 days which later recovered to normal condition within 3 to 5 days.

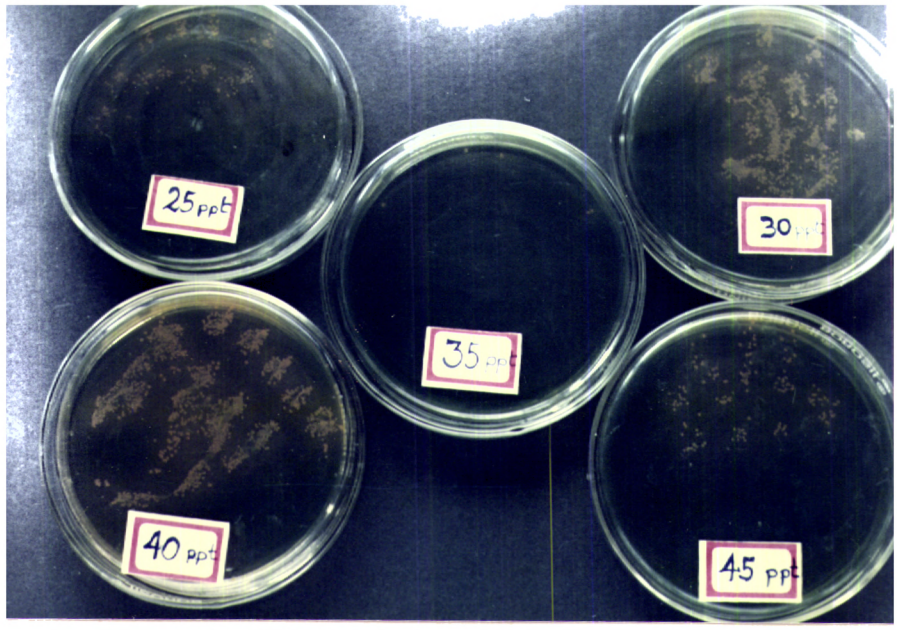


Plate V. TCBS Plates (impression smear) showing no vibrio invasion at 35 ppt salinity.

PATHOGENICITY IN STRESS CONDITIONS

pH alterations as stress factor

Both test and control juvenile shrimps reported 100% mortality at pH 5.0 and 5.5, within 5 to 8 hrs of inoculation following erratic swimming and jumping. Invasion of *V. parahaemolyticus* was not observed at pH 5. But at pH 5.5 high invasion of *Aeromonas* sp. was reported along with few numbers of *V. parahaemolyticus.*

TABLE 3.4. *Pathogenicity experiment - Intra-muscular injection*
Dose - 0.05 ml of bacterial suspension/juvenile
Pathogen - Vibrio parahaemolyticus

Injection dose (bacterial cells /juvenile)	% of martality	Duration in hrs
30.5×10^9	100	1-2
30.5×10^8	100	1-2
30.5×10^7	100	3-4
30.5×10^6	-	168
30.5×10^5	-	168
30.5×10^4	-	168
30.5×10^3	-	168
30.5×10^2	-	168
30.5×10^1	-	168

Though pH 6 and 6.5 did not result in mortality, high invasion of *Aeromonas* sp. along with low incidence of *V. parahaemolyticus* was noticed.

At pH 7, all the inoculated juvenile shrimps showed erratic movement and resulted in 30 % mortality within 16 hrs since the inoculation. Whereas the control juvenile shrimps behaved normal. Bacterial assay of the moribund and dead juvenile shrimps revealed the invasion of *Aeromonas* sp. into the muscle. However, the haemolymph exhibited high invasion of *V. parahaemolyticus.*

Test and control juvenile shrimps were healthy and normal at pH 7.5, 8.0 and 8.5 and the bacterial assay did not reveal any incidence of bacteria in muscle or haemolymph. Whereas at pH 9.0 the animals were normal initially, but resulted in 80% mortality in the test juvenile shrimps within 72 hrs followingabnormal swimming and other clinical sign. The bacterial assay of the juveniles revealed the high invasion of *V. parahaemolyticus* into the body muscle.

TABLE 3.5. *Pathogenicity experiemnt – pH as stress*

Pathogen - V. parahaemolyticus

pH	% of mortality	
	Test juveniles	Control juveniles
5	100	100
5.5	100	100
6	0	0
6.5	0	0
7	30*	0
7.5	0	0
8	0	0
8.5	0	0
9	80	0
9.5	100	100
10	100	100

* Invasion of *Aeromonas* spp. *into the body muscle was reported.*

V. parahaemolyticus was isolated from haemolymph only.

100% mortality was observed at pH 9.5 and 10 within 3 to 4 hrs of inoculation, both in test and control juvenile shrimps. Bacterial assay of both inoculated and control juvenile shrimps showed invasion of *Aeromonas* sp. in the body muscle at pH 9.5 but at pH 10 no bacterial invasion was observed.

In general, invasion of *V. parahaemolyticus* into the muscle was observed at pH 9.0 resulting in 80% mortality and at pH 7 invasion of *Aeromonas* sp. a resident of the juvenile shrimp intestine, into the muscle and invasion of *V. parahaemolyticus* into the haemolymph was observed resulting in 30% mortality in the test juvenile shrimps (Table 3.5).

The behavioural abnormalities, clinical signs of infection and pathogenicity were noticed common for both *V. anguillarum* and *V. parahaemolyticus*.

Alterations in salinity as stress factor

100% mortality was noticed within 3 hrs at 0 ppt and no bacterial occurrence was reported from muscle or haemolymph. Though high level behavioural abnormality was exhibited by both inoculated and control juvenile shrimps at 5 and 10 ppt, it subsided and the animals recovered to normal condition within 3 to 5 hrs. Inoculated bacteria was not reported either in muscle or in haemolymph subsequently.

Eventhough *V. parahaemolyticus* was orally administered to the juveniles they did not exhibit any external abnormality or mortality at 15, 20 and 25 ppt and the muscle and haemolymph was found free of pathogen.

At 30 ppt and 40 ppt salinity, test juvenile shrimps exhibited erratic swimming and other clinical signs and the bacterial assay revealed high invasion of *V. parahaemolyticus* into the body muscle, but no mortality was noticed. The test juvenile shrimps recovered within 5 to 6 hrs at 30 ppt and within 40 to 48 hours at 40 ppt. Control animals were quite normal at 30 ppt, but at 40 ppt control also exhibited erratic movements, but subsided within 12 to 14 hours.

As in the case of *V. anguillarum*, at 35 ppt both the juvenile shrimp muscle and haemolymph remained free from pathogenic invasion though the immediate lower and higher levels resulted in pathogenic invasion. Though the test and control juvenile shrimps exhibited behavioural abnormalities, all became to normal within 2 to 3 hrs.

TABLE 3.6. *Pathogenicity experiment - Salinity as stress*

Pathogen - V. parahaemolyticus

Salinity (ppt)	% of mortality	
	Test juvenile	Control juvenile
0	100	100
5	0	0
10	0	0
15	0	0
20	0	0
25	0	0
30	0*	0
35	0	0
40	0*	0
45	100	0
50	100	100

* Invasion of *V.parahaemolyticus* into the body muscle was reported, but no mortality.

At 45 ppt, 100% mortality was observed in test juvenile shrimps within 8 to 12 hrs. But the control animals were found surviving even on the seventh day. *V. parahaemolyticus* were isolated from the muscle of inoculated juvenile shrimps in pure culture. Whereas at 50 ppt 100% mortality was noticed within 3 to 4 hrs of inoculation, but muscle and haemolymph of juvenile shrimp were free of any invasion.

The pathogenicity of *V. parahaemolyticus* in juvenile *P. indicus* stressed by salinity changes revealed to be maximum at higher salinities such as 30, 40 and 45 ppt, whereas no

mortality or invasion was reported at 35 ppt, as has been observed in the experiments with *V. anguillarum* (Table 3.6).

In general, 'vibriosis' in juvenile *P. indicus* was characterised by the external clinical signs such as erratic swimming, sudden ascending and descending behaviour, occasional sedentary habit, occasional jumping, opacity of body musculature, delay in clotting of haemolymph, high level liquefaction and crimson reddening of hepatopancreas and reddening of uropods, telson and pleopods.

The characters of the *Aeromonas* sp., a resident flora of intestine of *P. indicus* which caused death in the juvenile shrimp are the following.

Motility	+
Gram's staining	-
Glucose fermentation	+
Sucrose fermentation	+
Cytochrome oxidase	+
Argenine dihydrolase	-
0/129 sensitivity	-

DISCUSSION

Pathogenicity experiments were conducted with a view to demonstrate the vibrio infections in juvenile shrimp and the significant role played by physico-chemical parameters.

Although many workers have suggested 'vibriosis' in shrimps as a stress related disease (Delves-Broughton and Poupard, 1976; Lightner, 1977; Bowser *et al.*,1981; Sindermann, 1990 b; Anon., 1993) due to adverse or unstable environment, they did not give definite experimental evidence. However Newman and Feng (1982) has observed high level of

mortality in the experimentally infected Rock-crab *Cancer irroratus* with *Vibrio* spp. at high temperature in which the inoculation of pathogen was done by intramuscular injection.

INTRAMUSCULAR INJECTION

In intramuscular injection method no manipulation of water salinity and pH was employed in the experimental tank and this was selected inorder to test the number of bacterial cells required to result mortality in the juvenile shrimp.

In the experiment with *V. anguillarum* a concentration of 22.5 x 10^6 Cells /animal was observed to be a threshold level for infection that leads to death (70% mortality in 48-52 hrs) and higher number of bacterial cells in the inoculation resulted in 100% mortality with in short time. In the case of intramuscular injection with *V. parahaemolyticus* the minimum number of bacteria required to result mortality in juvenile shrimp was observed to be 30.5 x 10^7 cells/animal. which induced 100% death within 3-4 hrs and as in the case of *V. anguillarum* higher number of the pathogen resulted mortality more quickly.

In the pathogenicity experiments Nash (1990) reported mortality within 12-13 hrs in juvenile *P. monodon* by inoculating 10^5 cfu/animal of *V. parahaemolyticus* and at higher concentration of pathogen, death was reported to occur in lesser duration of time. Vera *et al.* (1992) reported 100% mortality in 4 hrs in juveniles of *P. japonicus* inoculated with *V. parahaemolyticus* (10^8 cfu/shrimp) and similar doses of *V. alginolyticus* and *V. anguillarum* resulted only 80 % and 40% mortality respectively. Low doses (10^3-10^5 cfu/shrimp) of *V. parahaemolyticus and V. anguillarum* did not resulted in mortality, but the same dose of *V. alginolyticus* induced 40% mortality in the juvenile shrimp within 92 hrs. Similarly Takahashi *et al.* (1984) reported 100% mortality in *P. japonicus* inoculated with *Vibrio* spp. with of 10^{10} cfu/ gram wt of shrimp. Whereas with low doses, the rate of

mortality was less and below 10^6 cfu/ gram wt of shrimp no mortality was reported. Takahashi *et al.* (1985) was reported 100% mortality in *P. japonicus* inoculated with another unidentified *Vibrio* spp. inoculated with doses above 10^7 cfu/gram body wt of shrimp and 90% mortality with lower doses such as 10^3 and 10^2.

This indicates that the number of cells of bacteria require to cause death in a host juvenile shrimp varies for different species though they belong to same genus and showing similar clinical signs. Similarly the rate of mortality and duration of infection before death also are specific for different pathogen.

PATHOGENICITY IN STRESS CONDITIONS

Juvenile shrimps were fed with inoculated shrimp meat and assayed for 7 days with out making any pH or salinity alteration in the water in experimental tanks. But the inoculum could neither induce mortality nor any behavioural abnormality in the animal. Similar observations have been reported by Lightner (1975) and Delves-Broughton and Poupard (1976) also.

Since vibrios form part of normal microbial flora of shrimp intestine, an oral inoculation of vibrio could not induce any infection in the juvenile shrimp. The intestinal microbial flora of a healthy juvenile could accommodate the orally inoculated population of vibrios along with them in the intestine. But since vibrios are opportunistic pathogens which cause infection in a weak host under prolonged stress, the chances for infection during such conditions can not be neglected.

So inorder to study the role of some physico-chemical factor such as pH and salinity in vibrionic infection, altered levels of these parameters were employed in experimental tanks to induce physiological stress to the juvenile shrimp and the pathogen was introduced by oral route.

pH

Water pH was observed as an important parameter in vibrio infections. In experiments with *V. anguillarum* the bacteria was found infecting the juvenile shrimp when the inoculated juveniles were transferred to experimental tanks with altered pH levels of 6.0, 6.5 and 7.0 and infection leading to mortality was observed at pH 6.0 and 6.5.

Similarly in experiments with *V. parahaemolyticus*, the altered pH that triggering infection was 9.0 which resulted in 80% mortality of the inoculated juveniles. pH of 6.5, 7.0 were reported to be favouring the growth of *V. anguillarum* (pH 7.0 reported highest growth), but pH 6.0 was not that much conducive for the growth. Still the invasion and multiplication of the pathogen in the host were reported at pH 6.0, 6.5 and 7.0. Similarly though pH 9.0 was not that congenial for the growth of *V. parahaemolyticus* when compared to other levels of pH 7.5, 8.0 or 8.5 the invasion and multiplication of *V. parahaemolyticus* inducing mortality in the host juvenile shrimp was observed at pH 9.0. This indicates that the most favourable condition for growth and multiplication of the pathogen is not the factor that govern the infection, but the health of the host is the important factor which plays a significant role in the disease manifestation. It is also evident that the survival of the pathogen at such stressful situations is also a must for infection.

At test levels of pH 7.5, 8.0, 8.5 no behavioural abnormality was found, even in inoculated shrimps. This indicates the incapability of the pathogen to infect a healthy host which does not experience any stress by any physico-chemical parameters.

At pH test levels 5.0, 5.5 and 9.5, 100% death was reported in both test juvenile shrimps as well as in controls. Invasion of *Aeromonas* sp. into the body muscle was observed at these pH levels. Though no *Aeromonas* sp. was inoculated, the environmental stress due to the altered pH may probably be attributed to the invasion of *Aeromonas* sp. The presence

of this bacteria was reported in the gut of healthy juvenile shrimp which were reared in the laboratory for experiment. Its occurrence in the rearing water in small numbers also was reported. So it is suggested that the same bacterium is invading the host body under stress conditions. Since both controls and test juvenile shrimps reported 100% mortality, it is difficult to conclude specifically whether the mortality is due to the *Aeromonas* spp. which showed invasion into the body musculature or due to the extreme level of pH of the water. But the role of a physiological stress (due to the low pH of water) in the invasion of the resident *Aeromonas* sp. was so evident.

In the experiments with *V. parahaemolyticus* at pH 7.0, 30% mortality was reported. The bacterial assay of the test juvenile shrimps revealed invasion of the resident *Aeromonas* sp. into the body musculature of the juvenile shrimp and high septicemia with *V. parahaemolyticus* also was observed. This suggests a synergistic role of the *Aeromonas* sp. and *V. parahaemolyticus* in causing infection which induces mortality. This observation also agrees with the reports of earlier workers who suggest the occasional involvement of *Aeromonas* sp. in disease outbreaks along with *Vibrio* spp. (Lewis, 1973; Lightner, 1975; 1977; 1983; Lightner and Lewis, 1975; Nash 1990; Sindermann, 1990b).

Along with vibrios and other microbial flora, *Aeromonas* spp. also form part of gut flora of penaeid shrimps and play a significant role in shrimp's digestion (Dempsey and Kitting, 1987; Dempsey *et al.*, 1989). Since *Aeromonas* sp. is another opportunistic or facultative pathogen (Lightner, 1975), an environmental stress which debilitates the host can facilitate their invasion and multiplication in the host body.

SALINITY

Salinity was another important physico-chemical parameter that plays a vital role in vibrio infections in the juvenile shrimp.

In pathogenicity experiments with *V. anguillarum* and *V. parahaemolyticus*, both juvenile test shrimps and control animals reported 100% mortality at 0 ppt and the bacterial assay of the juvenile shrimp did not reveal any bacterial invasion. This indicates that a sudden salinity drop from 20 ppt to 0 ppt itself causes an extreme physiological stress to the shrimp juveniles inducing mortality even without any infection. Shylaja (1989) reported 100% mortality of juvenile *P. indicus* when they were acutely transferred from water with 20 ppt salinity to 0 ppt. Though behavioural abnormalities and stress signs were shown by both inoculated and control juvenile shrimp at salinity test levels of 5 ppt,10 ppt and 15 ppt, no bacterial infection was observed and the animal became normal within few hours. At 20 ppt, no behavioural abnormality was exhibited even by the inoculated juveniles. This again indicates the incapability of the pathogens to infect a healthy host, which is not under any physiological stress.

Invasion of *V. anguillarum* into the test shrimp juvenile's body muscle was observed at 25 ppt, 30 ppt, 40 ppt and 45 ppt, and mortality was reported at 30 ppt, 40 ppt and 45 ppt Whereas in the case of *V. parahaemolyticus* invasion to the body muscle was observed at 30 ppt, 40 ppt and 45 ppt inwhich infection that induces mortality (100%) was reported at 45 ppt level only.

Euryhaline shrimps regulates the osmolarity of body fluid as an adaptation to changing salinities. But sudden changes in salinity obviously require high expenditure of energy for osmoregulation (Dall *et al.*, 1990). So the juvenile shrimp under salinity stress will have to spend high amount of energy for osmoregulation and became physiologically weak. Since the opportunistic pathogen like vibrios are capable of invading the body system of the weak host, the salinity stress culminate in the infection, but the level of invasion is always depended on the level of physiological stress suffered by the juvenile shrimp.

Salinity that was reported to give highest growth of *V. anguillarum* in broth culture was 45 ppt and for *V. parahaemolyticus* it was 30 ppt. However, the stress that caused 100% mortality in the juvenile shrimps inoculated with both *V. anguillarum* and *V. parahaemolyticus* was 45 ppt. This again shows that most favourable condition for the growth and multiplication of the pathogen is not the factor that govern the infection instead the the physiological stress suffered by the host has the crucial role in invasion of the pathogen.

The very interesting phenomena observed in the pathogenicity experiments was the behaviour of the animal at 35 ppt. At this salinity *V. anguillarum* and *V. parahaemolyticus* did not cause any infection in the host juvenile shrimp, eventhough the immediate higher and lower stress levels at 30 ppt and 40 ppt could lead to an infectious death. Like many other penaeids, juvenile *P. indicus* also migrates to sea for maturation and spawning (Silas *et al.*, 1985) and sexual maturity is attained in waters of 35 ppt. This indicates that the physiological system of juveniles is at the tune with the sea water salinity and the juvenile shrimp needs to spend very little energy for the osmoregulation. In case of any pathogenic attack they will be capable to utilize more energy for their cellular and humoral defense. This may be the reason for the reported observation inwhich the pathogen could not infect the juvenile shrimp at 35 ppt salinity though it could at 30 ppt and 40 ppt stress levels. But this requires confirmation through extensive research.

Aeromonas sp. which was noted for their infection at different pH stress levels had not appeared at all in the salinity stress experiments inducing any sort of infection in the test juvenile shrimp. Similarly *V. anguillarum* and *V. parahaemolyticus* could infect the test juvenile shrimp only at certain specific stress levels. This suggests that an opportunistic pathogens can not infect the host juvenile shrimp in all stress conditions instead it requires a host which is under certain specific level of physiological stress for inducing infection.

In this study on stress induced pathogenicity experiments it is proved that the opportunistic pathogen which form normal gut flora of juvenile shrimps could trigger infection under prolonged specific physiological stress due to physico-chemical parameters.

Addition of bacterial isolates to the food given to the shrimps has been reported to be an unsuccessful means of infection (Lewis, 1973; Lightner and Lewis, 1975). In the present experiments also oral inoculation of the pathogens to healthy juvenile shrimps failed to induce infection in the host, but oral inoculation of the pathogens along with generation of physiological stress in the host juvenile shrimp could effectively induce infection. This gives a very clear picture about the mode of infection of opportunistic pathogen which form normal gut flora of juvenile shrimp.

Since it was proved difficult to induce infection in healthy shrimps (other than by direct injection), Delves-Broughton and Poupard (1976) suggested a most probable close link of environmental parameters in the 'vibriosis' of penaeid shrimp. The present investigation proved this assumption through the pathogenicity experiments by inducing physiological stress to the juvenile shrimps by the alteration of environmental parameter coupled with oral administration of the pathogen.

The resident *Aeromonas* sp. which is reported to have caused infection in the test juvenile shrimps was encountered all through the year as a part of normal gut flora of juvenile shrimps in the culture systems. Year round occurrence of this opportunistic pathogen in the juvenile shrimps and its culture system should be considered a serious issue since it caused septicemic infection which induced 100% mortality in the laboratory reared juvenile *P. indicus*.

The affected test juvenile shrimps showed behavioural abnormalities such as reduced (lethargic) swimming, disorientation while swimming, occasional jumping and lying motionless at the bottom. But control juvenile shrimps also exhibited similar behavioural abnormalities at extreme stress levels. So these symptoms cannot be considered as an exclusive behavioural signs of vibrios instead it can be considered as stress signs.

Clinical signs shown by the affected juvenile test shrimps were opacity of body musculature, reddening of telson, uropod and telson and delay in clotting of haemolymph. Similar clinical symptoms in vibrios have been reported by earlier workers also (Lightner, 1975; 1977; Lightner and Lewis, 1975; Vera *et al.*, 1992; de la Pena, 1993). The opacity of body musculature was shown by control shrimps also at extreme stress levels. It is rather a sign of stress than of "vibriosis". In the studies on tolerance limit of salinity, temperature, oxygen and pH by the juveniles of *P. indicus*, Shylaja (1989) also has reported similar observations. Reddening of body parts is suggested as due to the expansion of integumental erythrophores as has been reported by Lightner and Lewis (1975). Clotting of haemolymph is carried out by coagulin released from haemocytes. The delayed clotting of haemolymph in affected shrimps has been suggested to be due to the reduction in the number of haemocytes caused by continuous phagocytosis and disruption of phagocytes (Stewart, 1980).

Through the pathogenicity experiments, 'vibriosis' was demonstrated to be a consequence of interaction of the facultative pathogen, environmental stressor and the host shrimp. Since the *Vibrio* spp. that infect shrimp are ubiquitous and have been reported from all the culture system studied for this work, they can be facultatively pathogenic to the stressed shrimp population under prolonged unfavourable environmental conditions. So care has to be taken in the management of culture system to avoid different stress factors to the possible minimum inorder to bring down the chances of vibrio infections.

SUMMARY

SUMMARY

1. The results of this research work provide very important information regarding i. the population structure and ecology of *Vibrio* spp. in the shrimp culture systems, ii. seasonal fluctuations in the incidence and distribution,iii. effect of unprecedented rain on vibrio population, iv. optimum growth requirement of vibrios, v. antibiotic sensitivity, vi. minimal inhibitory concentration of two important antibiotics Furazolidone and Perfuran, vii. pathogenicity of two major pathogens isolated from the culture system and viii. the important role of stress in vibrio infection.

2. The incidence of vibrios in the culture system was significantly correlated to the environmental parameters such as temperature, salinity, total hardness and alkalinity of the pond water.

3. The incidence of vibrios was lowest during Monsoon and it increased to the highest level during Summer through Postmonsoon and Transition seasons.

4. The gradual increase observed in temperature, salinity, total hardness and alkalinity of the water resulted in an increase in the vibrio population with greater species diversity.

5. Juvenile shrimps seem to provide a suitable micro-environment for vibrios to attach and multiply.

6. Among the body parts of the juvenile shrimps assayed, the incidence pattern of vibrios observed was digestive system (54.62%), gill (26.89%) and body surface (18.49%).

7. The digestive system along with food provides a very suitable and nutrient-rich environment for the higher incidence and abundance of vibrios with greater species diversity.

8. In the culture pond the incidence of vibrios in water was higher (62.22%) than that in the sediment (38.78%).

9. *V. anguillarum* was the predominant species in the culture system with 24.40% followed by *V. fischeri* like bacteria (19.05%), *V. parahaemolyticus* (17.26%), *V. neries* (11.90%), *V. ordalii* (9.52%), *V. fluvialis* biovar II (8.33%), *V.cholerae* non-O1 (5.36%), *V. proteolyticus* (4.17%).

10. A sudden decline in the vibrio population leading to their disappearance in the culture system immediately after the unprecedented and sudden rain was noticed as an interesting observation. The separate or combined effect of environmental changes in the system and the entry of an alien bacteria *Bacillus* sp. into the system by the rain water has suggested as the probable causative factor for this unusual phenomenon.

11. Optimum growth requirement tests revealed that the ideal temperature, pH and salinity required for the highest growth of vibrios was specific for each species, but over a range of temperature (25°-35°C), pH (7.0-8.5) and salinity (15-30 ppt) they generally demonstrated good growth.

12. *V. proteolyticus* showed certain degree of fastidiousness by exhibiting a lesser growth in the culture broth and agar plates and isolates of this species showed the widest spectrum of drug resistance (resistance to 21 antibiotics out of the 40 tested).

13. Multiple drug resistance was seen from isolates of all vibrios encountered from the shrimp culture system.

14. Among 40 antibiotic tested, Co-trimoxazole was the most effective drug against vibrios. All the isolates were sensitive to co-trimoxazole forming a clear zone of 16-25 mm dia. around the test disc.

15. The resistance pattern observed was Bacitracin, Methicillin, Oxacillin, Penicillin > Colistin > Cephaloridine > Augmentin > Lincomycin, Vancomycin > Ampicillin > Furazolidone > Amikacin, Cephalexin, Oleandomycin > Oxytetracycline > Kanamycin > Strep- tomycin > Tetracycline, Cephalothin > Tobramycine >Chlortetra-cycline > Carbenicillin > Cephotaxime > Chloramphenicol > Co-trimazine, Nitrofurantoin > Amoxycillin, Cefazoline > Poly- myxin-B> Gentamycin > Nalidixic acid > Norfloxacin > Cipro- floxacin, Methanamine mandalate, Neomycine > Clindamycin, Cloxacillin, Erythromycin, Novobiocin > Co-trimoxazole.

16. In the minimal inhibitory concentration test, Perfuran proved more effective than Furazolidone against vibrios. But *V. proteolyticus* was resistant to both these antibiotics, even at 1000 µg/l concentration.

18. The minimum dose of intra-muscular injection required to induce mortality in the juvenile shrimp was found to be 22.5×10^6 cells/animal for *V. anguillarum* and 30.5×10^7 Cells/animal for *V. parahaemolyticus* which indicated that the number of cells of pathogen required to cause death in juvenile shrimp varies from species to species and the incubation period also was found varying.

19. Pathogenicity tests at stress conditions proved that the opportunistic pathogen which form part of normal gut flora of juvenile shrimp could cause infection in a host which is under prolonged specific physiological stress due to physico-chemical parameters.

20. *Aeromonas* sp. which was observed as a resident flora of the juvenile shrimp used for the experiments caused invasive death in the host at stress condition, though it was not the pathogen inoculated for the assay. This again infers that a prolonged stress condition in the system can trigger an infection of the resident opportunistic pathogen present in the gut.

21. Though some earlier workers reported inoculation of the pathogen through food as an unsuccessful means to induce infection, the present study revealed that even such a method of inoculation can cause infection when the host is under prolonged physiological stress.

22. It was found that the most favourable condition for the growth and multiplication of pathogen is not the factor that governs the infection, instead the physiological stress suffered by the host has the crucial role in infection.

23. All stress levels which cause physiological stress were not capable of inducing infection in the juvenile shrimps.

24. At 35 ppt salinity the stress level an unusual behaviour of the animal, was noticed in the pathogenicity experiment. Eventhough 30 ppt and 40 ppt salinity could induce stress in the animal leading to vibrio infection, 35 ppt did not result in any disease conditions in juvenile shrimps. 35 ppt being the true marine salinity which is ideal for the maturation of the juvenile shrimps at best of their health, has the capacity to enable them to resist against infection.

25. Behavioural signs exhibited by the infected juveniles were reduced (lethargic) swimming, disorientation while swimming, occasional jumping and motionless lying at the bottom. But since these symptoms were also showed by control shrimps which were under stress, it can be considered as stress signs.

26. Clinical signs shown by the infected juvenile shrimps were opacity of body musculature, reddening of telson, uropods and pereopods, and delay in the clotting of haemolymph.

27. Through the pathogenicity experiments, 'vibriosis' was demonstrated to be a consequence of interaction between facultative or opportunistic pathogen, environmental factors that induce stress and the host shrimps.

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