PREVALENCE, RISK ASSESSMENT AND SURVIVAL KINETICS OF INDICATOR AND PATHOGENIC BACTERIA IN WATER, SEDIMENT AND BIVALVE SHELLFISH FROM COCHIN ESTUARY

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By

ALLY C. ANTONY
Reg. No: 3947

DEPARTMENT OF MARINE BIOLOGY, MICROBIOLOGY AND BIOCHEMISTRY
SCHOOL OF MARINE SCIENCES
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
KOCHI –682 016, INDIA

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Ph.D. Thesis under the Faculty of Marine Sciences

Author

Ally C. Antony
Research Scholar
Department of Marine Biology, Microbiology and Biochemistry
School of Marine Sciences
Cochin University of Science and Technology
Kochi – 682 016

Supervising Guide

Dr. A. A. Mohamed Hatha
Professor
Department of Marine Biology, Microbiology and Biochemistry
School of Marine Sciences
Cochin University of Science and Technology
Kochi – 682 016

Department of Marine Biology, Microbiology and Biochemistry
School of Marine Sciences
Cochin University of Science and Technology
Kochi – 682 016

May 2017

Department of Marine Biology, Microbiology and Biochemistry
School of Marine Sciences
Cochin University of Science and Technology
Kochi – 682 016

Dr. A. A Mohamed Hatha
Professor

Certificate

This is to certify that the thesis entitled **"Prevalence, Risk Assessment and Survival Kinetics of Indicator and Pathogenic Bacteria in Water, Sediment and Bivalve Shellfish from Cochin Estuary"** is an authentic record of research work carried out by **Ms. Ally C. Antony** under my supervision and guidance in the Department of the Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy in Microbiology of Cochin University of Science and Technology, and no part thereof has been presented for the award of any other degree, diploma or associateship in any other University or Institution. All the relevant corrections and modifications suggested by the audience during the pre-submission seminar and recommended by the Doctoral Committee have been incorporated in the thesis.

Kochi - 682 016
May 2017

Prof. (Dr.) A. A. Mohamed Hatha
(Supervising Guide)

Declaration

I hereby declare that the thesis entitled **"Prevalence, Risk Assessment and Survival Kinetics of Indicator and Pathogenic Bacteria in Water, Sediment and Bivalve Shellfish from Cochin Estuary"** is a genuine record of research work done by me under the supervision and guidance of Dr. A. A. Mohamed Hatha, Professor, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology and no part thereof has been presented for the award of any other degree, diploma or associateship in any other University or Institution earlier.

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Ally C. Antony

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Ally C. Antony

Contents

List of Abbreviations

A/E	-	Attaching and effacing
AA	-	Aggregative adherence
ADB	-	Azide dextrose broth
ANOVA	-	Analysis of Variance
APHA	-	American Public Health Association
ARG	-	Antibiotic resistance gene
ASQAP	-	Australian Shellfish Quality Assurance Programme
BAM	-	Bacteriological analytical manual
BEA	-	Bile esculin azide agar
BHI	-	Brain heart infusion
bp	-	Base pair
CDC	-	Centre for Disease Control and Prevention
CDDEP	-	Center for Disease Dynamics, Economics and Policy
CFU	-	Colony forming unit
CLSI	-	Clinical and Laboratory Standards Institute
COVIS	-	Cholera and other *Vibrio* illnesses surveillance system
°C	-	Degree celsius
DEC	-	Diarrheagenic *E. coli*
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxy ribonucleotide
EAEC	-	Enteroaggregative *E. coli*
EARS-Net	-	European Antimicrobial Resistance Surveillance Network
EC	-	European commission
EFSA	-	European Food Safety Authority
EHEC	-	Enterohemorrhagic *E. coli*
EIEC	-	Enteroinvasive *E. coli*
ELISA	-	Enzyme linked immunosorbent assay
EMB	-	Eosin methylene blue
EPEC	-	Enteropathogenic *E. coli*
ESBL	-	Extended-spectrum beta-lactamase
ETEC	-	Enterotoxigenic *E. coli*
EU	-	European Union
EXPEC	-	Extraintestinal pathogenic *E. coli*
FAO	-	Food and Agricultural Organization

FC	-	Faecal coliforms
FIB	-	Faecal indicator bacteria
FS	-	Faecal streptococci
HC	-	Hemorrhagic colitis
HEA	-	Hektoen enteric agar
HLG	-	High level gentamycin
HLS	-	High level streptomycin
HUS	-	Hemolytic uraemic syndrome
kb	-	Kilo base pair
KF	-	Kenner faecal streptococcal agar
KMFDS	-	Korea Ministry of Food and Drug Safety
LAP	-	Leucine aminopeptidase
LEE	-	Locus of enterocyte effacement
LIA	-	Lysine Iron Agar
MAEC	-	Meningitis-associated *E. coli*
MAR	-	Multiple antibiotic resistance
MDE	-	Maryland Department of Environment
MDR	-	Multidrug-resistant
Mg	-	Milligram
mL	-	Millilitre
mM	-	Millimolar
MOF	-	Ministry of Oceans and Fisheries
MPN	-	Most probable number
MRSA	-	Methicillin resistant *Staphylococcus aureus*
NaCl	-	Sodium chloride
NHMRC	-	National Health and Medical Research Council
NSEC	-	National *Salmonella* and *Escherichia* center
NSSP	-	National Shellfish Sanitation Program
NZFSA	-	New Zealand Food Safety Authority
PCR	-	Polymerase chain reaction
PFU	-	Plaque forming unit
ppt	-	Parts per thousand
PYR	-	Pyrrolidonylarylamidase
R	-	Rough type
RS	-	Raw sediment
RTE	-	Ready to eat

RVS	-	Rappaport-Vassiliadis soy broth
RW	-	Raw water
SHV	-	Sulfhydryl variable
SPSS	-	Statistical Package for the Social Science
STEC	-	Shiga toxin-producing *E. coli*
TBE	-	Tris-borate-EDTA buffer
TC	-	Total coliforms
TCBS	-	Thiosulphate citrate bile salt sucrose agar
TEM	-	Temoneira
TSA	-	Trypticase soy agar
TSI	-	Triple sugar iron agar
TTB	-	Tetrathionate broth
UPEC	-	Uropathogenic *E. coli*
US EPA	-	United States Environmental Protection Agency
US FDA	-	United States Food and Drug Administration
UT	-	Untypable
UTI	-	Urinary tract infection
UV	-	Ultraviolet
VBNC	-	Viable but nonculturable
VDH	-	Virginia Department of Health
VP	-	Voges Proskauer
VRE	-	Vancomycin resistant enterococci
VTEC	-	Verotoxin-producing *E. coli*
WHO	-	World Health Organization
XLD	-	Xylose Lysine Deoxycholate
%	-	Percentage
µL	-	Microlitre
µm	-	Micrometer

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Chapter 1

GENERAL INTRODUCTION

Contents

1.1 Introduction

Seafood constitutes an important food component for a large portion of the world population, especially in the developing countries. Fish and shellfish have constituted an important cheap source of protein from time immemorial. Bivalve molluscs form an important natural fishery resource of India. Bivalve shellfish include clams, oysters, mussels, scallops and other members of the phylum Mollusca. Among the molluscan shellfish, clams provide livelihood for the local population, who exploits them for meat and shells. Clam meat is a rich source of protein and essential nutrients including essential fatty acids, minerals and vitamins such as A and D. It is also cheap, when compared to other seafood such as crabs, prawns and fishes; hence it is relished by the rich and poor alike. There are many other health benefits derived from fish consumption such as reduced incidence of coronary heart diseases, improved brain development and eyesight (Sioen *et al*., 2007) which makes it an important part of healthy diet.

1.1.1 Food safety concerns associated with bivalves

In spite of all the aforesaid nutritional and health benefits, food safety remains the primary concern associated with bivalve shellfish consumption. This can be attributed to their filter feeding nature by which, they accumulate all environmental contaminants. Incidentally, most of the shellfish growing areas are situated in shallow, nutrient rich, sheltered near-shore waters, which often receive river and land run-off and domestic and other industrial sewage discharges. Hence, they have been often proposed as bio-indicators for assessment of faecal contamination in recreational waters (Roslev *et al*., 2010).

1.1.2 Shellfish associated disease outbreaks

Many shellfish associated food borne outbreaks have been reported in various parts of the world. According to FAO reports (FAO, 2014) 10% of all food borne disease outbreaks are caused by seafood. However, only less than 1% of the true incidences are actually reported (Mossel, 1982). As per recent CDC reports, in 2013 (CDC, 2013b), the food category mostly implicated in food borne disease was related to molluscs (11%) and the most implicated bacterial pathogen coupled with this food source was *Vibrio parahaemolyticus*. In 2013, *Vibrio parahaemolyticus was* responsible for 4 outbreaks associated with raw oysters and clams. In addition, 2 out of the total 14 product recalls that happened in 2013 were due to oyster related disease outbreaks.

Most of the reports of shellfish related disease outbreaks are from US and Europe, whereas Asia and Africa has very less reporting. Reports of shellfish related disease outbreaks from developing countries are less, and least from African countries, possible reason being lack of prompt surveillance and reporting systems rather than non-occurrence of such events (Potasman

et al., 2002). Bivalves are notorious as the vehicles for many food borne diseases ranging from viral Hepatitis to the deadly Cholera (Costa, 2013; Iwamoto *et al*., 2010; Rippey, 1994). Shellfish consumption has been associated with many viral diseases like Hepatitis and bacterial diseases like vibriosis, salmonellosis etc. Poor sanitary quality of seafood is a serious health hazard, which may also exert negative impact on seafood exports, thus affecting the economy of the country.

1.1.3 Sanitary indicators of shellfish growing areas

1.1.3.1 Faecal coliforms as sanitary indicators

Faecal coliforms and faecal streptococci are the universally accepted indicators of faecal contamination (NHMRC, 2008; Edberg *et al*., 2000; USEPA, 1986). Sanitary water qualities of shellfish growing areas have been evaluated based on the faecal colifom levels of the growing areas. Estimation of faecal coliforms in the water bodies as well as shellfish will provide an idea about the level of faecal contamination the aquatic environment as well as shellfish has undergone (Chinnadurai *et al*., 2016; Mok *et al*., 2016) .

1.1.3.2 Enterococci as sanitary indicators

Though enterococci are shown to survive better in the environment than other Enterobacteriaceae members (Noble *et al*., 2004), they are not considered for the assessment of microbiological quality of growing areas, as a function of their suitability for bivalve cultivation. However, a preliminary study by Sobsey (1989), has suggested that there may be a correlation between illness and enterococcus counts in shellfish flesh at harvest. Study of indicator bacteria has been useful in microbial source tracking which will help to identify the sources of non point-source faecal pollution in natural waters.

Apart from their role as faecal indicators, there are many reports stating that enterococci are opportunistic or rather emerging pathogens responsible for many community-acquired, hospital-acquired (nosocomial) super infections such as endocarditis, bacteraemia etc (Morrison *et al*., 1997; Murray, 1990).

1.1.4 Classification of shellfish growing areas

Realising the importance of maintaining good sanitation levels in shellfish growing areas, regulatory bodies of several countries have made monitoring and certification of shellfish growing areas mandatory, failing which the areas are declared closed for harvesting until the required sanitation levels are attained. Statutory bodies of various countries such as United States (NSSP, 2015), European Union (EC No. 854/2004), New Zealand (New Zealand Food Safety Authority (NZFSA), 2006) Korea (Korea Ministry of Food and Drug Safety (KMFDS), 2015; Ministry of Oceans and Fisheries (MOF), 2015), Australian Shellfish Sanitation Program (ASQAP, 2016) etc. have formulated guidelines to classify growing areas, based on which post harvest treatments have to be planned.

1.1.5 Prevalence of pathogens in shellfish growing areas

1.1.5.1 Pathogenic *E. coli*

Apart from their role as faecal indicators, there are some potentially pathogenic or enterovirulent strains also among *E. coli*, hence their presence in shellfish growing areas is a matter of great concern which cannot be overlooked. Though not very frequent, there are a few reports regarding prevalence of shiga toxin producing *E. coli* (Baliere *et al*., 2015; Bennani *et al*., 2012) and other pathogenic groups such as enteropathogenic and enterohemorrhagic *E. coli* strains from shellfish growing areas worldwide (Brandao *et al*., 2017; Bennani *et al*., 2012).

1.1.5.2 *V. parahaemolyticus*

V. parahaemolyticus, an indigenous bacterium associated with estuarine, marine and coastal surroundings, has been recognized as the leading cause of human gastroenteritis associated with seafood consumption. There were 1280 reports of infection due to the organism in 1994-95 (Anon, 1999). Several researchers have reported the role of *V. parahaemolyticus* in shellfish associated disease outbreaks (Newton *et al*., 2012; Fuenzalida *et al*., 2007) worldwide.

1.1.5.3 *Salmonella*

Though *Salmonella* has been identified as one of major cause of seafood related outbreaks worldwide, recent reports suggest that it is no longer a major safety hazard associated with shellfish growing areas nowadays (Lunestad *et al*., 2016; Mok *et al*., 2016). However, being a potential pathogen, its absence in 25 g shellfish sample has been set as the regulatory limits for human consumption. Before 1950s, it was one of the common illnesses associated with raw bivalve consumption in United States, causing outbreaks involving more than 1500 cases and 150 deaths. However, as a result of stringent regulatory interventions, since 1950 no shellfish associated typhoid cases have been reported (Rippey, 1994). However, incidences of non-typhoidal *Salmonella* associated illnesses associated with bivalve consumption have been occasionally reported worldwide (Rahimi *et al*., 2013; Martinez-Urtaza *et al*., 2003).

1.1.6 Antibiotic resistance among pathogenic bacteria

Antibiotic resistance is nowadays considered as one of the paramount public health challenges globally. Emergence of more and more resistant bacteria to newer classes of antibiotics is a real challenge in chemotherapy,

as the once commonly used life saving drugs become no longer useful and even the smallest infections sometimes prove lethal. Indiscriminate use of antibiotics, result in increased antibiotic residues discharged into natural environments, exposure to which leads to the selection of drug resistant mutants of bacteria (Berglund, 2015; Chattopadhyay *et al*., 2015). Once developed resistance, there is high risk of horizontal as well as vertical transmission of resistance rendering more and more organisms resistant. Emergence of multiple drug resistant (MDR) strains of *E. coli*, enterococci, *V. parahaemolyticus* and *Salmonella* have been widely reported.

E. coli resistant to third generation cephalosporins, carbapenem and colistin have been reported worldwide. Several studies have revealed increasing antibiotic resistance among *E. coli* isolated from food producing animals (Filioussis *et al*., 2013; Jones-Dias *et al*., 2013).

There are several reports about antibiotic resistance of *V. parahaemolyticus* isolated from seafood from various geographical locations (Hu and Chen, 2016; Xu *et al*., 2016). Resistance to penicillin and ampicillin have been widely reported in *V. parahaemolyticus* from a number of sources such as coastal waters, wastewater, fish farms, shrimp farms, shellfish samples etc. (Silvester *et al*., 2015; Sudha *et al*., 2014; Ottaviani *et al*., 2013).

Enterococci pose potential challenge to chemotherapy, because of their inherent as well as acquired resistance to a number of antibiotics. Enterococci demonstrate intrinsic resistance towards penicillins, cephalosporins, nalidixic acid, macrolides, aztreonam, trimethoprim-sulfamethoxazole and low levels of clindamycin and aminoglycosides (Chajecka-Wierzchowska *et al*., 2014; Morrison *et al*., 1997). In addition to intrinsic resistance, enterococci also

have acquired resistance to penicillin by beta-lactamases, chloramphenicol, tetracyclines, rifampin, fluoroquinolones, aminoglycosides (high levels) and vancomycin.

1.1.7 Presence of antibiotic resistance genes

The genes encoding drug resistance may be chromosomal or present on some mobile genetic elements such as plasmids or transposons. If the genetic determinants of resistance are present on mobile genetic elements, they may be transferred to other commensal bacteria or bacteria of clinical significance (Schuurmans *et al*., 2014).

E. coli has been found to harbour several antibiotic resistance genes such as extended spectrum beta lactamases (ESBLs), *mcr-1* etc. ESBL producing *E. coli* are resistant to penicillins as well as first, second and third-generation cephalosporins and aztreonam. There are many recent reports regarding transmission of ESBL producing Enterobacteriaceae from farm animals to humans especially through food chain (Lazarus *et al*., 2015; Valentin *et al*., 2014) which is identified as a serious threat for public health. *Mcr-1*, the first reported plasmid-mediated colistin resistance gene was first identified in *E.coli* from food animals, food and humans in China (Liu *et al*., 2016b). Recent studies have revealed the spread of *mcr-1* gene across most of the continents (Battisti, 2016; Falgenhauer *et al*., 2016), and has been isolated from various food animals.

Vancomycin resistant enterococci (VRE) harbour vancomycin resistance genes; thus posing potential therapeutic challenge. There are evidences of horizontal transmission of these genes beyond the genus *Enterococcus* which render other bacteria also drug resistant.

1.1.8 Virulence factors/ genes among pathogenic bacteria

1.1.8.1 Toxigenic genes in *E. coli*

Pathogenic *E. coli* have been detected harbouring several toxigenic genes such as shiga like toxin (stx_1 and stx_2) coding genes, *eaeA* gene coding for cell adhesion protein intimin, enterohemolysin EHEC-*hlyA* gene etc. Shiga-like toxin-producing *E. coli* (STEC) with various seropathotypes have been reported from various parts of the world, from diverse sources ranging from environmental to food and clinical samples (Khalil and Gomaa 2016; Loukiadis *et al*., 2006).

1.1.8.2 Virulence genes in *V. parahaemolyticus*

Tdh and *trh* were the virulent genes first identified in *V. parahaemolyticus* associated with disease outbreaks. Strains producing virulence genes, *tdh* and *trh* are generally considered pathogenic to man. Majority of clinical strains produce virulence genes, *tdh* and *trh*, while very less environmental strains produce this haemolysin and thus are not usually pathogenic (Ceccarelli *et al*., 2013; US FDA, 2005).

1.1.9 Factors affecting bacterial survival in natural aquatic environments

Natural environments are dynamic systems influenced by an array of variables, and hence the survival of indicator as well as pathogenic bacteria is largely affected by the conditions that prevail in such environments. Survival of these organisms is largely influenced by a number of physical, chemical, and biological factors such as solar radiation, temperature, salinity, nutrients, other competing bacterial flora, bacteriophages, protozoan predation etc.

1.1.9.1 Effect of temperature on survival

Though the optimum growth temperature required for enteric bacteria is found to be 37 °C, bacterial survival has been found to occur at wide temperature ranges as it exists in natural environments. Some researchers have reported that, lower temperatures favour increased growth of bacteria in fresh and marine waters (An *et al*., 2002; Rozen and Belkin, 2001); some others have reported contradictory findings that higher temperatures favour increase in bacterial survival (Crabill *et al*., 1999), whereas Alkan *et al*. (1995) noted no significant impact of temperature on FC survival.

1.1.9.2 Effect of salinity on survival

Salinity has been found to be an important factor influencing the survival of bacteria in marine and estuarine environments. Saline conditions impact the survival of bacteria by posing unwanted stress on cells and hence an inverse relationship exists between osmotic stress and survival of faecal indicator bacteria (FIB). Rapid death rate of coliforms has been reported in marine environments whereas enterococci are believed to survive well in marine waters (Anderson *et al*., 2005).

1.1.9.3 Role of biotic factors on survival

Natural microbiota (predation and competition) plays significant role in survival of bacteria in natural environments. Diverse groups of the food web which includes protozoa, lytic phages and predatory bacteria have been found to significantly regulate bacterial concentrations in marine and fresh water environments (Barcina *et al*., 1997). Protozoan predation accounts for majority of the bacterial mortality in aquatic habitats. Competing autochthonous bacteria has been reported to have significant impact on bacterial survival in natural environments (Staley *et al*., 2011). Bacteriophages form an important

component of the self-purifying ability of aquatic habitats and they regulate bacterial population by cell lysis.

1.1.10 Extended bacterial survival in sediments compared to overlaying water

Increased bacterial survival rates have been observed in sediments compared to overlying water, due to the increased availability of nutrients, better protection due to attachment to particles, reduced protozoal predation compared to water, better protection from UV radiation etc. (Suter *et al*., 2011; An *et al*., 2002). Sediment may also remain as a permanent source of contamination to the above water body releasing pathogens when re-suspended due to natural or anthropogenic activities.

1.1.11 Reduction of microbial load in bivalve shellfish by depuration

Depuration is the process by which harvested shellfish is placed in land-based plants containing clean sea water, to permit the purging of their gastrointestinal contents under controlled conditions (Jackson and Ogburn, 2009; Richards, 1998). This process has been found to be effective in reducing the microbial load to regulatory limits and thus safe for human consumption.

1.2 Significance of the study

Cochin estuary is a part of the Vembanad Lake, which is one of the important Ramsar sites of India. It is a rich natural resource of fish and shellfish and forms life line of thousands of local fishermen in the area. In addition, a number of seafood export companies are also located in this area. Hence, the sanitary quality of the aquaculture products harvested from the area calls for special attention. Several previous studies have revealed the extensive pollution the estuary has undergone, mainly due to the proximity to point sources of effluents such as the satellite town ships along the

Cochin estuary as significant amount of partially or improperly treated domestic sewage from these places finds their way to the estuary. Other point source effluents include the drains from market places and abattoirs, seafood factories etc. besides several non-point sources of pollution.

Present study envisages to assess the microbial quality of water, sediment as well as shellfish (*Villorita cyprinoides* var. *cochinensis*) collected from shellfish harvesting areas of Cochin estuary, in terms of faecal indicators as well as pathogens themselves. Risk assessment of the isolated bacterial species in terms of their antibiotic resistance, resistance genes and other pathogenic/virulence genes is also undertaken. This will help to understand if any possibility for cycling of resistance/virulence genes exists between environment, food and human sources. Since no previous sanitary surveys of shellfish harvesting areas in the study area have been conducted, present findings may form a baseline reference for regulatory interventions required, if any.

1.3 Broad objectives

- To monitor the prevalence of faecal indicator bacteria such as faecal coliforms, faecal streptococci and specific pathogens such as *V. parahaemolyticus* and *Salmonella* in shellfish (*Villorita cyprinoides* var. *cochinensis*), sediment and water from various shellfish harvesting areas in Cochin estuary.
- To study the serological diversity of *Escherichia coli* isolated from shellfish (*Villorita cyprinoides* var. *cochinensis*), sediment and water collected from different shellfish harvesting areas in Cochin estuary and to determine the prevalence of pathogenic serotypes.

- To study the antibiotic resistance profiles of *E. coli,* enterococci, and *V. parahaemolyticus* isolated from shellfish growing areas of Cochin estuary.
- To analyze the prevalence of antibiotic resistance genes in *E. coli* and enterococci.
- To assess the prevalence of toxigenic genes/virulence genes in *E. coli* and *V. parahaemolyticus* isolated from shellfish growing areas of Cochin estuary.
- To study the survival of *E. coli*, *E. faecalis* and *V. parahaemolyticus* in estuarine water as a function of varying temperatures and salinities.
- To study the survival of *E. coli*, *E. faecalis* and *V. parahaemolyticus* in estuarine water and sediment as a function of biological factors (protozoan predation, autochthonous bacteria and bacteriophages)
- To assess the efficacy of a closed water depuration system fitted with filter containing activated carbon from locally available material (coconut shell), to reduce the number of total coliforms (TC), faecal coliforms (FC) and faecal streptococci (FS) from naturally contaminated shellfish (*Villorita cyprinoides*) harvested from Cochin estuary to regulatory limits.

Chapter 2

PREVALENCE OF FAECAL INDICATOR AND PATHOGENIC BACTERIA IN SHELLFISH (*VILLORITA CYPRINOIDES* VAR. *COCHINENSIS*), SEDIMENT AND HARVESTING WATERS FROM COCHIN ESTUARY

Contents

2.1 Introduction

Pollution is the most important hazard affecting all environments including marine and estuarine ecosystems. Industrialization, urbanization, inadequate sewage treatment systems, poor hygienic practices and either lack of regulations or lapse in their implementation, has resulted in increased microbial contamination of aquatic bodies worldwide. Sanitary water quality attracts worldwide attention due its substantial role in fish and shellfish consumption associated disease outbreaks, recreation disruptions and contamination of crops eaten raw (Costa, 2013; Painter *et al*., 2013; Rippey, 1994). These water bodies are the biggest wild resources of seafood, and faecal contamination adversely affects the sanitary quality of the commodities posing potential health hazards to the consumers. The magnitude of these faecally polluted urban discharges often exceeds the self-purifying limits of

the receiving water bodies, as a result of which these water bodies become unfit for all the aforesaid beneficial purposes.

Bivalves are filter feeders, and during their feeding process they tend to accumulate all sorts of contaminants in the surrounding water including microbial pathogens (Pavoni *et al*., 2013; Pinto and Bosch, 2013). Hence, they have been proposed as bio-indicators for assessment of faecal contamination in recreational waters (Roslev *et al*., 2010). Contamination of shellfish growing areas in particular, is a matter of great concern, since shellfish is a delicacy preferred to be eaten either raw or partially cooked in many parts of the world (Iwamoto *et al*., 2010; Potasman *et al*., 2002). Many infectious disease outbreaks like typhoid and cholera associated with shellfish consumption have been reported worldwide (Costa, 2013; Rippey, 1994). Realising the importance of maintaining good sanitation levels in shellfish growing areas, regulatory bodies of several countries have made monitoring and certification of shellfish growing areas mandatory, failing which the areas are declared closed for harvesting. Poor sanitary quality of shellfish growing areas have resulted in huge economic losses due to closure of such harvesting areas until the required sanitation levels are attained (Chigbu *et al*., 2005; Rabinovici *et al*., 2004). Due to elevated fecal coliforms 176 shellfishing areas in Virginia were indefinitely closed (US EPA, 2013; VDH, 2012) and 77 shellfish beds in Maryland were occasionally or permanently closed (MDE, 2016).

Cochin estuary, located towards the southwest coast of India, is a rich wild resource of shellfish and finfish fishery. Due to its proximity to the town ship, the estuary is highly polluted which may adversely affect the sanitary quality of the fish and shellfish resources harvested from it. There

are several recent reports about the extensive pollution the estuary has undergone (Sukumaran and Hatha, 2015; Sukumaran *et al*., 2012; Hatha *et al*., 2004). However, there are no previous reports or systematic study being undertaken to assess the sanitary quality of the shellfish harvesting areas located along this estuary, which has serious public health implications. In this context, assessing the prevalence of faecal coliforms and pathogenic bacteria in this estuarine environment is of great public significance and hence a potential topic of extensive research.

2.2 Review of Literature

2.2.1 Food safety concerns associated with bivalves

Food safety is the primary issue associated with shellfish consumption. Bivalves are notorious as the vehicles for many food borne diseases, ranging from viral Hepatitis to the deadly Cholera (Costa, 2013; Iwamoto *et al*., 2010; Rippey, 1994). Reports of shellfish related disease outbreaks from developing countries are less, and least from African countries, possible reason being lack of prompt surveillance and reporting systems rather than non-occurrence of such events (Potasman *et al*., 2002).

Among the inhabitants of coastal ecosystems, sedentary bivalve fauna is highly prone for contamination due to their peculiar filter feeding nature and bio-accumulation potential (Chigbu *et al*., 2005; Burkhardt, and Calci, 2000). Incidentally, most of the shellfish growing areas are situated in shallow, nutrient rich, near-shore waters which often receive river and land run-off and domestic and other industrial sewage discharges. Microbial load in the near shore areas are high compared with that off shore waters. The incidence of enteric bacteria like *E. coli*, *Shigella*, *Salmonella* and *Vibrio* have been found to be high in near shore waters (Robin *et al*., 2012). These

filter feeders being exposed to all sorts of contaminants for extended periods, tend to concentrate the contaminants in their tissues many times higher than the surrounding water, at levels hazardous to the consumers. They filter large volumes of water to trap the food particles, during which the contaminating microorganisms including pathogens accumulate especially in the liver like digestive glands (Potasman *et al*., 2002).

The hazards associated with bivalve consumption are compounded by the traditional consumption of raw or partially cooked shellfish in many parts of the world (Iwamoto *et al*., 2010; Potasman *et al*., 2002). Hence the chances of the pathogens getting eliminated before consumption is less, unless efficient depuration strategies are strictly followed (Almeida and Soares, 2012; Brands *et al*., 2005; Butt *et al*., 2004).

2.2.2 Faecal indicator bacteria (FIB) and other bacterial pathogens in shellfish growing areas

Consumption of contaminated food and water often causes food related disease outbreaks. Diseases like typhoid and cholera are mainly spread through water contaminated with faecal wastes and hence, it is important to determine the suitability of water for the intended purposes. For this simple, reliable, and rapid methods for detection and enumeration of microorganisms are necessary. Pathogens are not detected easily and their routine culturing is not practical or rather impossible. Hence, methods have been developed to determine the presence of other faecal indicator microorganisms whose presence indicates the possibility of the presence of other bacterial pathogens. Faecal coliforms and faecal streptococci are the universally accepted indicators of faecal contamination (NHMRC, 2008; Edberg *et al*., 2000; US EPA, 1986).

The presence of indicator bacteria does not mean the water contains pathogenic microorganisms, but rather the possibility exists for the presence of pathogens, since the indicator bacteria point to faecal contamination in the sample. The number of pathogens that might be associated with the concentration of the indicator will be a function of the disease incidence in the community at the time the faecal material was disposed. A correlation between *E. coli* levels and presence of pathogens such as *Salmonella* has been established (Prato *et al*., 2013). Even though coliforms are the widely accepted indicator bacteria, their reliability is often questioned, as they are found to be occasionally present when pathogens are absent (Rivera *et al.,* 1988) and absent when other pathogens are present (Harwood *et al*., 2005). There is another opinion that they do not reliably predict the occurrence and survival of enteric viruses and other pathogens in the marine environment (Hernroth *et al*., 2002; Lees, 2000). Estimation of faecal indicator bacteria in the water bodies as well as shellfish will provide an idea about the level of contamination the aquatic environment as well as the shellfish has undergone.

2.2.3 Prevalence of faecal coliforms and *Escherichia coli* in shellfish growing areas

Coliforms are bacterial species that may inhabit the intestines of warm-blooded animals, or occur naturally in soil, vegetation, and water. They are Gram-negative, rod-shaped facultatively anaerobic bacteria which produce gas from glucose (and other sugars) and ferment lactose to acid and gas within 48 h at 35 ºC (Hitchin *et al*., 1995). The coliform group includes species from the genera *Escherichia, Klebsiella, Enterobacter,* and *Citrobacter*, and principally includes *E. coli*. Coliforms were historically used as indicator microorganisms to serve as a measure of faecal contamination, and thus potentially, of the presence of enteric pathogens in fresh water. Although

some coliforms are found in the intestinal tract of man, most are found throughout the environment and have little sanitary significance (Greenberg and Hunt, 1985). Though coliforms are not usually pathogenic themselves, their presence in large numbers in foods is highly undesirable, but it would be rather impossible to eliminate all of them (Jay *et al*., 2005). They enter the water bodies as a result of faecal contamination; once introduced into the water, they survive in the water body depending on the environmental conditions.

Faecal coliforms, a subcategory of coliforms are better indicators of faecal contamination than total coliforms because, they are more directly associated with faecal contamination from warm-blooded vertebrates, than are other members of the group. *E. coli*, representative species of the faecal coliform group, is always found in faeces and is therefore, a more direct indicator of faecal contamination and thus the possible presence of enteric pathogens. *E. coli* usually makes up 75-95% of the faecal coliform count in shellfish growing areas, but at times can represent less than 1% of the coliform count (Campos *et al*., 2011; Paille *et al*., 1987; Greenberg and Hunt, 1985).

There are several sanitary survey reports of shellfish growing areas from various parts of the world. Mok *et al*. (2016) conducted a full sanitary survey of pollution sources in proximity to Hansan-Geojeman region, a prominent shellfish growing area in Korea. Even though several potential sources of pollution existed in the proximity, the dilution was sufficient, and hence no pathogens were detected and the FC levels of sea water and shellfish met the regulation limits of most of the countries. Campos *et al*. (2013) examined the temporal trends in 57 shellfish production areas along the coast of England and Wales during 1999-2008 and found that microbial

quality of majority of the harvesting areas improved largely may be due to the sewerage improvement schemes implemented. Similar observations were made Prato *et al*. (2013) in shellfish harvesting areas of Italy based on three studies. FC levels in shellfish progressively declined during the studies, so also the incidence of *Salmonella* and Hepatitis A cases. Lalitha and Surendran (2005) studied the microbiological quality of black clam *Villorita cyprinoides* var. *cochinensis* and water samples collected from three clam harvesting areas in Vembanad lake. The faecal contamination levels were high as indicated by high levels of *E. coli* and enterococci which did not conform to legal standards.

FC levels of shellfish harvesting areas have been found to be closely influenced by seasonal changes and rainfall. Several studies have revealed several fold increase in FIB numbers in the surface water bodies after storm events (Sidhu *et al*., 2012; Parker *et al*., 2010; Brownell *et al*., 2007). FC levels are usually high and often above the regulatory limits during monsoon seasons whereas during dry season in most areas the levels were within acceptable regulatory limits. This is mainly due to contamination of water bodies from nonpoint sources during rainfall events whereas during dry months point sources are responsible for pollution (Chinnadurai *et al*., 2016; Campos *et al*., 2011; Sasikumar and Krishnamoorthy, 2010; Lipp *et al*., 2001; Rose *et al*., 2001).

Chinnadurai *et al*., (2016) conducted a study in oyster growing areas of Ashtamudi Lake in India and made similar findings. FC levels were highest during monsoon months while it fell below the threshold limits during pre-monsoon and post-monsoon periods. Sasikumar and Krishnamoorthy (2010) also made similar findings from southern coastal district of Karnataka, where highest FC levels were observed in seawater, sediments and mussel

tissue during monsoon season. Fortunately, the harvesting seasons were during the pre-monsoon and post-monsoon seasons where the FC levels conformed to regulatory standards. Chigbu *et al*. (2005) also reported that FC geometric mean levels exhibited a positive relationship with rainfall in the Mississippi Sound.

Seasonal temperature variation also affected the abundance and survival of bacteria (Rippey, 1994). Bacteria including coliforms may proliferate in nutrient-rich waters during warmer months and survive in colder months (Anderson *et al*., 1983). Avila *et al*. (1989) described the monitoring of seawater quality on the southern coast of Spain and reported values of microbial counts around eight-fold higher in summer compared to that in winter. The incidence of increased number of *E. coli* in warmer months is confirmed by the results of DePaola *et al*. (2010), where highest geometric mean levels of *E. coli* (~200 MPN/100g) were found in the Gulf region oysters during the summer.

Several researchers recorded the bioaccumulation of contaminants by mussels which resulted in elevated microbial levels in mussel tissue compared to overlying waters. Mok *et al*. (2016) reported bioaccumulation factors ranging from 7.4 to 12.8 fold for FC in oyster samples compared to seawater. The accumulation rates differed among various filter-feeders, depending on their ability to accumulate microorganisms which varied from a few fold to more than hundreds-fold (Burkhardt and Calci, 2000; Dore and Lees, 1995).

Chinnadurai *et al*. (2016) reported mean accumulation factor of 10.67 for faecal coliforms and 5.20 for *E. coli* in oysters. Significant variation in accumulation with various seasons was observed; bioaccumulation was high

during the pre-monsoon season which characterized transition from the cooler post-monsoon months to the warmer summer months. However, no significant correlation between accumulation and environmental parameters such as water temperature, salinity, pH and rainfall was observed during study.

Hatha *et al*. (2003) evaluated the microbiological of shrimp products for export trade, produced from cultured shrimp and observed high prevalence of *E. coli* in headless shell-on shrimps. *E. coli* was also detected in cooked, peeled tail-on shrimp samples. Environmental variables also influenced significantly like semidiurnal tidal cycles; clear correlation was found between high river run off, low salinity and high levels of *E. coli* in shellfish and seawater.

Venkataraman and Sreenivasan (1954) studied the mussel pollution at korapuzha estuary (Malabar), and found seasonal variation in the coliform counts. They found a parallelism in the coliform numbers of overlaying water, mussels from bed, and mussels from the market. The total bacterial counts were the lowest in October, but highest in July.

Lalitha and Surendran (2005) assessed the bacterial flora of black clam (*Villorita cyprinoides* var. *cochinensis*) and water samples collected from three clam harvesting areas in Vembanad lake (Kerala, India). The shellfish collected from Vembanad lake showed faecal contamination at levels which did not conform to legal standards. High prevalence of *E. coli*, faecal streptococci and *C. perfringens* in water and clam indicated high degree of faecal pollution of the harvesting areas.

Sediments in shellfish growing areas often act as repository of indicator and pathogenic bacteria releasing them to the overlaying water column when disturbed by natural phenomena like rainwater runoff, storms,

tidal events (Gerba and Schaiberger, 1975) or by anthropogenic activities such as dredging, boating etc (Grimes, 1975). The microorganisms entering the aquatic bodies may settle in the sediment layer by adsorption to particulate matter. Higher concentrations (LaBelle *et al*., 1980; Chen *et al*., 1979; Gerba *et al*., 1979) and extended survival (Lear, 1985; Rao *et al*., 1984; Smith *et al*., 1978; Gerba and McLeod, 1976) of faecal indicators and pathogens such as *Salmonella* have been demonstrated in sediments compared to overlaying water column, which serve as a permanent source of contamination to these shellfish growing areas. Martinez-Manzanares *et al*. (1992) conducted a 19 months study in an estuary in Spain and reported presence of maximum number of all the microorganisms studied, in sediments followed by shellfish and water demonstrated least. This was in agreement with the findings of Bennani *et al*. (2012) who studied the prevalence of *E. coli* in shellfish and Mediterranean coastal environments of Morocco. Higher prevalence of *E. coli* strains was reported in sediment samples (40%) followed by shellfish samples (30%) and sea water samples (14%) demonstrated the least.

2.2.3.1 Classification of shellfish growing areas

Faecal contamination of shellfish growing areas poses serious threat to consumer safety. Statutory bodies of various countries such as National Shellfish Sanitation Programme of US FDA (NSSP, 2015), European Union (EC No. 854/2004), New Zealand (New Zealand Food Safety Authority (NZFSA), 2006) Korea (Korea Ministry of Food and Drug Safety (KMFDS), 2015; Ministry of Oceans and Fisheries (MOF), 2015), Australian Shellfish Sanitation Program (ASQAP) etc. have formulated guidelines to classify growing areas, based on which post harvest treatments have to be planned.

The classification is based on either faecal coliform or *E. coli* levels in shellfish harvesting waters or bivalve tissue and intravalvular fluids. The shellfish growing area classification as per two important regulations are as follows:

Table 2.1 European Union (EU) criteria (EC directive No. 854/2004)

Class	Microbiological standard	Post-harvest treatment required
A	Live bivalve molluscs from these areas must not exceed 230 MPN E. coli per 100 g of flesh and intra-valvular fluid.	None.
B	Live bivalve molluscs from these areas must not exceed , in 90% of the samples, 4600 E. coli per 100 g of flesh and intra-valvular liquid. In the remaining 10% of samples, live bivalve molluscs must not exceed 46,000 E. coli per 100 g of flesh and intra-valvular fluid.	Purification, relaying or cooking by an approved method.
C	Live bivalve molluscs from these areas must not exceed the limits of a five-tube, three dilution MPN test of 46000 E. coli per 100 g of flesh and intra-valvular fluid.	Relaying or cooking by an approved method.

Table 2.2 National Shellfish Sanitation Programme of US FDA (NSSP, 2015)

	Total coliforms per 100 mL water		Faecal coliform per 100 mL water		
Classification	**Geometric Mean**	**90% compliance**	**Geometric Mean**	**90% compliance**	**Treatment required**
Approved areas	≤70	≤230	≤14	≤43	None
Restricted areas	≤700	≤2300	≤88	≤260	Purification or relaying in an approved area
Prohibited areas	No sanitary survey or conditions for approved/restricted areas not met				Harvesting not permitted

2.2.3.2 Incidence of pathogenic *E. coli* in shellfish growing areas

Apart from their role as faecal indicators, there are some potentially pathogenic or enterovirulent strains also among *E. coli*, hence their presence in shellfish growing areas is a matter of great concern, which cannot be overlooked. Pathogenic strains of *E. coli* are transferred to seafood either through primary contamination of the coastal environment by sewage pollution or by secondary contamination after harvest. Food borne *E. coli* infection causes abdominal cramps, water or bloody diarrhoea, fever, nausea and vomiting and sometimes serious cases such as haemolytic uremic syndrome (HUS) or even kidney failure and death may happen (Ward *et al*., 1997).

The species *Escherichia coli* is serologically divided into several serogroups (based on somatic O antigens) and serotypes (based on flagellar or H antigens). The species comprises of both intestinal and extraintestinal pathogens. The intestinal pathogens also known as diarrheagenic *E. coli* (DEC), have been characterized into six various categories: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adhering *E. coli* (DAEC) (Campos *et al*., 2004; Nataro and Kaper, 1998).

Among the various pathogenic subgroups, the major subgroup is enterohaemorrhagic *E. coli* (EHEC), which causes haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS). They are also called Shigatoxigenic (STEC) or Verocytotoxigenic (VTEC) *E. coli* because of their ability to produce shiga like toxins. Six verotoxins have been identified within this group, but only stx_1 and stx_2 seem to be important in human infections.

E. coli O157:H7 is the principal serotype of this group. Enteroinvasive *E. coli* (EIEC) causes a diarrheal illness similar to shigellosis. Enterotoxigenic *E. coli* (ETEC) is a major cause of travellers' diarrhoea and infant diarrhoea in developing countries, produce a heat-labile toxin and/or a heat-stable toxin. Enteropathogenic *E. coli* (EPEC) is an important cause of infant diarrhoea in developing countries. Attaching and effacing (A/E) lesions is the key feature of EPEC pathogenesis (Trabulsi *et al*., 2002; Nataro and Kaper, 1998). Enteroaggregative *E. coli* (EAEC) displays a typical aggregative adherence (AA) pattern when associated with cultured epithelial cells and inert surfaces. EAEC have intrinsic ability to produce biofilms (Hitchins *et al*., 1998; Nataro *et al*., 1992). Diffusely adherent *E. coli* (DAEC) may cause diarrhoea in very young children (Scaletsky *et al*., 2002). They have a characteristic distinct adherence to Hep-2 cell monolayers (Bilge *et al*., 1989; Nataro *et al*., 1987). The extraintestinal pathogenic *E. coli* strains (EXPEC) includes the strains associated with urinary tract infections (UPEC), neonatal meningitis (MAEC), and bacteremia (Campos *et al*., 2004).

The possibility of contamination of shellfish growing areas with pathogenic *E. coli* such as O157:H7 exists, as those areas often receive sewerage overflow or runoff from farm fields. Ruminants have been identified as the principal reservoir of *E. coli* O157:H7, among which cattle is the most important source of human infections (Caprioli *et al*., 2005).

There are very few reports regarding presence of shiga toxin producing *E. coli* from shellfish growing areas worldwide. Gourmelon *et al*. (2006) reported the presence of several strains of STEC, including *E. coli* O157:H7, in shellfish collected from coastal areas of France. *Stx* genes were detected in 27.8% sample enrichments from mussels, oysters or cockles. Five strains

carrying stx1 or stx_{1d} genes and one *stx*-negative, *eae* and *ehxA*-positive *E. coli* O157:H7 were also isolated from the samples analysed. However, no relation was found between the total *E. coli* counts and the presence of STEC strains in the shellfish samples. Guyon *et al*. (2000) also reported one potentially pathogenic strain of *E. coli* O157 out of 150 French oysters tested.

Baliere *et al*. (2015) examined the presence of shiga toxin producing *E. coli* (STEC) in shellfish, upstream waters and sediment from coastal shellfish sites and reported 33% prevalence in shellfish, 91% in water samples and 28% in sediment samples. Bennani *et al*. (2012) detected shiga toxin *E. coli* (STEC) for the first time in shellfish and marine environment in the Mediterranean coast of Morocco. Shiga toxin-producing *E. coli* O157:H7 (STEC) was detected in 1.9% samples; Shiga toxin producing non-O157 *E. coli* strains were also detected. Brandao *et al*. (2017) also confirmed the presence of enteropathogenic and enterohemorrhagic *E. coli* strains in oysters harvested from urban estuaries. Surendraraj *et al*. (2010) first reported the presence of enterohemorrhagic *E. coli* O157:H7 in shrimp from India, which the authors inferred, was due to lapse in adherence to hygienic handling methods.

However, there are contradictory reports that *E. coli* O157:H7 is not a significant contaminant of shellfish (Ristori *et al*., 2007; MacRae *et al*., 2005). Ristori *et al*. (2007) analysed pathogenic bacteria associated with oysters (*Crassostrea brasiliana*) and estuarine water along the south coast of Brazil. Even though pathogens such as *V. parahaemolyticus* and *Salmonella* were detected, none of *E. coli* O157:H7 could be detected.

2.2.4 Prevalence of faecal streptococci

The faecal streptococci are another category of faecal indicator bacteria similar to coliforms. These bacteria are spherical, facultative, Gram positive, aerotolerant anaerobes and grow in chains. The enterococcal sub group of faecal streptococci have been shown to be of value in assessing the pollution status of both marine and freshwaters, and as secondary indicators of potable water contamination (Leclerc *et al*., 1996; Murray, 1990; US EPA, 1986; Dufour, 1984). Study of indicator bacteria has been useful in microbial source tracking, which will help to identify the sources of non point-source faecal pollution in natural waters.

Apart from their role as faecal indicators, there are many review reports stating that enterococci are opportunistic or rather emerging pathogens responsible for many community-acquired, hospital-acquired (nosocomial) super infections such as endocarditis, bacteraemia etc (Morrison *et al*.,1997; Murray, 1990).

Considering the fact that, the ratio of faecal coliforms to faecal streptococci (FC: FS ratio) varied with different animal species including humans, it was proposed as a good index to determine the source of faecal contamination (human *vs* animal) (Winslow and Palmer, 1910; US EPA, 1970). The FC: FS ratio in human faeces was calculated to be greater than 4 (Geldreich and Kenner, 1969) whereas in animal faeces, particularly cattle, FC: FS ratio is lesser than 0.7 (Raibaud *et al*., 1961; Kjellander 1960). Hence, even though it sounds theoretically possible to determine the source to faecal pollution as human or animal based on the evidence of FC : FS ratios, practically it is not so, since FC and FS has differential die-off rates. The FC: FS ratio has been found to change under sample storage,

and hence not a reliable indication of source of faecal contamination. Hence, it is no longer recommended as a reliable indicator of source of faecal contamination (human *vs* animal) and has been abandoned (APHA, 1992; US EPA, 1986).

Faecal streptococci have been suggested as better indicators of potential health hazard in recreational waters, as their densities correlated well with the possibility of contracting gastrointestinal diseases while swimming in both fresh and marine waters (Berg and Metcalf, 1978; Cabelli, 1983). This later led to the establishment of recreational freshwater quality criteria by the US Environmental Protection Agency (US EPA) as geometric mean densities not to exceed 33 Enterococci or 126 *Escherichia coli* per 100 mL respectively for recreational fresh water whereas for marine water the Enterococci limit is 35/100mL. (Leclerc *et al*., 1996; US EPA, 1986; Dufour, 1984; Cabelli, 1983).

Sinton et al. (2016) reported that the survival of the faecal streptococci paralleled that of enteric viruses, better than the coliforms. Thus, faecal streptococci may in certain cases provide a better estimate of the probable virus content in lightly contaminated water than the other indicators. Berg and Metcalf (1978) evaluated the validity of faecal coliforms, total coliforms, and faecal streptococci as indicators of viruses in chlorinated sewage effluents and found that chlorination destroyed most of the faecal and total coliforms and also faecal streptococci but the viruses showed better survival. Hence the absence of currently monitored faecal indicator bacteria may not truly indicate the absence of viruses.

2.2.4.1 Enterococci as indicators of faecal contamination in shellfish growing areas

Though enterococci are shown to survive better in the environment than other Enterobacteriaceae members (Noble *et al*., 2004), they are not considered for the assessment of growing areas for bivalve cultivation, as per the current EU or NSSP regulations. Most of the regulatory agencies rely on FC levels for assessing microbiological quality of shellfish harvesting areas. However, a preliminary study by Sobsey (1989) has suggested that there may be a correlation between illness and enterococcus counts in shellfish flesh at harvest.

Compared to the available data on the accumulation of coliforms in shellfish, there have been very few reports regarding the accumulation of faecal streptococci in shellfish tissues. Slanetz *et al*. (1964) correlated coliform, faecal coliform, and faecal streptococci concentrations in shellfish with the presence of *Salmonella* spp. and some enteric viruses. In another study, Slanetz *et al*. (1968) found that concentrations of coliforms, faecal coliforms, and faecal streptococci per 100 g of oyster tissue were 10-20 folds higher than in the surrounding sea water. Slanetz *et al*. (1965) found that the ratio of *E. coli* to faecal streptococci was 2.4 : 1 in seawater whereas the equivalent ratio in oysters were 3 : 1. The faecal streptococci isolated included *Streptococcus faecalis*, *S. faecalis* var. *liquefaciens*, *S. faecium,* and various biotypes. The species of coliforms and faecal streptococci isolated from sea water above the oyster beds were similar to those isolated from the oysters.

Lunestad *et al*. (2016), evaluated the role of enterococci as an indicator of viral faecal contamination in bivalves, as an alternative to *E. coli*.

For the evaluation, the data of shellfish samples available from 152 localities along the coast of Norway collected during the six-year period from 2007 to 2012 was analysed. They reported that, for most of the samples analysed, the concentration of enterococci was less than the corresponding *E. coli* concentrations. They reported that in 81.6% samples *E. coli* concentrations were 230 MPN per 100 g shellfish sample, whereas the corresponding concentration of enterococci were below the limit of detection of 100 CFU per g. The lesser concentrations of enterococci, as compared to *E. coli*, can be partially explained by their lesser concentrations (100 to 1000 fold less numerous than *E. coli*) in faecal material from homoeothermic animals (Edberg *et al*., 2000; Geldreich and Kenner, 1969). Bennani *et al*. (2012) studied the occurrence and antimicrobial resistance of enterococcal species isolated from seawater, shellfish and sediments at three marine sites and found that the *Enterococcus faecalis* and *Enterococcus faecium* were the predominant species. Pollution from sewage and urban runoff may be attributed for the increased incidence and resistance. Lalitha and Surendran (2005) studied the microbiological quality of black clam *Villorita cyprinoides* var. *cochinensis* and water samples collected from three clam harvesting areas in Vembanad Lake. The faecal contamination levels were high as indicated by high levels of *E. coli* and enterococci which did not conform to legal standards.

Seasonal variation was found in prevalence of faecal streptococci. Hennani *et al*. (2011) reported increased FS during winter and decreased FS during summer depending on rainfall, while the reverse trend was found in case of faecal coliforms. Bouchriti *et al*. (1992) studied microbial contamination in a shellfish growing area in Morocco and detected FS in

oyster and water samples year round while the counts increased from winter to summer.

Chinnadurai *et al*. (2016) studied the bioaccumulation of bacteria in oysters from Ashtamudi Lake, Kerala, India. Maximum level of FS (1.75 log 10 cfu/100 g) was found during monsoon season in oyster tissue while in harvesting water a lower maximum count (1.65 log $_{10}$ cfu/100 mL) was observed during the pre-monsoon season. The bio-accumulation factor was found to range between 0.93 and 9.5.

Increased prevalence and survival of microorganisms in bottom sediments compared to the overlying water column have been reported by many researchers. Many previous studies have revealed elevated levels of indicators and pathogens in sediments compared to the overlying water column (Legnani *et al*., 1998; LaBelle *et al*., 1980; Gerba *et al*., 1979). Prolonged survival also has been demonstrated in the sediment layer compared to water column (Lear, 1985; Rao *et al*., 1984). Microorganisms discharged into marine environments will tend to settle in the sediment bottom layer after attaching to particles.

Martinez-Manzanares *et al*. (1992) studied the incidence of faecal pollution in shellfish growing area in Spain and found that the levels of FS was maximum in sediments followed by shellfish samples whereas water samples demonstrated the least. Legnani *et al*. (1998) studied the microbiological quality of shellfish growing area in Italy and found similar observations. FS levels were higher in sediment samples (59%) compared to water samples (39%). However, mussels and clams recorded the maximum of 80% and 100% respectively. On the contrary, Bennani *et al*. (2012) reported maximum enterococci concentrations in sediments of shellfish

growing areas in Mediterranean coast of Morocco followed by sediments and least was recorded in seawater. Among the various indicators analysed, Anderson *et al*. (2005) observed that faecal coliform decay rates were significantly lower than those of enterococci in freshwater, but were not significantly different in saltwater. Ferguson *et al*. (2005) studied the relative prevalence of faecal indicators in shoreline and near offshore sediments of California and found that, *Enterococcus* was present more often and at higher concentrations in sediment samples, when compared with total and faecal coliforms. Approximately 57.5% sediment samples were positive for *Enterococcus* whereas only 22.9% was positive for faecal coliforms. The study of microbial content of bottom sediments is important, since there are possibilities that the microorganisms present in the sediment layer get re-suspended by natural or anthropogenic activities, thus acting as a persistent source of contamination.

2.2.5 Incidence of *Vibrio parahaemolyticus* in shellfish growing areas

V. parahaemolyticus is a Gram-negative halophilic bacterium, that is widely disseminated in estuarine, marine and coastal surroundings (Letchumanan *et al*., 2014; Ceccarelli *et al*., 2013; Zhang and Orth, 2013; Baker-Austin *et al*., 2010; Su and Liu, 2007). It has been recognized as the leading cause of human gastroenteritis associated with seafood consumption (Odeyemi, 2016; Odeyemi and Stratev, 2016; Letchumanan *et al*., 2014; Newton *et al*., 2012; Zarei *et al*., 2012). The organism native to estuarine waters throughout the world was first identified as a food borne pathogen in Japan in the 1950s (Fujino *et al*., 1953). The Cholera and Other *Vibrio* Illness Surveillance system (COVIS), initiated by CDC, FDA, and the Gulf Coast states regularly monitor and provide obtain reliable information on

vibriosis. Chowdhury *et al*. (2004) has reported a significant increase in infections caused by *V. parahaemolyticus* globally. There were 1280 reports of infection due to the organism in 1994-95 (Anon, 1999a). In Taiwan, some 197 outbreaks of foodborne disease were caused by *V. parahaemolyticus*, between 1986 and 1995 (Pan *et al*., 1997). However, the number of *V. parahaemolyticus* illnesses is very much under reported and it is estimated that only 1 in 142 cases is actually detected (Scallan *et al*., 2011).

Consumption of raw or undercooked seafood, is one of the primary causes of gastroenteritis due to *V. parahaemolyticus* characterised by diarrhoea, vomiting, abdominal cramps, nausea etc (Shimohata and Takahashi, 2010; Su and Liu, 2007; DePaola *et al*, 2003). The Centre for Disease Control and Prevention (CDC, 1998) has reported approximately 40 disease outbreaks of *V. parahaemolyticus* infections from 1973 to 1998 (Daniels *et al*., 2000a). Out of the 40 outbreaks, four involved over 700 cases of diseases associated with consumption of raw oyster in the Gulf Coast, Pacific Northwest, and Atlantic Northeast regions during the years 1997 and 1998. During the summer of 1997, there were 209 cases of *V. parahaemolyticus* infections (including one death) reported, involving raw oyster consumption in the Pacific North-west (CDC, 1998). Another minor outbreak of eight cases of *V. parahaemolyticus* illnesses was reported in New Jersey and NewYork in 1998, as a result of consumption of oysters and clams harvested at Long Island Sound of NewYork (CDC, 1999). There are several other reports of several disease outbreaks due to *V. parahaemolyticus* linked to raw oyster consumption in United States (McLaughlin *et al*., 2005; DePaola *et al*., 2000). *V. parahaemolyticus* has been isolated from 32% of the environmental samples collected from 17 Alaskan oyster farms in 2004. The most frequently

occurring serotypes were O1:K9, O4:K63, and O6:K18 (Newton *et al*., 2012).

Several researchers have reported the role of *V. parahaemolyticus* in shellfish associated disease outbreaks. In an earlier study, a high prevalence and accumulation of *V. parahaemolyticus* in local shellfish implicating a potential public health hazard has been reported (Chan *et al*., 1989). Consumption of raw or undercooked shellfish from sewage contaminated areas have been responsible for several food-borne disease outbreaks in different parts of the world. (Fuenzalida *et al*., 2007; Deepanjali *et al*., 2005; Daniels *et al*., 2000; DePaola *et al*., 2000). The presence of *V. parahaemolyticus* was confirmed in shellfish and clinical samples during epidemics (Fuenzalida *et al*., 2006). Ristori *et al*. (2007) detected high prevalence of *V. parahaemolyticus* in oyster and estuarine water samples from south coast of São Paulo, Brazil. Yu *et al*. (2013), carried out a study on five oyster and clam growing areas in Taiwan and detected *V. parahaemolyticus* in 77.5, 77.5, 70.8 and 68.8% of the oyster, clam, sediment and water samples respectively. *V. parahaemolyticus* levels were significantly higher in shellfish compared to those in sediment or water samples. In contrary, Mannas *et al*. (2014) reported a low prevalence (7.7%) of *V. parahaemolyticus* in 52 samples of *Mytilus galloprovincialis* collected from four sites along the Atlantic coast. Schets *et al*. (2010) surveyed the levels of *Vibrio* species in oysters and mussels in Dutch production areas. The *V. parahaemolyticus* MPN levels in oysters ranged from 6 to 622 most probable number (MPN) per g in oysters and 6 to 62 MPN/g in mussels.

There are many reports of *V. parahaemolyticus* associated food borne illnesses mainly due to consumption of seafood from various Asian countries. *V. parahaemolyticus* was first recognized as a food borne pathogen in the

year 1951 in Osaka, Japan due to consumption of raw or uncooked seafood (Daniels *et al*., 2000b). There are a few other reports of *V. parahaemolyticus* related disease outbreaks from Japan (Hara-Kudo *et al*., 2012; Kubota *et al*., 2011; Aberoumand, 2010). Ever since then there are reports from other Asian countries, Taiwan (Yu *et al*., 2013; Anon, 2005), China (Li *et al*., 2014; Liu *et al*., 2004), Bangladesh (Bhuiyan *et al*., 2002), Hong Kong, and Indonesia (Matsumoto *et al*., 2000)

In India, there are reports of *V. parahaemolyticus* isolations both from clinical and environmental samples. In a clinical study, 178 *V. parahaemolyticus* strains were isolated from 13,607 diarrheal patients admitted in Infectious Diseases Hospital, Kolkata from 2001 to 2012 (Pazhani *et al*., 2014). *V. parahaemolyticus* diarrheal cases were also reported from the urban slums of Kolkata, India (Kanungo *et al*., 2012). Reyhanath and Kutty, (2014) have reported the detection of multidrug resistant strains of *V. parahaemolyticus* from a fishing land at South India. There are recent reports of multiple drug resistant *V. parahaemolyticus* strains from seafood and backwater samples from the present study area, Cochin (Silvester *et al*., 2015; Sudha *et al*., 2014).

Seasonal influence on prevalence of *V. parahaemolyticus* in shellfish and coastal waters has been reported by a number of researchers (Urquhart *et al*., 2015; Johnson *et al*., 2010; Magny *et al*., 2009; Parveen *et al*., 2008; DePaola *et al*., 2003). Warmer temperatures are found to increase the number of *V. parahaemolyticus* density in water samples and their corresponding isolations from shellfish (Schets *et al*., 2010; Fletcher, 1985). Thus, it is more likely to detect *V. parahaemolyticus* in oysters harvested in the spring and summer, than in the winter (Su and Liu *et al*., 2007; DePaola

et al., 2000). Seasonal distribution was observed during summer months in *V. parahaemolyticus* numbers in market oysters with high densities sometimes exceeding even 1000 MPN/g (Cook *et al*., 2002). *V. parahaemolyticus* density in water samples depended significantly on precipitation, while that in the sediment or shellfish samples depended significantly on the salinity of the seawater (Yu *et al*., 2013). Cantet *et al*. (2013), investigated seasonal distribution of 3 pathogenic *Vibrio* species i.e *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* in water, shellfish (mussel and clams) and sediment of three French Mediterranean coastal lagoons. In summer, *V. parahaemolyticus* could be isolated from all the above samples; highest numbers were detected in mussels, followed by clams, sediments and water. In winter, *V. parahaemolyticus* could be isolated only from mussels, but at very low densities. However, some researchers could not find any correlation between temperature, salinity and *V. parahaemolyticus* prevalence (Ristori *et al*., 2007).

Diverse *V. parahaemolyticus* serotypes may be associated with human infections, but recently, strains belonging to the O3:K6 serotype, a pandemic strain has been found to be the causative agent of several outbreaks in different countries. Currently there are more than 20 serovariants reported including 03:K6, 04:K68, 01:K25, and 01: KUT (Nair *et al*., 2007). It was first reported from Kolkata, India, and later food borne outbreaks due to this strain have been reported from South east Asia, United States, Europe, Africa and Central and South America (Velazkuez-Roman *et al*., 2012; Okuda *et al*., 1997; Ansaruzzaman *et al*., 2005;).

Varying levels of occurrence of pathogenic genes have been reported in clinical as well as environmental *V. parahaemolyticus* strains. Strains

producing virulence genes, *tdh* and *trh* are generally considered pathogenic to man. Higher prevalence (more than 90%) of virulence gene, *tdh* gene has been reported in clinical *V. parahaemolyticus* strains whereas very less environmental strains (1%) produce these haemolysins (Ceccarelli *et al*., 2013; DePaola *et al*., 2000; Sakazaki *et al*., 1968,). In environmental samples and seafoods the frequency of *tdh* detection ranged from 0-6% (Cook *et al*., 2002; Kaysner *et al*., 1990; Kiiyukia *et al*., 1989).

However, non-toxigenic *V. parahaemolyticus* strains lacking these toxin producing genes, but capable of causing acute gastroenteritis have been reported. When the virulence properties of four *V. parahaemolyticus* strains causing acute gastroenteritis following consumption of indigenous mussels in Italy was investigated, it was found that the strains were cytotoxic and adhesive, however lacked *tdh*, *trh*, and type three secretion system 2 (T3SS2) genes, which are found to be associated with pathogenic strains (Ottaviani *et al*., 2012). Study of virulent genes by Vongxay *et al*. (2008) suggested that hemolysins TDH and/or TRH may not be necessarily the only virulence factors of pathogenic *V. parahaemolyticus* isolates. This suggested the complexity of *V. parahaemolyticus* pathogenicity as well as its dynamic genomic features, which have to be explored.

2.2.6 Incidence of *Salmonella* in bivalves

Salmonella has been identified as one of major cause of seafood related outbreaks worldwide (Amagliani *et al*., 2012; Brands *et al*., 2005; Duran and Marshall, 2005; Heinitz *et al.*, 2000). *Salmonella* is a facultatively anaerobic, non-sporulating, mesophilic, Gram-negative bacterium of the family Enterobacteriaceae. Salmonellosis is one of the most commonly reported enteric illnesses worldwide. *Salmonella* spp. are transmitted by the

faecal-oral route by either consumption of contaminated food or water, person-to-person contact, or from direct contact with infected animals (Jay *et al*., 2003). There are formally two species of *Salmonella*, *S. enterica* (comprises of six subspecies) and *S. bongori*.

Salmonella strains can be characterised serologically (into serovars) based on the presence and/or absence of somatic (O) and flagellar (H) antigens. More than 2500 serovars have been identified which are potentially pathogenic to both animals and humans (Norhana *et al*., 2010). Over 99% of human *Salmonella* spp. infections are caused by *S. enterica* subsp. *enterica* (Crum-Cianflone, 2008; Bell and Kyriakides, 2002).

The symptoms of salmoellosis may vary from having no effect to acute gastroenteritis characterised by abdominal cramps, nausea, diarrhoea, fever, vomiting, head ache etc and in severe cases septicaemia may occur leading to fatality. The incubation period is usually 8-72 hours and symptoms may last for 2-7 days (Darby and Sheorey, 2008; Hanes, 2003).

The primary reservoir of *Salmonella* spp. is the intestinal tract of warm and cold-blooded vertebrates and faecal shedding of *Salmonella* spp. leads to contamination of the surrounding environment. *Salmonella* and other bacteria may contaminate seafood either from polluted environment like water contaminated with sewage from various sources or during processing, and may cross-contaminate products during various stages of preparation. Bacteria naturally present in seawater can be found in limited numbers in live and raw fish and shellfish, they can also be concentrated by filter-feeding molluscs which are often eaten raw or partially cooked (Amagliani *et al*., 2012; Jones *et al*., 2012).

Outbreaks attributed to *Salmonella* spp. have predominantly been associated with animal products such as eggs, poultry, raw meat, milk and dairy products, but also include fresh produce, salad dressing, fruit juice, peanut butter and chocolate (Montville and Matthews, 2005; Jay *et al*., 2003). The presence of *Salmonella* has been demonstrated in a variety of fish and shellfish, including ready-to-eat (RTE) seafood products, seafood products requiring minimal cooking, and shellfish eaten raw (Brands *et al*., 2005; Duran and Marshall, 2005). Seafoods including such as fish and shellfish can acquire *Salmonella* from polluted waters or they can be contaminated with *Salmonella* during storage or processing (Panisello *et al*., 2000).

There are reports of *Salmonella* in shellfish from various geographic locations worldwide. Ristori *et al*. (2007) detected 10% prevalence of *Salmonella* estuarine water samples and untreated oyster samples from south coast of Sao Paulo, Brazil. However, treated oyster samples did not show the presence of *Salmonella*. Lunestad *et al*. (2016) investigated the prevalence of *Salmonella* in blue mussels harvested from 152 localities along the coast of Norway during six-year period from 2007 to 2012. The incidence was very less, only one horse mussel was positive (0.3%) and the strain was identified as *Salmonella enterica* serovar *infantis*, seroprofile: 6,7: r:1,5. *Salmonella* spp. were detected in 1.8% of shellfish samples which included mussels, clams, oysters and cockles collected off the coast of Spain (Martinez-Urtaza *et al*., 2003). Rahimi *et al*. (2013) also reported low prevalence of *Salmonella* in seafood in Iran. Overall *Salmonella* prevalence was found to be 5%. Highest isolation was made from fish followed by shrimp, whereas lobster and crab samples were tested negative for *Salmonella***.** Prato *et al*. (2012) conducted three studies on enterically transmitted diseases

through shellfish in Italy. For the study, different samples of *Mitylus edulis* lamellibranch collected over a period of 20 years between 1989 and 2009 were used. *Salmonella* spp. was detected in 3.1% of samples examined during the first study in 1987, whereas it was not detected in the following two studies. Mok *et al*. (2016) could not detect pathogens such as *Salmonella* or *Shigella* in any oyster samples collected from Hansan-Geojeman area, a prominent shellfish growing area in Korea.

In a study conducted by US FDA the presence of *Salmonella* in imported and domestic seafood samples collected over a 9-year period (1990 to 1998) was assessed. The study revealed very low overall incidence of *Salmonella* in imported (7.2%) and domestic seafood (1.3%). In ready-to-eat seafood and shellfish eaten raw, the overall incidence of *Salmonella* was 0.47% for domestic, while in imported shellfish consumed raw, the prevalence was 1% in oyster, 3.4% in clams, and 0% in mussels (Heinitz *et al*., 2000).

There are a few reports of *Salmonella* prevalence in seafood from India. Kumar (2009) has reported the distribution trends of *Salmonella* serovars in India between 2001 and 2005 as obtained from the National Salmonella and Escherichia Center (NSEC), Central Research Institute (Kasauli), India. During the study period, 70 non-typhoidal serovars were isolated from seafood.

Kumar *et al*. (2008) investigated the presence of *Salmonella* in seafood samples from the harbours and retail fish markets of Cochin. Cultural, ELISA and PCR methods were used to detect *Salmonella* in shellfish including clam, mussel and oyster and observed incidences of 21.4%, 16.6% and 35.7% respectively. Several serotypes of *Salmonella* like *S. weltevreden,*

S. rissen, S. typhimurium and *S. derby* were isolated. Hatha *et al*. (2003) isolated *Salmonella* from only one sample of raw, peeled tail-on shrimp while analysing the bacteriological quality of individually quick frozen (IQF) shrimp products produced from cultured tiger shrimp (*Penaeus monodon*). Bujjamma *et al*. (2015) studied the prevalence of *Salmonella* in fish and shellfish from a domestic fish market, Guntur city (Andhra Pradesh, India), collected over a period of one year (2010-11). *Salmonella* was detected in 5.72% of fishes and 9.72% of shellfish.

Many researchers have reported increased relative prevalence of *Salmonella* in shellfish and sediments compared to seawater. Shellfish has been reported to show increased prevalence of *Salmonella* compared to surrounding seawater (Simental and Martinez-Urtaza, 2008; Martinez-Urtaza *et al*., 2004). In Morocco, Setti *et al*. (2009) detected highest incidence of *Salmonella* in mussels (10%) followed by sediments (6.8%) and least was reported in seawater (4.1%). Remarkable seasonal variation in the incidence pattern was observed in both fin fishes and shellfishes with higher incidence during monsoon season followed by post-monsoon and pre-monsoon seasons respectively (Balfour *et al*., 2012).

Two interesting studies by Brands *et al*. (2005) and DePaola *et al*. (2010), reported the presence of *Salmonella* in live oysters collected from waters approved for shellfish harvesting in the US. These findings questions the reliability of the system of approving areas for shellfish growing based on faecal coliform levels. These results suggest that *Salmonella* contamination may not only be linked to poor hygiene, and the system of approving areas for shellfish growing solely based on faecal coliform levels may be insufficient to guarantee the absence of the pathogen in fish and fishery products.

2.3 Objectives

- To study the prevalence of faecal coliforms and faecal streptococci in shellfish (*Villorita cyprinoides* var. *cochinensis*), sediment and water from different shellfish harvesting areas in Cochin estuary.
- To study the prevalence of pathogens such as *Vibrio parahaemolyticus* and *Salmonella* spp. in shellfish (*Villorita cyprinoides* var. *cochinensis*), sediment and water from different shellfish harvesting areas in Cochin estuary.
- To find out the serological diversity of *Escherichia coli* isolated from shellfish (*Villorita cyprinoides* var. *cochinensis*), sediment and water collected from different shellfish harvesting areas in Cochin estuary and to determine the prevalence of pathogenic serotypes.

2.4 Materials and Methods

2.4.1 Description of the study area

Cochin estuary is a part of Vembanad Lake, which is an important Ramsar site and the largest brackish tropical wetland ecosystem, located along the south-west coast of India. Eight different shellfish harvesting areas located along Cochin estuary were selected for the study, based on their closeness to satellite townships, sea food industries, hospitals, markets with increased probable levels of contamination (Figure 2.1). The sampling stations selected were the following: Aroor (station 1), Valanthakad (station 2), Cheppanam (station 3), Valappu (station 4), South Malipuram (station 5), Kumbalangi (station 6), South Vaduthala (station 7) and Perumbalam (station 8) as shown in Figure 2.2.

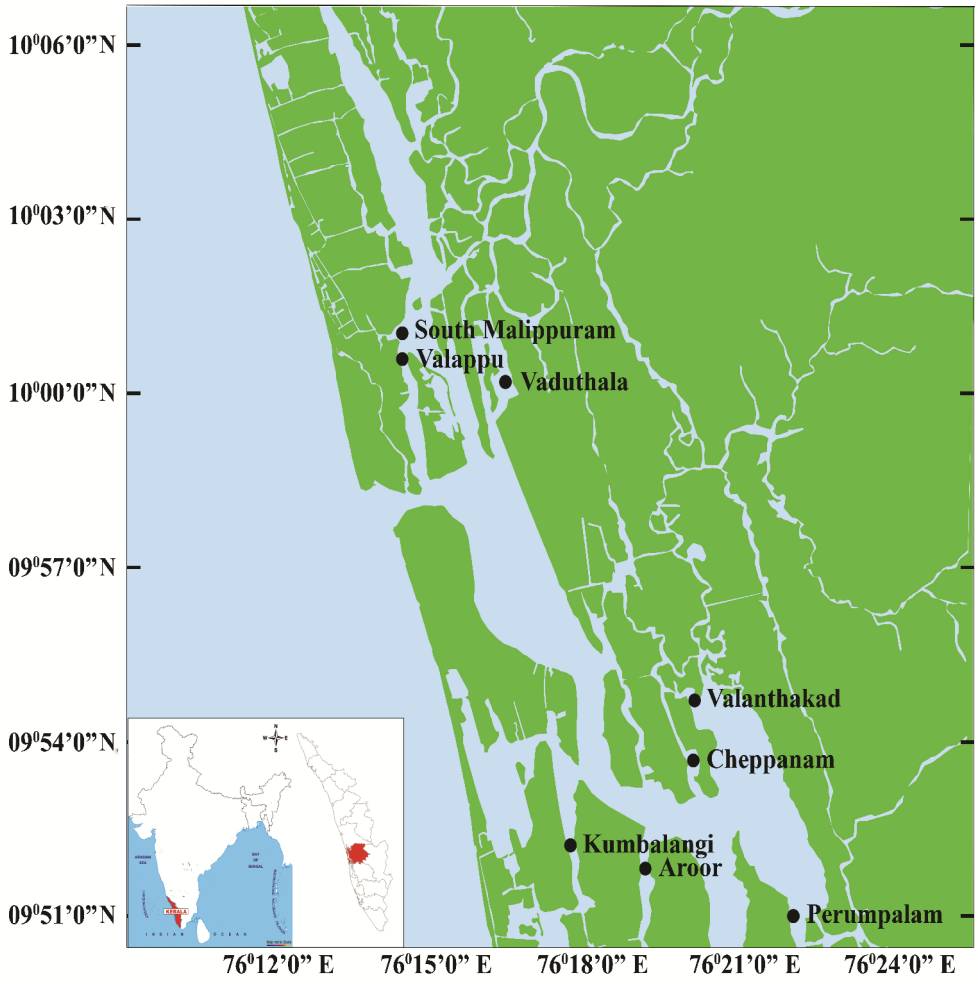

Figure 2.1 Cochin estuary map showing various sampling locations

Aroor (station 1)

Valanthakad (station 2)

Cheppanam (station 3)

Valappu (station 4)

South Malipuram (station 5)

Kumbalangi (station 6)

South Vaduthala (station 7)

Perumbalam (station 8)

Figure 2.2 Shellfish harvesting areas located along Cochin estuary selected for the study

2.4.2 Collection of the samples

Live Indian black clams, *Villorita cyprinoides* var. *cochinensis* endemic to the study area, sediment and water samples from the eight respective harvesting areas were aseptically collected for a period of one year from January 2013 to December 2013. Season-wise sampling was done, to study the variation during the three seasons *viz.* pre-monsoon (February to May), monsoon (June to September) and post-monsoon (October to January).

2.4.2.1 Shellfish samples

Live clams from each station were collected on a seasonal basis from the eight stations under study. Collections were made in the early morning hours with the help of local fishermen. Samples were collected in sterile polythene bags and transported to the laboratory in live condition in an icebox. Bacteriological analysis was done within 1-2 hours of sample collection.

2.4.2.2 Sediment samples

Sediment samples were collected using a sterile scoop, from the 8 stations in sterile polythene bags and transported to the laboratory in an icebox. Bacteriological analysis was done within 1-2 hours of sample collection.

2.4.2.3 Water samples

Season-wise collection of water samples were taken in sterile 1 litre plastic bottles (Tarson, India) from one foot below the surface to get better representation of the water column. Water samples were transported to the laboratory in an ice box and bacteriological analysis was done within 1-2 hours of sample collection.

2.4.3 Bacteriological analysis of samples

2.4.3.1 Enumeration of faecal coliform levels and isolation of *Escherichia coli* from shellfish, harvesting waters and sediments

Faecal coliform levels in shellfish, sediment and harvesting waters were enumerated by three decimal dilution 5 tube most probable number (MPN) method using EC broth (Hi-media, India), as described in detail elsewhere (BAM, 2011; Hitchin *et al*., 1995; APHA, 1970). Approximately 15-20 medium sized shellfishes were surface cleaned, aseptically shucked and about 25 g of meat and liquor was transferred to a sterile stomacher bag and blended with 225 mL of sterile peptone water in a stomacher (IUL Instruments, Spain). Ten mL samples were inoculated into 10 mL double strength EC broth; 1 mL and 0.1 mL samples were inoculated into single strength EC broth of 9 mL and 9.9 mL respectively, all containing inverted Durham's tubes. Similarly, appropriately diluted sediment and water samples (1: 9 ratio) in 10 mL, 1 mL and 0.1 mL quantities were also inoculated into respective dilution tubes with inverted Durham's tubes, as mentioned above. The inoculated tubes were incubated at 44.5 °C for 24 h and observed for growth and gas production.

Tubes showing growth and gas production were recorded as FC positive and compared with the MPN table to calculate the MPN index. The results were expressed as MPN index/100 g of shellfish/sediment or MPN index/100 mL of harvesting waters. One loopful from positive EC broth tubes showing growth and gas production was streaked simultaneously onto Eosin methylene blue (EMB) agar (Hi-media, India) and Hicrome *E. coli* agar (Hi-media, India) plates and incubated at 37 °C for 24 h. After incubation, the plates were examined, typical colonies from EMB (green metallic sheen) and Hicrome *E. coli* agar (blue-green) were re-streaked and

purified and transferred to nutrient agar (Hi-media, India) slants, for further characterization.

2.4.3.1.1 Biochemical characterization of *Escherichia coli*

Typical colonies isolated from EMB agar and Hicrome agar plates and maintained on nutrient agar vials were subjected IMViC test i.e., indole production in tryptone broth, the ability to produce various organic acids from mixed acid fermentation of dextrose in Methyl red test, ability to produce non- acidic by products in Voges-Proskauer test and ability to utilize sodium citrate as the sole carbon source in citrate test. The cultures giving + + - - reaction were confirmed as *E. coli*.

2.4.3.1.1.1 *Indole test:-* Indole test checked the production of indole from the breakdown of tryptophan by *E. coli*. Presumptive *E. coli* culture from nutrient agar was inoculated into tryptone broth and incubated at 37 °C for 24 h. After incubation, 1 mL of Kovac's reagent was added to the medium. Development of a red ring in the reagent layer is considered as indole positive.

2.4.3.1.1.2 *Methyl Red (MR) test:-* In this test the ability of *E. coli* to produce various organic acids from mixed acid fermentation of dextrose and the subsequent reduction of pH to 4.6 or below was checked. Suspected *E. coli* cultures were inoculated into MR-VP broth and incubated at 37 °C for 24 h. After incubation a few drops of methyl red reagent was added. A persistent red colour on addition of methyl red indicated positive for methyl red test, whereas yellow or orange colour indicated negative for MR.

2.4.3.1.1.3 *Voges Proskauer (VP) test:-* Voges-Proskauer test determined the ability of *E. coli* to produce non- acidic byproducts such as acetyl methyl carbinol from dextrose. Suspected colonies were inoculated into MR-VP broth and incubated at 37 °C for 24 h. After incubation a few drops

of Barrits reagent 'A' and equal volume of Barrits reagent 'B' were added. The tubes were vigorously shaken after removing the cotton plugs and allowed to stand for 15 minutes. Development of cherry red colour was considered as positive for Voges-Proskauer test. *E. coli* was unable to produce acetyl methyl carbinol and hence showed negative result.

2.4.3.1.1.4 *Citrate test*:- Citrate test detects the ability of *E. coli* to utilize sodium citrate as the sole carbon source. Presumptive *E. coli* cultures were inoculated into Simmons citrate agar and incubated at 37 °C for 48 h. Development of prussian blue colour indicated positive reaction for citrate utilization test and lack of colour change indicated a negative result. *E. coli* was unable to utilize sodium citrate as the sole carbon source and hence gave negative result.

2.4.3.1.2 Confirmation of *E. coli* by detection of *uid A* gene by Polymerase chain reaction (PCR)

2.4.3.1.2.1 Extraction of genomic DNA from *E. coli*.

Genomic DNA was extracted by boiling method (Devi *et al*., 2009). Briefly, overnight culture incubated at 37° C with shaking (120 rpm) was centrifuged at 10,000 rpm at 4° C, for 1 min, to obtain a pellet. The cell pellet was washed with normal saline (0.8% NaCl w/v) and re-suspended with 0.5 mL sterile distilled water. The cell suspension was heated at 98° C ± in a boiling water bath for 15-20 min to enable cell lysis and release of DNA. The lysate was centrifuged (10,000 rpm, 4 °C, 5 min) to eliminate the cell debris and the supernatant was stored at -20° C, until further use.

2.4.3.1.2.2 Detection of species specific *uid A* gene in *E. coli* by PCR

The presence of *uid A* gene which codes for β-D- glucuronidase enzyme was detected by PCR amplification of a 147 bp coding region

using the primers UAL- 754 (5'-AAAACGGCAAGAAAAAGCAG-3') and UAR- 900 (5'ACGCGTGGTTACAGTCTTGCG-3') (Bej *et al*., 1991). The optimized reaction was carried out in a total reaction volume of 25 µL consisting of sterile Milli Q water (15.5 µL), 10x PCR buffer (2 µL), primer (1 µL each), dNTP mix (1 µL, 200 mM), template (4 µL) and Taq DNA polymerase (0.5 µL). The PCR conditions included an initial denaturation at 94 °C for 2 min followed by 25 cycles of denaturation (94 °C for 1 min), primer annealing (58 °C for 1.5 min) and primer extension (72 °C for 2 min) followed by a final extension (72 °C for 5 min). PCR products were then electrophoresed on an agarose (1.5% w/v) gel in 1xTBE buffer (Hi-media, India), stained with ethidium bromide (Genei, India) and visualized by Gel Documentation System (Bio-Rad Gel Doc EZ Imager, USA). The amplicon sizes were compared with a 100 bp DNA ladder.

2.4.3.2 Enumeration, isolation and identification of *Enterococcus* subgroup of faecal streptococci from shellfish, sediment and harvesting waters

Faecal streptococci levels in shellfish, harvesting waters and sediments were enumerated by three decimal dilution 3 tube most probable number (MPN) method using Azide dextrose broth (ADB) (Hi-media, India), as described in detail elsewhere (APHA, 1992). Samples were processed as described in section 2.4.3.1.1. Appropriately diluted aliquots of the three samples *viz*. shellfish, water and sediment were inoculated into ADB. Ten mL samples were inoculated into 10 mL double strength ADB; 1 mL and 0.1 mL samples were inoculated into tubes containing single strength ADB of 9 mL and 9.9 mL respectively. The inoculated tubes were incubated at 35 °C for 24-48 h and observed for growth and acid production indicated by turbidity and colour change from purple to yellow. From the presumptively

positive tubes, one loopful each were streaked onto Kenner faecal (KF) streptococcal agar (Hi-media, India) and Bile esculin azide (BEA) agar (Hi-media, India) plates and were incubated for 24 hours at 35 °C. Faecal streptococci formed characteristic pink coloured colonies on KF agar and blackish-brown colonies with brown halos on BEA agar. The characteristic streptococcal colonies developed were transferred to a tube containing Brain heart infusion (BHI) broth with 6.5% NaCl and incubated at 35 °C for 24-48 hours. Growth in BHI broth with 6.5% NaCl confirmed the isolate as a member of the *Enterococcus* genus. The total numbers of confirmed faecal enterococci/streptococci ADB tubes which showed corresponding positive reaction on Bile esculin azide agar and BHI broth with 6.5% NaCl were determined. The numbers of positive tubes were compared with the MPN table to calculate the MPN index. The results were expressed as MPN index/100 g of shellfish/sediment or MPN index/100 mL of harvesting waters.

2.4.3.2.1 Morphological and biochemical characterisation of enterococci

Enterococci when subjected to Gram staining appeared as Gram positive cocci or cocobacilli arranged in singles, pairs or short chains. The biochemical characteristics other than the aforementioned include catalase test (negative), growth at 10 °C (positive) and 45 °C positive), pyrrolidonylarylamidase (PYR) test (positive) and leucine aminopeptidase (LAP) test (positive).

2.4.3.2.1.1 Catalase test

The catalase test detected the catalase enzyme activity of bacteria. A few drops of 3% H_2O_2 solution was added to 24 h old bacterial culture grown on brain heart infusion agar and observed for production of oxygen

gas bubbles. The release of gas bubbles indicated a positive test and absence of gas production indicated a negative test. Enterococci are catalase negative.

2.4.3.2.1.2 Pyrrolidonylarylamidase (PYR test)

PYR test checked the presence of pyrrolidonylarylamidase activity by enterococci. Typical brown colonies picked from BEA were inoculated into small volume of PYR broth and incubated at 35 ^{o}C for 4 hours. The bacterial aminopeptidase enzyme if present will cleave the substrate L-naphthylamide-β-naphthylamide releasing β-naphthylamide, which in turn is detected by the addition of the reagent N, N-dimethylaminocinnamaldehyde. The reagent couples with naphthylamide forming a red coloured Schiff base which indicates a positive result.

2.4.3.2.1.3 Leucine aminopeptidase (LAP test)

The LAP test is used to check the presence of leucine aminopeptidase activity of enterococci. Leucine-beta-naphthylamide impregnated in the discs served as a substrate for the detection of leucine aminopeptidase. A small amount of the 24 hour old presumptive bacterial pure culture is rubbed onto a small area of the LAP disc and incubated at room temperature for 5 minutes. After incubation 1drop of cinnamaldehyde reagent is added. The beta-naphthylamine produced by the hydrolysis of the substrate will react with the cinnamaldehyde reagent producing red colour if the test is positive.

2.4.3.3 Isolation and identification of *Vibrio parahaemolyticus* from shellfish, sediment and harvesting waters

Approximately 15-20 medium sized clams were surface cleaned, aseptically shucked and about 25 g of meat and liquor was transferred to a sterile stomacher bag and blended with 225 mL of sterile alkaline peptone

water for pre-enrichment in a stomacher (IUL Instruments, Spain). For *V. parahaemolyticus* detection in sediment 10 g of sediment sample was added to 90 mL alkaline peptone water. For the detection of *V. parahaemolyticus* in water, about 1 or 2 litres of water were filtered through sterile 0.45 µm membrane filters. The filters were cut into pieces and placed into 100 mL of alkaline peptone water. The alkaline peptone waters were incubated at 37 °C for 24 h. After incubation, a loopful of culture from alkaline peptone water was streaked onto the selective media, Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) agar and incubated at 37 °C for 24-48 h. Typical blue-green colonies, characteristic of *V. parahaemolyticus* were isolated, re-streaked to ensure purity and maintained on nutrient agar slants for further biochemical characterisation. All the media were purchased from Hi-media laboratories, India.

2.4.3.3.1 Biochemical characterization of *V. parahaemolyticus*

The cultures were identified as per the standard protocol BAM, (2009). The confirmatory biochemical tests for *V. parahaemolyticus* included Cytochrome oxidase (+), Utilization of amino acids (arginine dihydrolase (-), lysine decarboxylase (+), Ornithine decarboxylase (+), nitrate reduction (+), Voges-proskauer (-), Carbohydrate fermentation tests (sucrose (-), lactose (-) and arabinose (+), salt tolerance test conducted in peptone water containing varying concentrations of NaCl such as 0% (-), 3% (+), 6% (+) and 8% (+) and growth at 42 °C (+).

2.4.3.3.1.1 Oxidase test:-

In this test the ability of bacteria to produce cytochrome oxidase is determined. Cytochrome oxidase uses O_2 as an electron acceptor during the oxidation of reduced cytochrome *C* to form water and gets cytochrome *C*

oxidised. A small amount of pure culture is placed on the dry filter paper soaked in freshly prepared oxidase test reagent, tetramethyl-*p*-phenylene diamine dihydrochloride. If the organism is oxidase positive, the reagent gets itself oxidised to deep purple compound by donating its electron in the presence of free O_2. A positive reaction is indicated by an intense deep purple blue colour appearing within 5-10 seconds. No color change or a light pink coloration indicate the absence of oxidase and is considered as a negative test.

2.4.3.3.1.2 Utilization of amino acids (Decarboxylase/dihydrolase test)

These tests checked the ability of microorganisms for enzymatic degradation of amino acids. The basic principle involved is removal of carboxyl groups of the amino acids which produced alkaline end products such as amines. The suspected cultures were inoculated into medium containing the specific amino acid substrate (arginine, lysine or ornithine) and a pH indicator bromocresol purple. Since decarboxylation requires an anaerobic environment, the culture tubes are overlaid with mineral oil to ensure the same. The tubes were incubated at 37 °C for up to 96 h. Turbidity with purple colour development is considered as a positive reaction for the presence of decarboxylase/dihydrolase.

2.4.3.3.1.3 Carbohydrate fermentation test

The ability of the bacteria to produce acid by the fermentation of lactose, sucrose and arabinose were tested. The suspected cultures were inoculated into carbohydrate fermentation basal medium containing lactose and sucrose and incubated at 37 °C for 24 h. The production of the acid was indicated by colour change from red to yellow and designated as positive while no colour change was considered as negative.

2.4.3.3.1.4 Nitrate reduction:-

The ability of bacteria to reduce nitrate NO_3 to nitrite NO_2 by the production of nitrate reductase is determined. Suspected cultures were inoculated into nitrate broth containing 0.5% potassium nitrate and incubated at 37 °C for 24 h. After incubation, the presence of nitrite ions was detected by the addition of sulfanilic acid and alpha naphthylamine (N, N – dimethyl-1-naphthylamine) to the culture. The development of a distinct red colour is indicated the presence of nitrite and considered as positive for nitrate reduction.

2.4.3.3.1.5 Voges Proskauer (VP) test:-

VP test has been carried out as described under section **2.4.3.1.1.3**

2.4.3.3.1.6 Salt tolerance test

The ability of the organism to grow at different concentrations of NaCl was checked. The cultures were inoculated in peptone water containing 0%, 3%, 6% and 8% NaCl and incubated at 37 °C for 24 h. The turbidity in the tube is considered as positive and lack of turbidity is considered as negative.

2.4.3.3.1.7 Growth at 42 °C

The suspected *Vibrio* cultures were inoculated into tryptone (1%) broth supplemented with 2% NaCl, and incubated for 24 h to check their ability to grow at 42 °C. Occurrence of turbidity in the tube after 24 h indicated their ability to grow, while no turbidity was considered as a negative reaction.

2.4.3.3.2 Confirmation of *V. parahaemolyticus* by PCR based amplification of species specific *tlh* and *toxR* genes.

Genomic DNA Extraction from V. parahaemolyticus has been carried out as described under section **2.4.3.1.2.1.** The optimized protocol was carried out with a PCR mix total reaction volume of 25 µL consisting of

sterile Milli Q water (11.5 µL), 10x PCR buffer (2 µL), primer (1 µL each of each primer; thus a total of 4 µL primer mix), dNTP mix (1 µL, 200 mM), template (4 µL), and Taq DNA polymerase (0.5 µL).

2.4.3.3.2.1 Detection of species specific *tlh* gene in *V. parahaemolyticus*

The presence of *tlh* gene which codes for thermolabile haemolysin was detected by amplification of a 450 bp coding region using the primers L-TL (5 ' AAA GCG GAT TAT GCA GAA GCA CTG 3 ') and R-TL (5 ' GCT ACT TTC TAG CAT TTT CTC TGC 3 ') (Bej *et al*., 1999). The PCR conditions included an initial denaturation at 94 °C for 3 min followed by 30 cycles of denaturation (94 °C for 1 min), primer annealing (60 °C for 1 min) and primer extension (72 °C for 2 min) followed by a final extension (72 °C for 3 min).

2.4.3.3.2.2 Detection of species specific *toxR* gene in *V. parahaemolyticus*

The presence of *toxR* gene was detected by PCR amplification of a 368 bp coding region using the primers F-5'GTCTTCTGACGCAATCGTTG-3' and R- 5 'ATACGAGTGGTTGCTGTCATG-3' (Kim *et al*., 1999). The PCR conditions included an initial denaturation at 94 °C for 3 min followed by 25 cycles of denaturation (94 °C for 1 min), primer annealing (63 °C for 1.5 min) and primer extension (72 °C for 1.5 min) followed by a final extension (72 °C for 5 min).

PCR products were then electrophoresed on an agarose (1.5% w/v) gel in 1xTBE buffer (Hi-media, India), stained with ethidium bromide (Genei, India) and visualized by Gel Documentation System (Bio-Rad Gel Doc EZ Imager, USA). The amplicon sizes were compared with a 100 bp DNA ladder.

2.4.3.4 Isolation and identification of *Salmonella* from shellfish, sediment and harvesting water

Approximately 15-20 medium sized clams were surface cleaned, aseptically shucked and about 25 g of meat and liquor was transferred to a sterile stomacher bag and blended with 225 mL of sterile buffered peptone water in a stomacher (IUL Instruments, Spain). For the detection of *Salmonella* in water, about 1 or 2 litres of water were filtered through sterile 0.45-µm membrane filters. The filters were cut into pieces and placed into 100 mL of buffered peptone water. For *Salmonella* detection in sediment, 10 g of sediment sample was added to 90 mL buffered peptone water. The buffered peptone waters were incubated at 37 °C for 24 h. After incubation 1mL and 0.1mL of samples from buffered peptone waters were inoculated into 10 mL each of selective enrichment broths- Tetrathionate Broth (TTB) and Rappaport-Vassiliadis Soybroth (RVS) respectively. After selective enrichment, a loopful of culture from both TTB and RVS were streaked onto selective media such as Xylose Lysine Deoxycholate (XLD) agar and Hektoen Enteric Agar (HEA) plates and incubated at 37 °C for 24 to 48 h. Typical *Salmonella* like colonies were picked up, re-streaked to ensure purity and were maintained on Tryptic Soy Agar (TSA) slants at room temperature for further biochemical testing. All the media were purchased from Hi-media laboratories, India.

2.4.3.4.1 Biochemical characterisation of *Salmonella*

The typical cultures isolated from XLD and HEA agars were subjected to preliminary biochemical screening, which included hydrogen sulphide (H2S) production in triple sugar iron Agar (TSI) slants and Lysine Iron Agar (LIA) slants, indole production in Tryptone broth and urease activity on Christensen's urea agar.

2.4.3.4.1.1 Reaction on Triple sugar iron (TSI) agar

In this test, the ability of the organism to ferment three different sugars such as lactose, sucrose and dextrose were analysed. Suspected *Salmonella* cultures were inoculated into TSI agar slants by stabbing the butt and streaking the slant and incubated at 37 °C for 24 h. The production of alkaline (red) slant and acid (yellow) butt indicated the glucose fermenting ability of the organism and inability to ferment lactose and sucrose. The glucose fermenting ability and the blackening of the medium by the production of (H_2S) is considered characteristic of *Salmonella*.

2.4.3.4.1.2 Lysine iron (LIA) agar

In this test, the ability of the organism to decarboxylate or deaminate lysine and form H_2S was assessed. The presumptive *Salmonella* cultures were inoculated into the medium by stabbing the butt and streaking the slant and incubated at 37 °C 24 h. The production of alkaline reaction (purple colour) in slant and butt by the decarboxylation of lysine and blackening of the medium by the production of H_2S is considered positive for *Salmonella*.

2.4.3.4.1.3 Indole test:-

Indole test was done using suspected *Salmonella* cultures as described in **2.4.3.1.1.1.** *Salmonella* showed negative reaction for indole production test.

2.4.3.4.1.4 Urease test:-

In this test, the urea splitting ability of *Salmonella* by the production of urease enzyme was checked. Suspected cultures were inoculated into the Christensen's urea agar by streaking on the surface of the slant and incubated at 37 °C for 24 h. If urea is hydrolysed by the bacteria, the ammonia produced accumulated in the medium making it alkaline. The increase in pH changed

the colour of the indicator from orange-red to deep pink and is considered as positive result for urease hydrolysis. As *Salmonella* does not utilize urea, deep pink color was not developed.

2.4.3.4.1.5 Identification of *Salmonella* by secondary biochemical screening

Suspected cultures which matched the typical reactions of *Salmonella* in preliminary biochemical screening were further subjected to secondary biochemical screening, which involved the fermentation of specific carbohydrates such as lactose, sucrose, dulcitol and salicin. Lactose and sucrose were employed at a final concentration of 1%, while dulcitol and salicin were used at a final concentration of 0.5%. The suspected cultures were inoculated into respective carbohydrate fermentation basal medium containing lactose, sucrose, dulcitol and salicin and incubated at 37 °C for 24 h. The fermentation of the carbohydrate resulted in reduction of pH which is evidenced by colour change from red to yellow. *Salmonella* does not have the ablility to ferment lactose, sucrose, and salicin but is capable of fermenting dulcitol. The cultures, which matched the biochemical reactions of *Salmonella*, were further confirmed by slide agglutination test using polyvalent 'O' serum. The confirmed cultures were serotyped at National Salmonella and Escherichia Center, Central Research Institute, Kasauli, Himachal Pradesh, India.

2.4.4 Statistical analysis

Statistical analysis in this study was performed using SPSS software 20 (Statistical Package for Social Science). The significance of the difference in the densities of the faecal coliforms and faecal streptococci from various sources and also during various seasons was analysed using one-way Analysis of Variance (ANOVA). Significance level was set at $\alpha = 0.05$.

2.5 Results

2.5.1 Prevalence of faecal coliforms in shellfish harvesting areas of Cochin estuary

The prevalence of faecal coliforms at 8 different shellfish harvesting areas located along Cochin estuary were analysed seasonally, i.e., pre-monsoon, monsoon and post-monsoon seasons. Two samples were taken from each station during each season and the values are presented as mean.

2.5.1.1 Prevalence of faecal coliforms in shellfish (*Villorita cyprinoides*)

Villorita cyprinoides harvested from various growing areas exhibited remarkable variation in faecal coliform MPN index during the pre-monsoon season. Stations 3, 4 and 7 recorded the maximum FC MPN index (4 logs) while stations 1, 5 and 6 exhibited medium MPN levels. The least FC MPN level was reported at station 2 with an index of 3.3 logs.

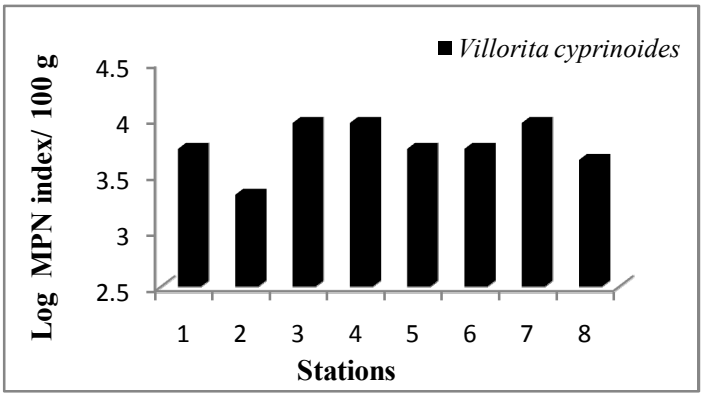

Figure 2.3 Prevalence of faecal coliforms in *Villorita cyprinoides* from different harvesting areas of Cochin estuary during pre-monsoon season

High faecal coliform MPN indices were demonstrated in shellfish during monsoon season at most of the stations. Out of the 8 shellfish harvesting areas under study, stations 1, 3, 4, 5, 6 and 7 exhibited maximum

MPN indices of 4.2 logs, whereas stations 2 and 8 showed lower MPN index of 3.9 logs.

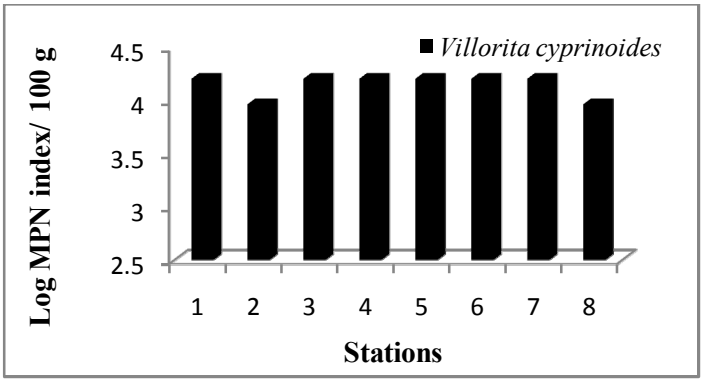

Figure 2.4 Prevalence of faecal coliforms in *Villorita cyprinoides* from different harvesting areas of Cochin estuary during monsoon season

Post-monsoon season also witnessed remarkably high levels of faecal coliforms in shellfish, at 6 out of the 8 harvesting areas under study. Stations 1, 3, 4, 5, 6 and 7 exhibited maximum MPN indices of 4.2 logs whereas stations 2 and 8 showed the lower MPN index of 3.96 logs.

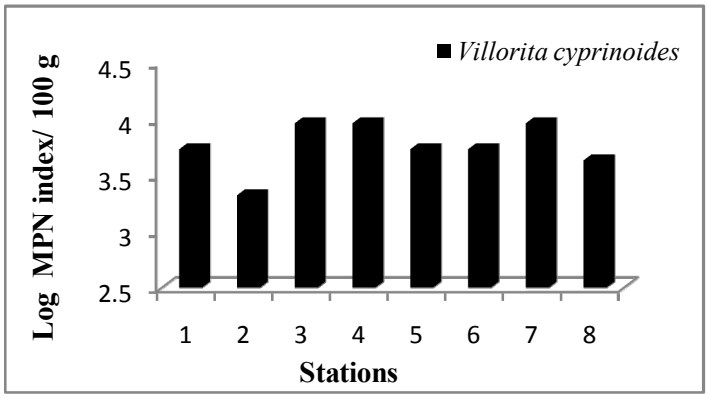

Figure 2.5 Prevalence of faecal coliforms in *Villorita cyprinoides* from different harvesting areas during post-monsoon season

2.5.1.2 Prevalence of faecal coliforms in sediment

Sediment samples exhibited remarkable variation in FC MPN indices at various shellfish harvesting areas during the pre-monsoon season. Station 4 showed the maximum FC index of 3.9 logs followed by stations 1,3,6,7 with MPN index of 3.73 logs. Station 2 showed the minimum MPN index of 3.32 logs.

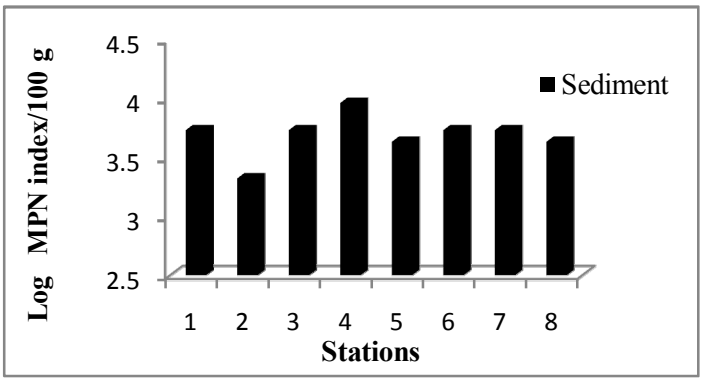

Figure 2.6 Prevalence of faecal coliforms in sediment from different shellfish harvesting areas of Cochin estuary during pre-monsoon season

In monsoon season, out of the 8 stations under study, sediments from 7 stations showed high faecal coliform MPN indices of 4.2 logs. Station 2 exhibited the least FC MPN index of 3.96 logs.

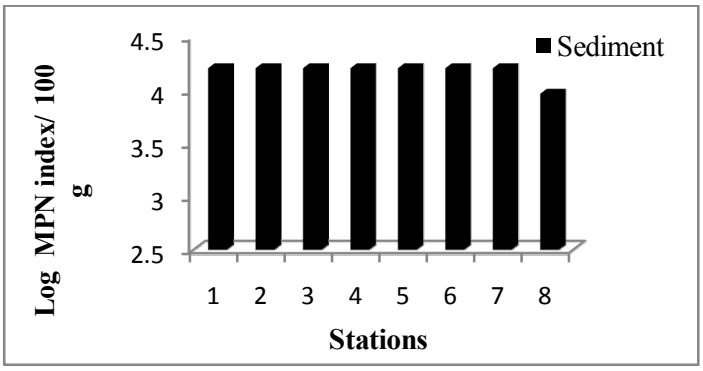

Figure 2.7 Prevalence of faecal coliforms in sediment from different shellfish harvesting areas of Cochin estuary during monsoon season

In post-monsoon season, out of 8 stations, sediments from 7 stations exhibited high faecal coliform MPN indices of 4.2 logs, except station 2, where comparatively less MPN of 3.96 logs was noticed.

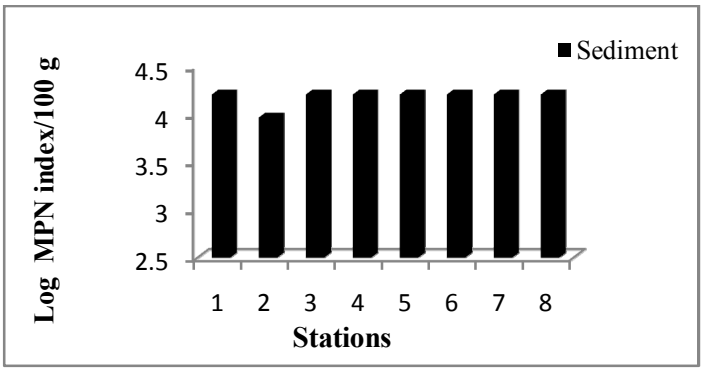

Figure 2.8 Prevalence of faecal coliforms in sediment from different shellfish harvesting areas of Cochin estuary during post-monsoon season

2.5.1.3 Prevalence of faecal coliforms in water

Estuarine water from shellfish harvesting areas showed marked variation in faecal coliform MPN indices during pre-monsoon season, ranging from 3.96-3.25 logs. Highest MPN index was noted at station 4 and lowest at station 2 with the other stations exhibiting intermediate faecal MPN indices.

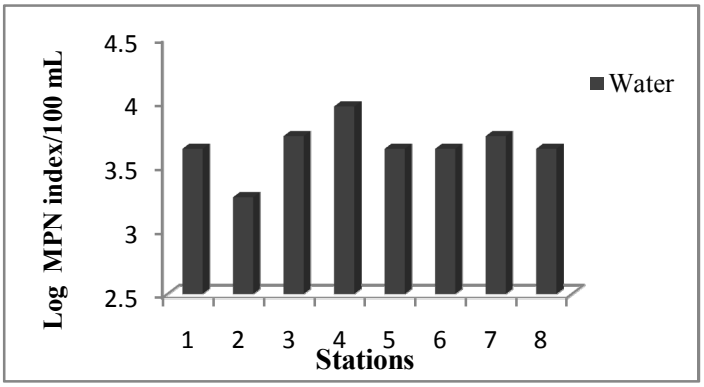

Figure 2.9 Prevalence of faecal coliforms in water from different shellfish harvesting areas of Cochin estuary during pre-monsoon season

In monsoon season, faecal coliform MPN levels were high at all the stations. Stations 1-6 exhibited MPN indices of 4.2 logs while only stations 7 and 8 exhibited slightly low MPN index of 3.96 logs.

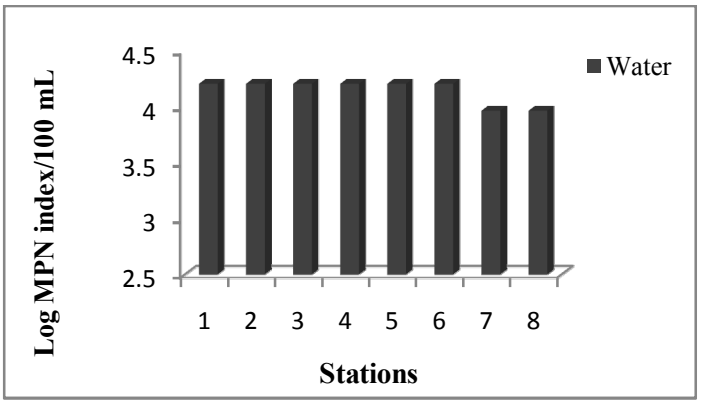

Figure 2.10 Prevalence of faecal coliforms in water from shellfish harvesting areas of Cochin estuary during monsoon season

The faecal coliform indices remained high in water from shellfish harvesting areas during post-monsoon season also. Stations 1-5 exhibited MPN indices of 4.2 logs while stations 6-8 exhibited lower MPN indices of 3.96 logs.

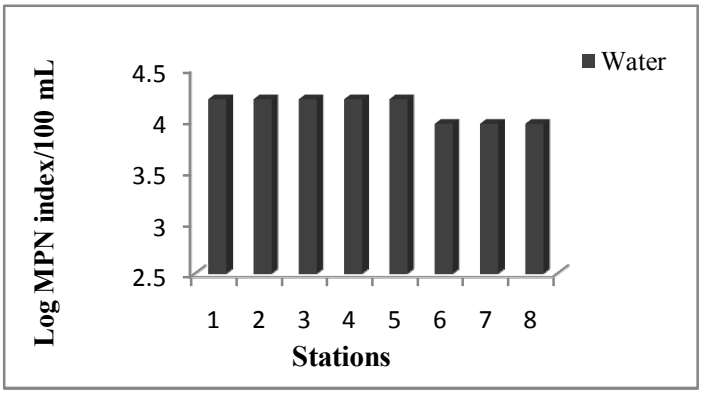

Figure 2.11 Prevalence of faecal coliforms in water from shellfish harvesting areas of Cochin estuary during post-monsoon season

2.5.1.4 Seasonal variation in the prevalence of faecal coliforms in *Villorita cyprinoides*, sediment and water from different shellfish harvesting areas in Cochin estuary

Shellfish samples exhibited significant seasonal variations in FC MPN indices; comparatively higher MPN indices were recorded during monsoon and post-monsoon seasons when compared to pre-monsoon season. In pre-monsoon season, the FC MPN indices in shellfish ranged from 3.32-3.96 logs. In monsoon as well as post-monsoon seasons, stations 1, 3, 4, 5, 6 and 7 showed higher MPN indices of 4.2 logs while stations 2 and 8 exhibited lower MPN index of 3.96 logs. The differences in MPN indices in shellfish between pre-monsoon, monsoon and post-monsoon seasons were found to be highly statistically significant ($p<0.001$).

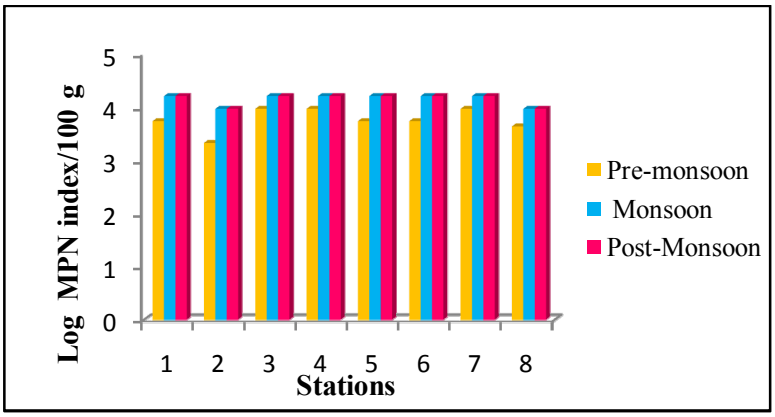

Figure 2.12 Seasonal variation in the prevalence of faecal coliforms in *Villorita cyprinoides* from shellfish harvesting areas in Cochin estuary

Sediment samples exhibited remarkable seasonal variations in FC levels; comparatively higher MPN indices were recorded during monsoon and post-monsoon seasons when compared to pre-monsoon season. The mean log FC MPN in sediments during pre-monsoon season ranged from 3.32-3.96 logs. In monsoon season, sediments from all stations showed

MPN of 4.2 logs except station 8, which exhibited MPN of 3.96 logs. In post-monsoon season all stations recorded elevated FC MPN index of 4.2 logs except stations 2 and 5 were lesser MPN index of 3.96 logs were recorded. The differences in MPN indices of sediment between pre-monsoon, monsoon and post-monsoon seasons were found to be statistically significant ($p<0.001$).

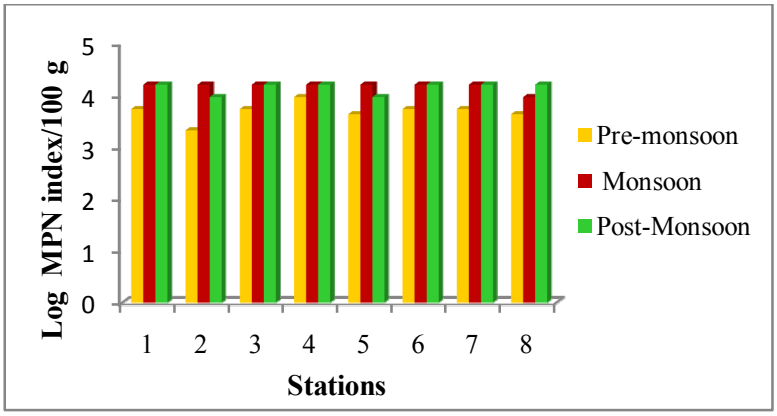

Figure 2.13 Seasonal variation in the prevalence of faecal coliforms in sediment from different shellfish harvesting areas of Cochin estuary

Water samples showed distinct seasonal variation in their FC MPN indices with lower MPN levels observed during pre-monsoon season when compared to monsoon and post-monsoon seasons as shown in figure 2.14. Water samples from all stations showed comparatively lower mean log FC MPN indices during pre-monsoon season, which ranged from 3.25-3.73 logs. However, there was no significant variation in MPN levels between monsoon and post-monsoon seasons. In monsoon, higher FC MPN indices of 4.2 logs were detected in water samples from stations 1-6; while stations 7 and 8 exhibited lesser MPN indices of 3.96 logs. In post-monsoon season also elevated FC MPN indices could be detected in water samples from stations

1-5; while stations 6, 7 and 8 exhibited lesser MPN indices of 3.96 logs. The differences in MPN indices of water between pre-monsoon, monsoon and post-monsoon seasons were found to be statistically significant ($p<0.001$).

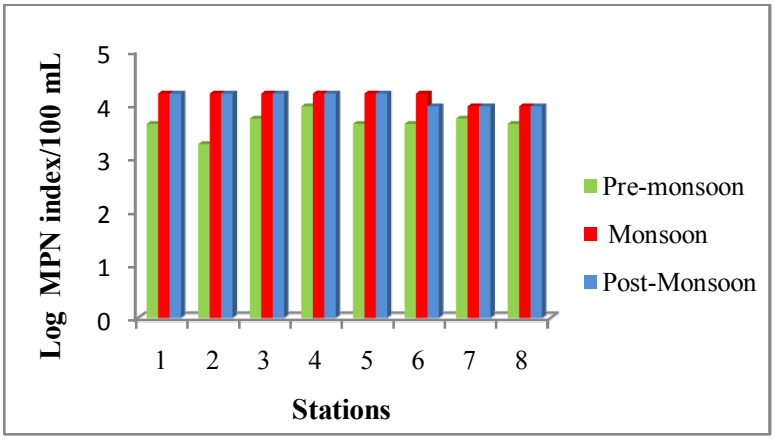

Figure 2.14 Seasonal variation in the prevalence of faecal coliforms in water from shellfish harvesting areas in Cochin estuary

2.5.1.5 Relative prevalence of faecal coliforms in *Villorita cyprinoides*, sediment and water from shellfish harvesting areas in Cochin estuary during various seasons

In pre-monsoon season, shellfish as well as sediment samples exhibited remarkably high FC MPN indices when compared to water samples as shown in Figure 2.15. In pre-monsoon season, the FC MPN indices in shellfish from stations 3, 5 and 7 were higher than those of sediment and water, whereas in stations 1, 2 and 6 the FC MPN of shellfish and sediment were equally higher than that of water. However stations 4 and 8 exhibited equal FC MPN levels for all the three samples *viz*. shellfish, sediment and water. In all stations except station 8, shellfish FC MPN indices were higher than that of water during pre-monsoon season; however the results were found to be statistically insignificant ($p=0.577$).

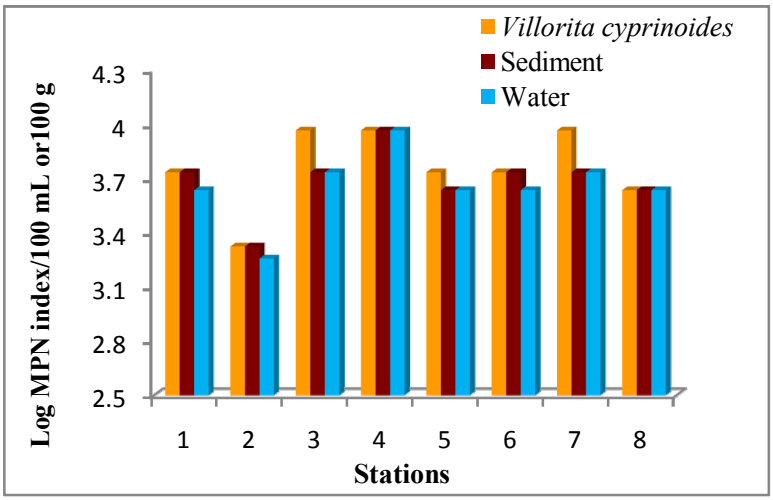

Figure 2.15 Relative prevalence of faecal coliforms in *Villorita cyprinoides*, sediment and water from different shellfish harvesting areas in Cochin estuary during pre-monsoon season

In monsoon season, the FC MPN levels increased considerably in all the three types of samples alike (Figure 2.16). In stations 1, 3, 4, 5 and 6 the MPN levels were equally high (4.2 logs) in all the 3 types of samples *viz*. shellfish, sediment and water. Compared to other stations, the FC MPN levels in all the three samples (3.96 logs) were lesser at station 8. In station 2, the FC MPN level in shellfish (3.96 logs) was comparatively lesser than the sediment and water samples. Similarly, in station 7, the FC MPN level was lower in water (3.96 logs) than those of sediment and shellfish samples. However, the difference in MPN levels were statistically insignificant (p=0.800).

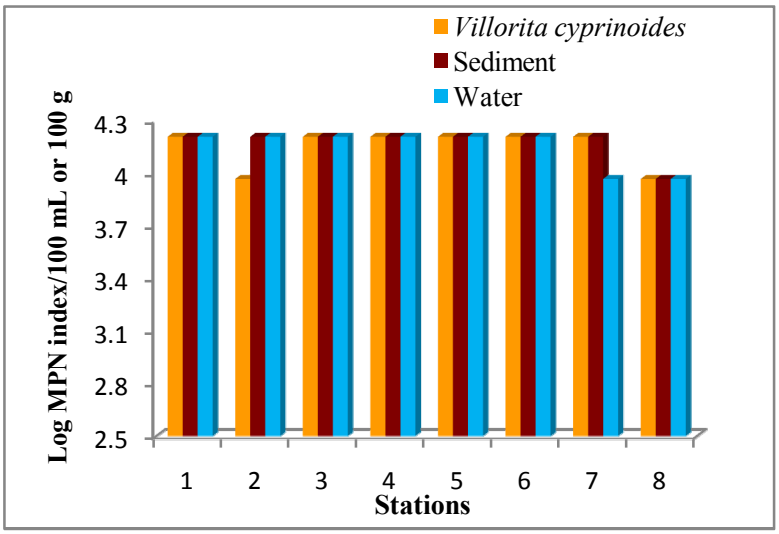

Figure 2.16 Relative prevalence of faecal coliforms in *Villorita cyprinoides*, sediment and water from different shellfish harvesting areas in Cochin estuary during monsoon season

In post-monsoon season, the FC MPN levels continued to remain high (4.2 logs) in all the three types of samples i.e. shellfish, sediment and water from the four stations 1, 3, 4 and 5 (Figure 2.17). The FC MPN levels in water reduced slightly to 3.96 logs in stations 6, 7 and 8 during post-monsoon season. In station 2, there was a slight reduction in FC MPN levels of both shellfish and sediment samples (3.96 logs) during the post-monsoon season. However, the differences in MPN levels in the three samples during post-monsoon season was found to be statistically insignificant ($p=0.549$).

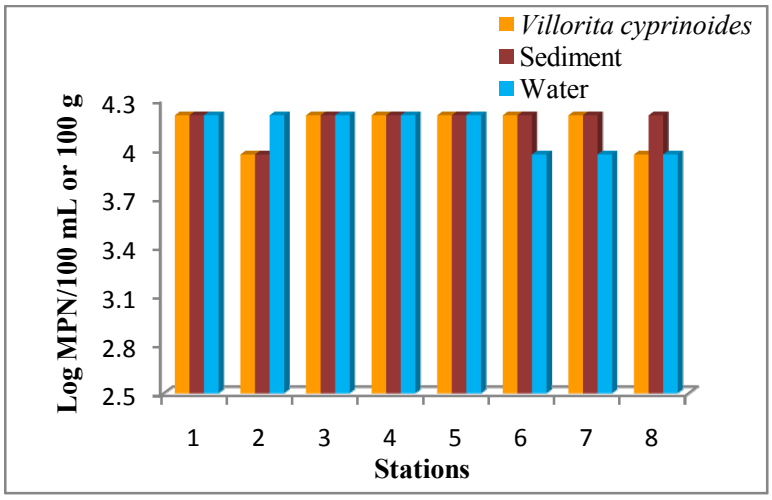

Figure 2.17 Relative prevalence of faecal coliforms in *Villorita cyprinoides*, sediment and water from different shellfish harvesting areas in Cochin estuary during post-monsoon season

2.5.1.6 Distribution of *E. coli* serotypes in different shellfish harvesting areas of Cochin estuary

All the biochemically identified *E. coli* were confirmed by PCR based amplification of uidA gene as shown in Figure 2.18. The overall distribution of *E. coli* serotypes in Cochin estuary is given in Figure 2.19. Out of the 125 strains isolated, 99 strains (79.2%) could be serotyped. Out of the 99 strains serotyped 35.35% was untypable and 6.06% was rough strains. Seventeen different serogroups of *E. coli* could be identified from Cochin estuary. Serogroup O8 constituted the maximum (16.1%), followed by O141 (10.1%), O121 (8.08%), O157:H7 (7.07%), O126 (5.05%), O120 (4.04%) and O118 (2.02%). Serogroups O2, O4, O17, O27, O33, O34, O84, O91, O124 and O156 constituted 1.01% each.

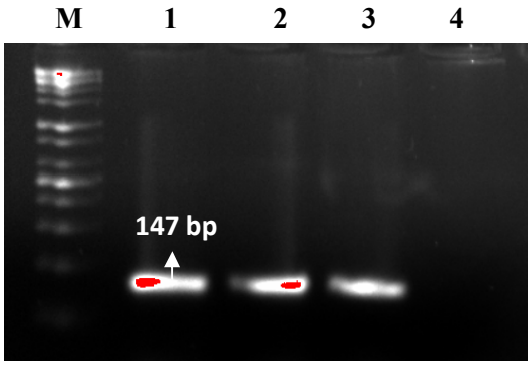

Figure 2.18 Gel image showing detection of *uidA* gene in *E. coli*

Lane M: 100 bp marker, lanes1-3: *uidA* gene of *E. coli*, lane 4: negative control

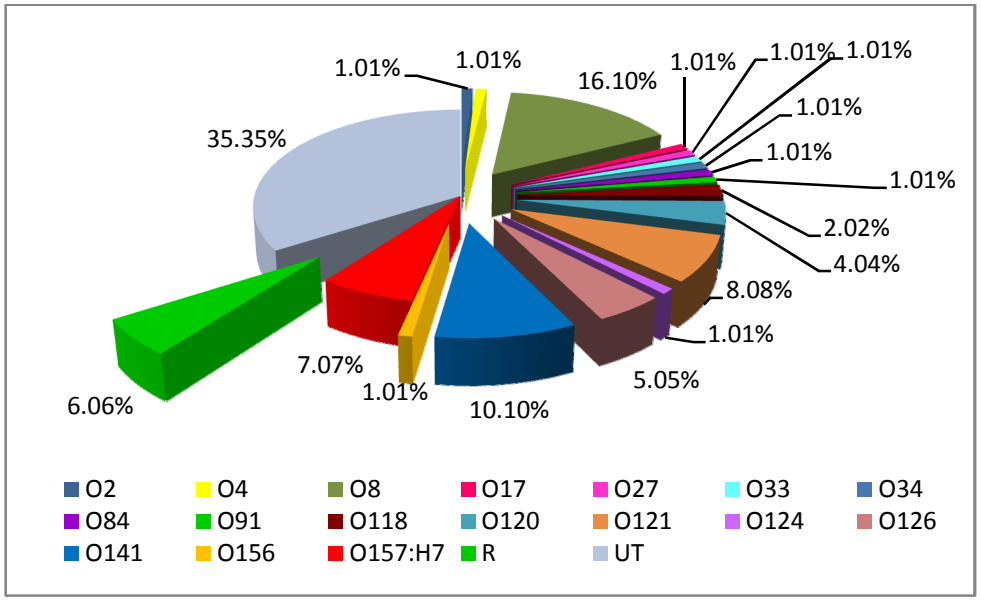

Figure 2.19 Overall diversity of *E. coli* serotypes in different shellfish harvesting areas of Cochin estuary

2.5.1.6.1 *E. coli* serotypes in shellfish (*Villortia cyprinoides*) from harvesting areas of Cochin estuary

The distribution of various *E. coli* serogroups in *Villortia cyprinoides* harvested from Cochin estuary is given in Fig. 2.20. Out of the 50 strains isolated 44 (88%) could be serotyped; out of which 38.63% was untypable and 2.27% was rough strains. *E. coli* isolated from shellfish from Cochin estuary comprised of 9 different serogroups. Serogroup O8 constituted the prominent group with 18.18%, followed by O157:H7 and O120 (9.09% each), O121 and O141 (6.81% each). Remaining serogroups O34, O118, O126 and O156 contributed 2.27% each to the total serogroup diversity.

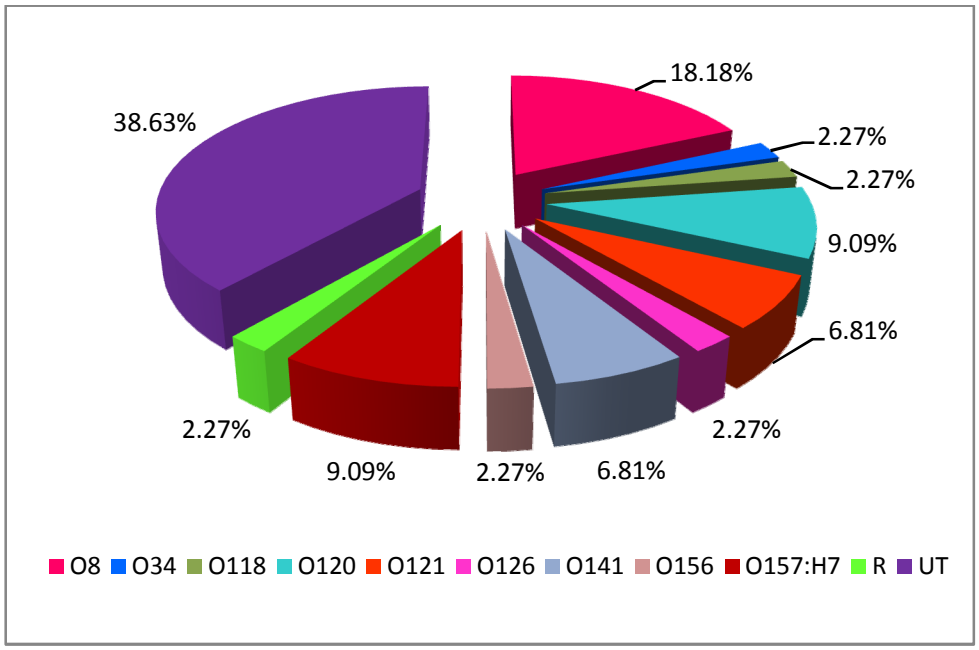

Figure 2.20 Serotype distribution of *E. coli* in shellfish from harvesting areas of Cochin estuary

2.5.1.6.2 *E. coli* serotypes in sediment from shellfish harvesting areas of Cochin estuary.

In sediment, out of the 38 strains of *E. coli* isolated, 27 (71%) could be serotyped; out of which 30% was untypable and 14.8% was rough strains (Figure 2.21). A total of 8 different serogroups of *E. coli* could be identified from sediment. Serogroups O157:H7 and O121 constituted maximum of 11.10% each, followed by serogroups O126 and O8 (7.40% each). Remaining serogroups O4, O17, O118 and O141 constituted 3.7% each.

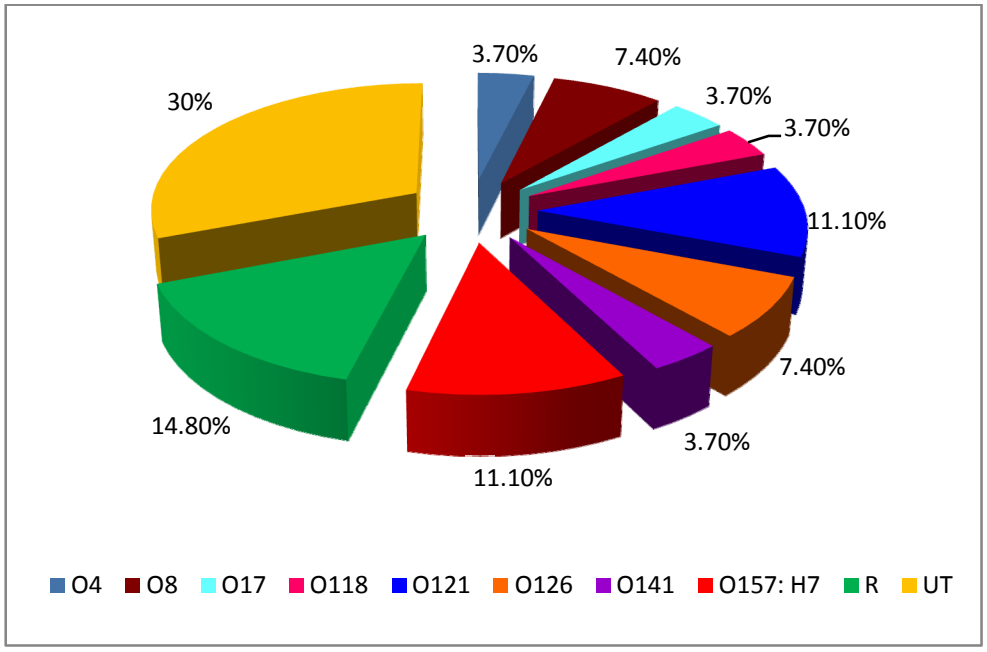

Figure 2.21 Serotype distribution of *E. coli* in sediment from different shellfish harvesting areas of Cochin estuary

2.5.1.6.3 *E. coli* serotypes in water from different shellfish harvesting areas of Cochin estuary

In water, out of 37 *E. coli* strains isolated, 28 strains (75.67%) could be serotyped, out of which 17.86% was untypable (Figure 2.22). A total of

10 different serogroups of *E. coli* could be identified from water. Serogroup O141 and O8 constituted the predominant group with 21.40% each, followed by O121 (14.29%). Serogroups O8, O27, O33, O84, O91, O124 and O126 constituted only 3.6% each.

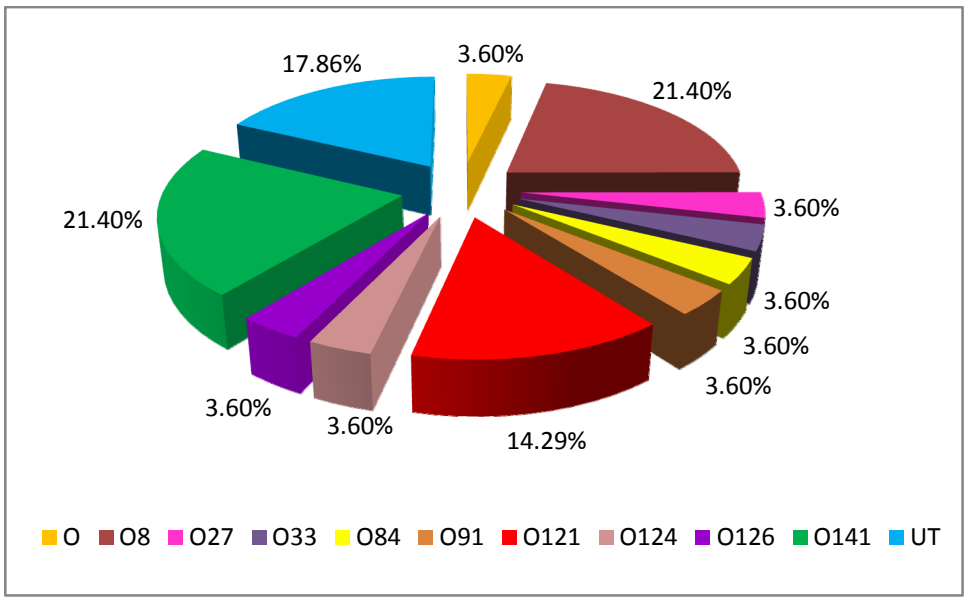

Figure 2.22 Serotype distribution of *E. coli* in water from different shellfish harvesting areas of Cochin estuary

2.5.1.7 Distribution of various pathogenic groups of *E. coli* in shellfish harvesting areas of Cochin estuary

Out of the total *E. coli* strains isolated from Cochin estuary 89.06% was found to be pathogenic, while only 10.94% was found to be nonpathogenic. Five different pathogenic *E. coli* groups were identified in total from Cochin estuary. Enterotoxigenic strains exhibited maximum prevalence (42.19%), followed by enterotoxigenic (40.62%) and uropathogenic strains (3.13%). The remaining pathogenic groups identified were enteropathogenic and enteroinvasive strains which exhibited the lowest prevalence of 1.56% each.

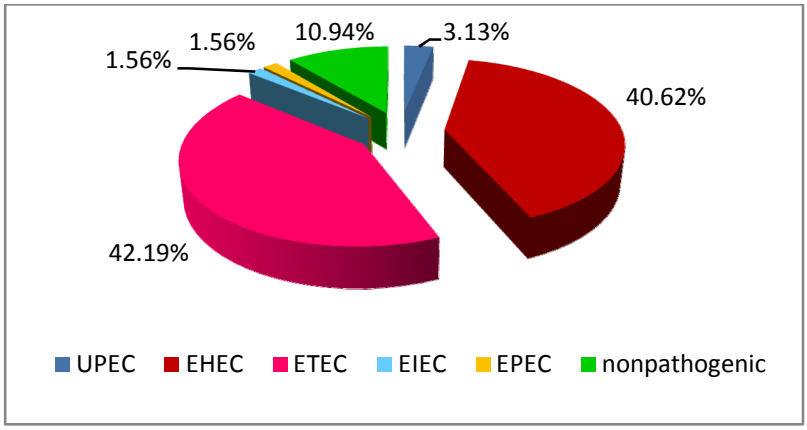

Figure 2.23 Overall distribution of various pathogenic groups of *E. coli* in different shellfish harvesting areas of Cochin estuary

2.5.1.7.1 Prevalence of pathogenic groups of *E. coli* in shellfish

In shellfish samples, 80.77% strains were found to be pathogenic while only 19.23% strains were nonpathogenic (Figure 2.24). Three different pathogenic groups were identified in shellfish. Enterotoxigenic strains exhibited maximum prevalence of 42.31%, followed by enterohaemorrhagic (34.6%) and enteropathogenic strains (3.86%).

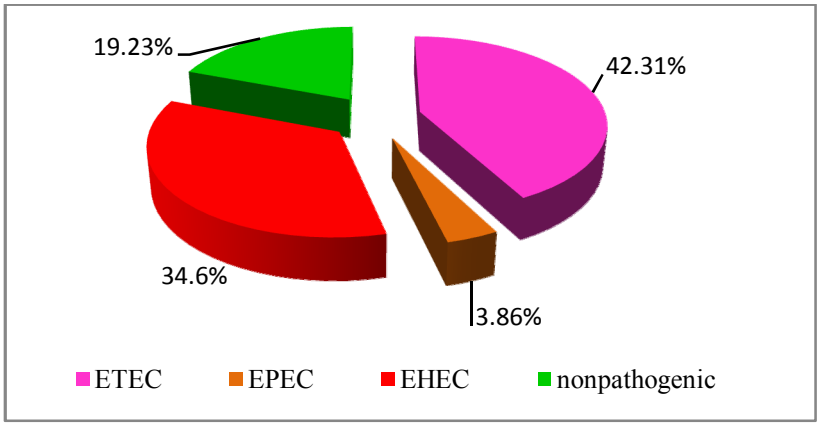

Figure 2.24 Distribution of different pathogenic groups of *E.coli* in *Villorita cyprinoides* from different harvesting areas of Cochin estuary

2.5.1.7.2 Prevalence of pathogenic groups of *E. coli* in sediment

All the *E. coli* strains isolated from sediment samples were found to be pathogenic. Enterohaemorrhagic strains constituted the prominent group with 66.67% prevalence, followed by enterotoxigenic (21.42%) and uropathogenic strains (13.3%).

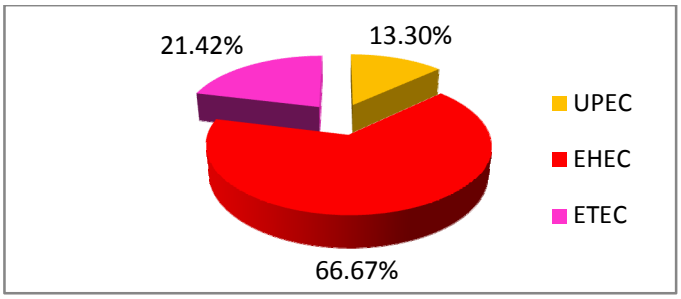

Figure 2.25 Distribution of different pathogenic groups of *E. coli* in sediment from different shellfish harvesting areas of Cochin estuary

2.5.1.7.3 Prevalence of pathogenic groups of *E. coli* in water

In water samples, 90.91% strains were pathogenic, while only 9.09% strains were nonpathogenic as shown in Figure 2.26. Three different pathogenic groups were identified; enterotoxigenic strains exhibited maximum prevalence of 59.1%, followed by enterohaemorrhagic (27.27%) and enteroinvasive (4.54%) strains.

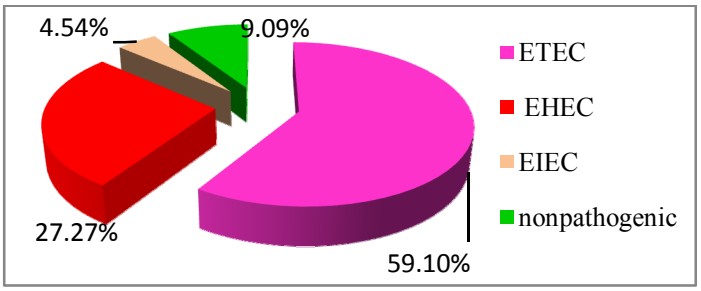

Figure 2.26 Distribution of different pathogenic groups of *E. coli* in water from different shellfish harvesting areas of Cochin estuary

2.5.2 Prevalence of faecal streptococci in shellfish harvesting areas of Cochin estuary

2.5.2.1 Prevalence of faecal streptococci in shellfish (*Villorita cyprinoides*)

Villorita cyprinoides harvested from various shellfish harvesting areas exhibited high faecal streptococci levels during the pre-monsoon season. Out of the 8 stations under study all the stations except station 2 exhibited FS MPN index of 4.04 logs. Station 2 showed least FS MPN index of 3.17 logs. The results were found to be statistically significant ($p<0.001$).

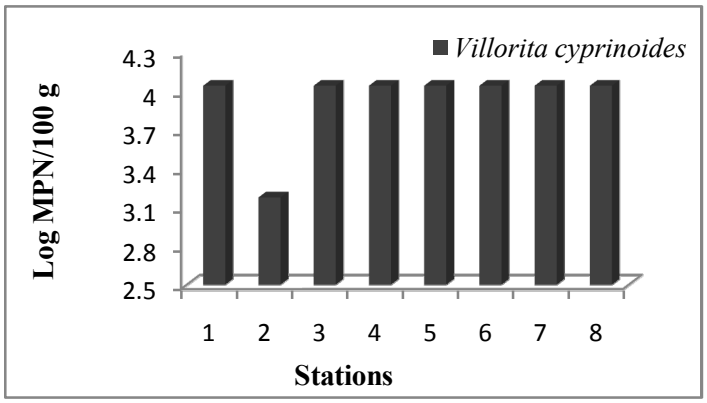

Figure 2.27 Prevalence of faecal streptococci in *Villorita cyprinoides* from different harvesting areas during pre-monsoon season

Monsoon season witnessed invariably high FS MPN indices in shellfish at all the stations. Shellfish (*Villorita cyprinoides*) harvested from all the 8 harvesting areas under study showed high FS MPN indices of 4.04 logs. The results were found to be statistically significant ($p<0.001$).

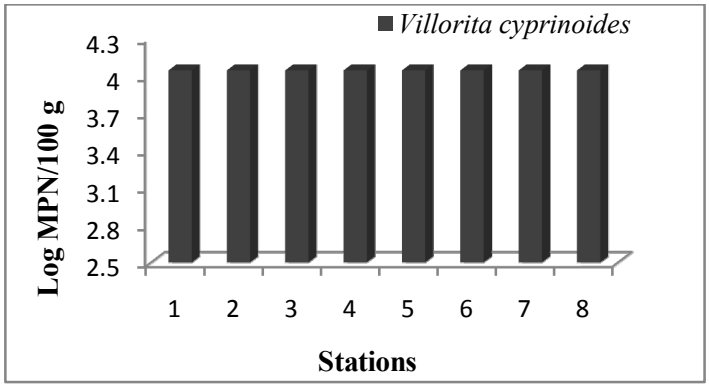

Figure 2.28 Prevalence of faecal streptococci in *Villorita cyprinoides* from different harvesting areas during monsoon season

In post-monsoon season also invariably high FS MPN indices were observed in shellfish at all the stations. Shellfish (*Villorita cyprinoides*) harvested from all the 8 harvesting areas under study showed high FS MPN indices of 4.04 logs as given in Figure 2.29. The results were found to be statistically significant ($p<0.001$).

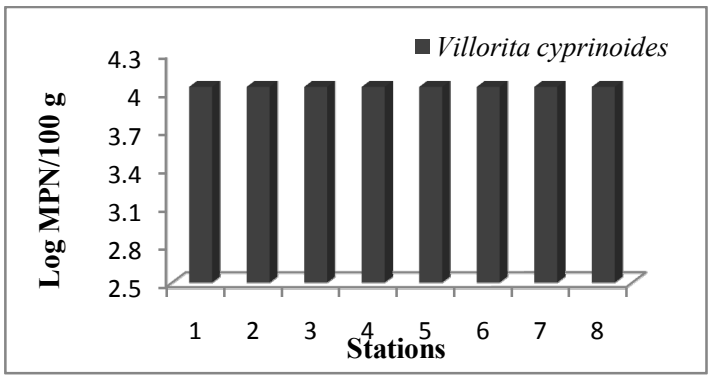

Figure 2.29 Prevalence of faecal streptococci in *Villorita cyprinoides* from different harvesting areas during post-monsoon season

2.5.2.2 Prevalence of faecal streptococci in sediment

Sediment samples from various shellfish harvesting areas exhibited remarkable variation in FS MPN indices during the pre-monsoon season as

shown in Figure 2.30. Stations 1, 6, 7 and 8 showed the maximum faecal coliform index of 4.04 logs followed by stations 2, 4 and 5 with MPN index of 3.96 logs. Station 3 showed the minimum MPN index of 3.66 logs.

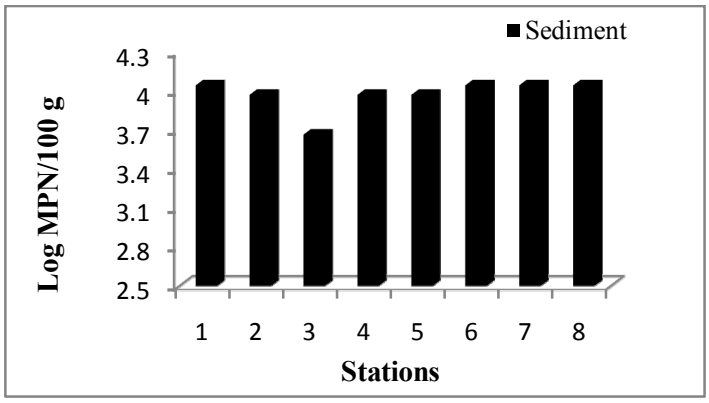

Figure 2.30 Prevalence of faecal streptococci in sediment from different harvesting areas during pre-monsoon season

In monsoon season, sediment samples from all the stations showed high FS MPN MPN indices. All stations except station 2 exhibited higher FS MPN indices of 4.04 logs; while station 2 exhibited the least FS MPN index of 3.96 logs (Figure 2.31).

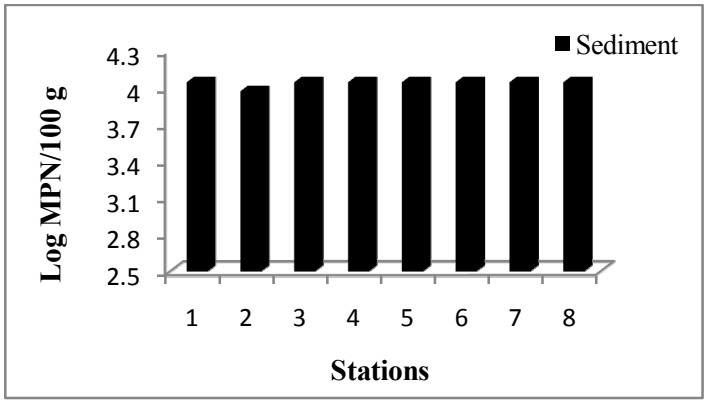

Figure 2.31 Prevalence of faecal streptococci in sediment from different harvesting areas during monsoon season

Sediment samples from various shellfish harvesting areas exhibited remarkable variation in FS MPN indices during the post-monsoon season (Figure 2.32). Stations 1, 3, 6, 7 and 8 showed the maximum faecal coliform index of 4.04 logs followed by stations 2 and 4 with MPN index of 3.96 logs. Station 5 showed the minimum FS MPN index of 3.66 logs.

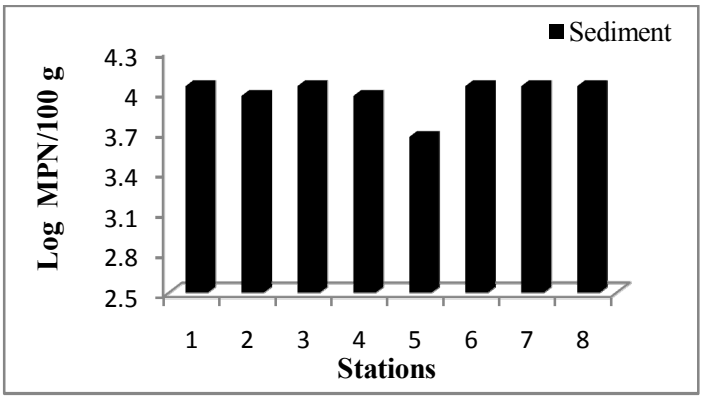

Figure 2.32 Prevalence of faecal streptococci in sediment from different harvesting areas during post-monsoon season

2.5.2.3 Prevalence of faecal streptococci in water

Water samples from various shellfish harvesting areas exhibited remarkable variation in FS MPN indices during the pre-monsoon season as shown in Figure 2.33. Maximum FS MPN index of 3.17 logs was observed at stations 8, followed by stations 1, 3, 4, 5, 6 and 7 with MPN index of 2.66 logs. Station 2 showed the minimum FS MPN index of 2.32 logs.

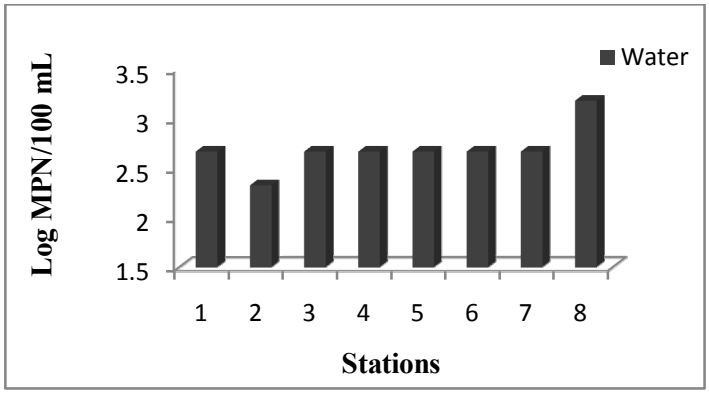

Figure 2.33 Prevalence of faecal streptococci in water from different shellfish harvesting areas during pre-monsoon season

In monsoon season also, water samples from various shellfish harvesting areas exhibited remarkable variation in FS MPN indices (Figure 2.34). Maximum FS MPN index of 3.66 logs was observed at stations 8, followed by stations 1, 4, 5, 6 and 7 with intermediate FS MPN levels. Stations 2 and 3 showed the least FS MPN index of 2.96 logs.

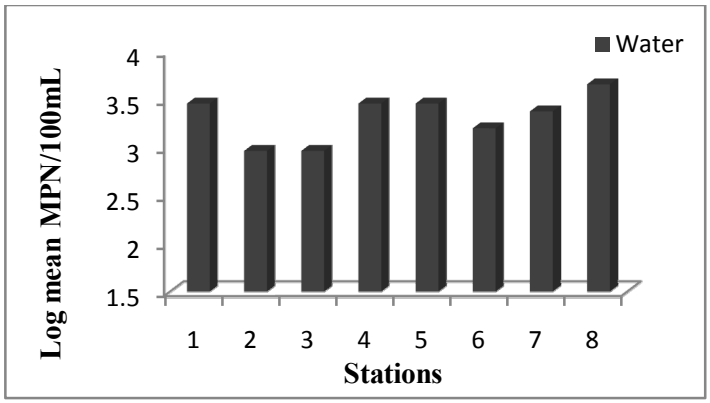

Figure 2.34 Prevalence of faecal streptococci in water from different shellfish harvesting areas during monsoon season

In post-monsoon season also water samples from various shellfish harvesting areas exhibited remarkable variation in FS MPN indices. Maximum

FS MPN index of 3.66 logs was observed at stations 8, followed by stations 1, 4, 5, 6 and 7 where intermediate FS MPN levels were recorded. Stations 2 and 3 showed the minimum FS MPN index of 2.96 logs.

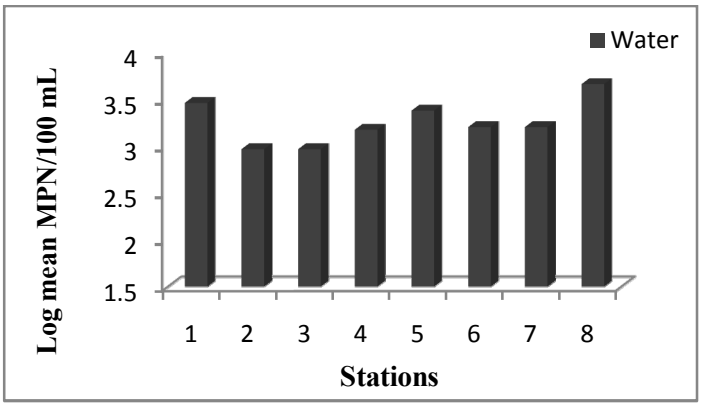

Figure 2.35 Prevalence of faecal streptococci in sediment from different harvesting areas during post-monsoon season

2.5.2.4 Seasonal variation in the prevalence of faecal streptococci in *Villorita cyprinoides*, sediment and water from different shellfish harvesting areas in Cochin estuary

Remarkable seasonal variation of faecal streptococci levels was observed only in water samples, while it was not so prominent in shellfish and sediment samples.

Shellfish samples did not show any significant seasonal differences in their FS MPN indices as given in Figure 2.36. In pre-monsoon season, station 2 exhibited reduced FS MPN index of 3.17 logs, whereas all other stations showed higher FS MPN indices of 4.04 logs. In monsoon and post-monsoon seasons, shellfish samples from all the stations invariably exhibited high MPN index of 4.04 logs. However, the results were found to be statistically insignificant (p=0.385).

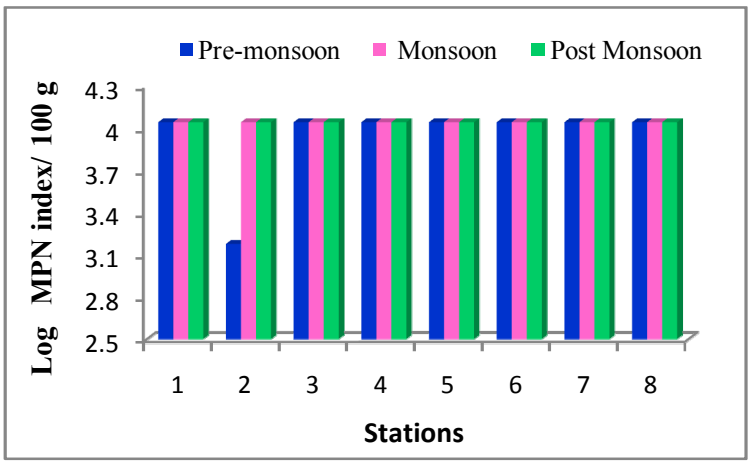

Figure 2.36 Seasonal variation in the prevalence of faecal streptococci in *Villorita cyprinoides* from different shellfish harvesting areas in Cochin estuary

In sediment samples, most of the stations did not show any significant seasonal differences in FS MPN indices (Figure 2.37). No significant seasonal variation could be observed between shellfish, sediment and water FS MPN indices at stations 1, 2, 6, 7 and 8. Stations 3, 4 and 5 exhibited variations in the FS MPN indices of the three samples, which however did not follow any significant pattern. In station 3, the MPN index (3.66 logs) in sediment was lower than that of monsoon and post-monsoon seasons; while monsoon and post-monsoon seasons recorded similar FS MPN indices (4.04 logs). Station 4 exhibited highest FS MPN index during monsoon season (4.04 logs), while pre-monsoon and post-monsoon seasons recorded similar sediment FS MPN levels (3.96 logs). In station 5, highest FS MPN (4.04 logs) was observed during monsoon season followed by pre-monsoon season (3.96 logs) and post-monsoon season (3.66 logs) recorded the least. However, the results were found to be statistically insignificant (p=0.427).

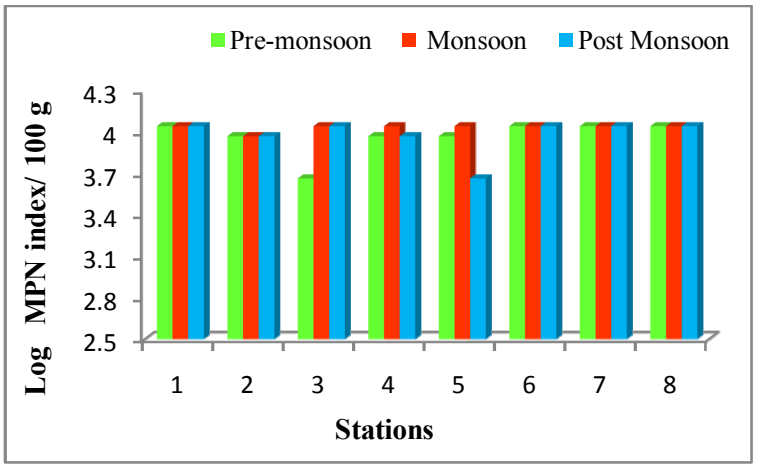

Figure 2.37 Seasonal variation in the prevalence of faecal streptococci in sediment from different shellfish harvesting areas in Cochin estuary

Water samples showed remarkable seasonal variation at all stations as shown in Fig. 2.38. Lowest FS MPN levels were recorded at all stations during the pre-monsoon season, compared to monsoon and post-monsoon seasons. Stations 1, 2, 3, 6 and 8 exhibited similar FS MPN indices during monsoon and post-monsoon seasons. Stations 4, 5 and 7 recorded higher FS MPN indices during monsoon season compared to post-monsoon season. In pre-monsoon season, the FS MPN of water at various stations ranged from 2.32-3.17 logs; the lowest MPN was observed at station 2 and highest at station 8. In monsoon as well as post-monsoon seasons, the FS MPN of water samples ranged from 2.96-3.66 logs. The differences in FS MPN indices of water samples between pre-monsoon, monsoon seasons were found to be statistically significant ($p<0.001$).

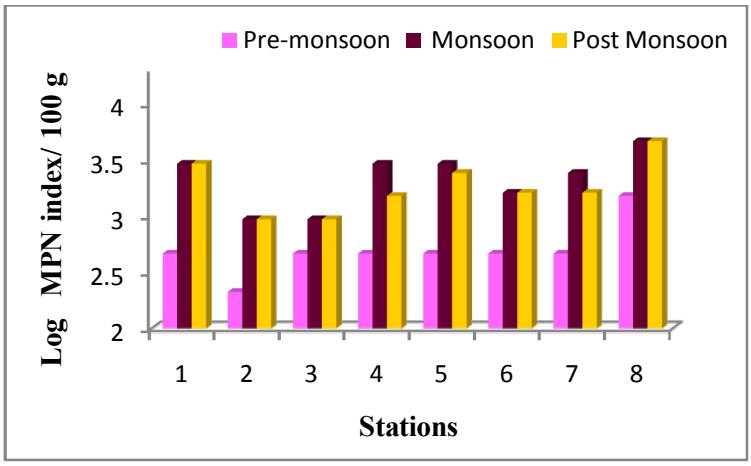

Figure 2.38 Seasonal variation in the prevalence of faecal streptococci in water from different shellfish harvesting areas in Cochin estuary

2.5.2.5 Relative prevalence of faecal streptococci in *Villorita cyprinoides*, sediment and water from shellfish harvesting areas in Cochin estuary during pre-monsoon, monsoon and post-monsoon seasons.

The shellfish, sediment and water samples collected from every station during pre-monsoon season showed significant differences in the FS MPN levels (Figure 2.39). In pre-monsoon season, the FS MPN levels were higher in shellfish and sediment samples compared to water samples. In all the eight stations FS MPN levels in shellfish were higher than that of water. The results were found to be highly statistically significant ($p<0.001$). Similarly the FS MPN values of sediment samples from all the stations were significantly ($p<0.001$) higher than that of water samples. The FS MPN values of shellfish samples, of all stations except station 2, were found to be higher than that of sediment samples and the results were found to be statistically significant ($p<0.001$).

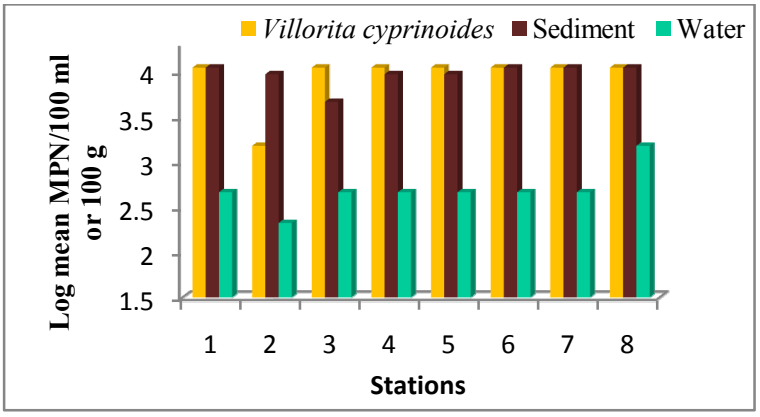

Figure 2.39 Relative prevalence of faecal streptococci in *villorita cyprinoides*, sediment and water from different shellfish harvesting areas of Cochin estuary during pre-monsoon season

Sample-wise variation could be observed at all stations under study during monsoon season as shown in Fig. 2.40. In monsoon season, the FS MPN values of shellfish samples and sediment samples were equally high (4.04 logs), except in station 2, where it was slightly lesser in sediment sample (3.96 logs) compared to shellfish sample (4.04 logs). However, in all the stations, the FS MPN loads in water samples were significantly lower than that of sediment as well as shellfish samples ($p<0.001$).

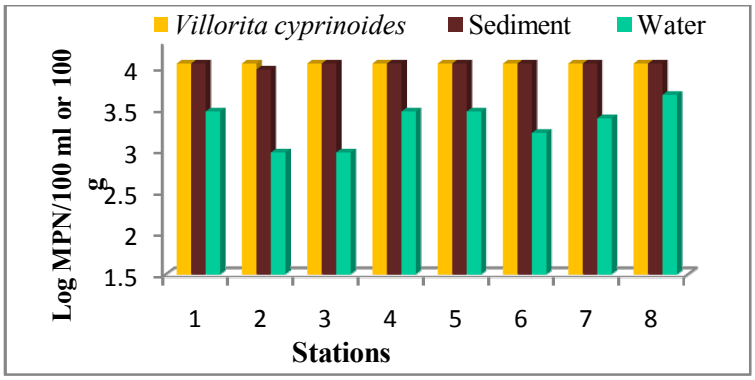

Figure 2.40 Relative prevalence of faecal streptococci in *villorita cyprinoides*, sediment and water from different shellfish harvesting areas of Cochin estuary during monsoon season

Sample-wise variation could be observed in FS MPN values during post-monsoon season also, as shown in Fig. 2.41. The FS MPN values of shellfish samples and sediment samples were equally high (4.04 logs), except in station 2, where it was slightly lesser in sediment sample (3.96 logs) compared to shellfish sample (4.04 logs). In all the stations, the FS MPN loads in water samples were significantly lower than that of sediment as well as shellfish samples ($p<0.001$).

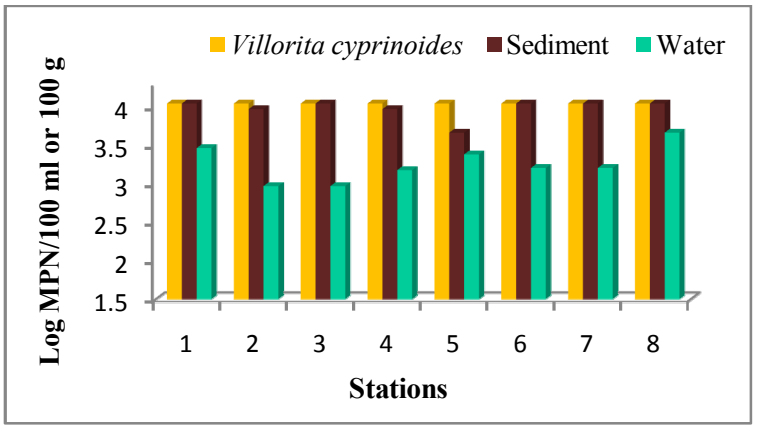

Figure 2.41 Relative prevalence of faecal streptococci in *villorita cyprinoides*, sediment and water from different shellfish harvesting areas of Cochin estuary during post-monsoon season

2.5.3 Prevalence of *V. parahaemolyticus* in shellfish harvesting areas of Cochin estuary

V. parahaemolyticus could be isolated from all the eight stations selected for the study; however stations showed variations in isolation during various seasons. Molecular confirmation of all the presumptive *V. parahaemolyticus* isolates were done by PCR based amplification of *tlh* (Figure 2.42) and *toxR* (Figure 2.43) genes respectively.

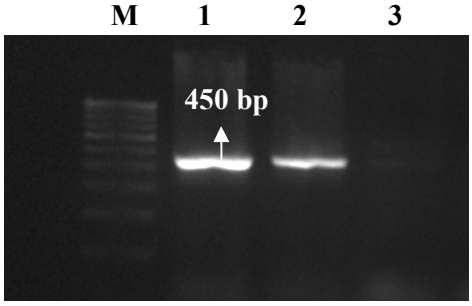

Figure 2.42 Gel image showing PCR products of *tlh* gene of *V. parahaemolyticus.* Lane M: 100 bp marker, lanes 1and 2: *tlh* gene of *V. parahaemolyticus,* lane 3: negative control

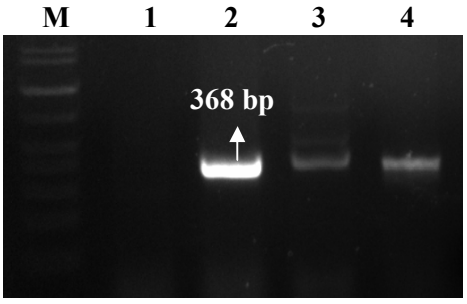

Figure 2.43 Gel image showing PCR products of *toxR* gene of *V. parahaemolyticus*. Lane M: 100 bp marker, lanes 1: negative control, lanes 2-4: *toxR* gene of *V. parahaemolyticus*

2.5.3.1 Prevalence of *V. parahaemolyticus* in shellfish during various seasons

In pre-monsoon season, *V. parahaemolyticus* could be isolated from *Villorita cyprinoides* from 7 out of the 8 stations under study. Station 1 did not show the presence of *V. parahaemolyticus*.

In monsoon season, shellfish harvested from 6 out of the 8 stations under study showed the presence of *V. parahaemolyticus* strains (Table 2.3). Shellfish harvested from stations 2 and 3 did not show the presence of *V. parahaemolyticus* while other 6 stations showed the presence of *V. parahaemolyticus*.

In post-monsoon season, 7 out of the 8 stations under study showed the presence of *V. parahaemolyticus*. Station 1 did not show the presence of *V. parahaemolyticus.*

There was no seasonal variation observed in *V. parahaemolyticus* prevalence in shellfish. In both pre-monsoon and post-monsoon seasons

V. parahaemolyticus could be detected in 7 out of 8 stations, whereas in monsoon season the prevalence was limited to 6 stations out of 8.

Table 2.3 Prevalence of *V. parahaemolyticus* in shellfish (*Villorita cyprinoides*) from different harvesting areas of Cochin estuary during various seasons

Stations	Pre-monsoon	Monsoon	Post-monsoon
1	-	+	-
2	+	-	+
3	+	-	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+

2.5.3.2 Prevalence of *V. parahaemolyticus* in sediment during various seasons

In pre-monsoon season, *V. parahaemolyticus* could be isolated from sediments collected from only 3 out of 8 stations under study (Table 2.4). Stations 5, 6 and 8 showed the presence of *V. parahaemolyticus*, while it could not be isolated from stations 1, 2, 3, 4 and 7.

In monsoon season, *V. parahaemolyticus* strains could be isolated from sediments from 6 out of 8 stations. Stations 3 and 4 did not show the presence of *V. parahaemolyticus.*

In post-monsoon season, *V. parahaemolyticus* could be isolated from sediments from stations 6, 7 and 8. Sediments from stations 1-5 did not show the presence of *V. parahaemolyticus.*

In sediment samples, seasonal variation in prevalence of *V. parahaemolyticus* could be observed as shown in Table 2.4. Highest incidence was observed during monsoon season compared to pre-monsoon and post-monsoon seasons.

In stations 1, 2, 5, 6, 7 and 8 maximum prevalence was found during monsoon season, compared to other seasons. During monsoon season, *V. parahaemolyticus* could be isolated from 7 out of 8 stations, whereas during pre-monsoon and post-monsoon seasons isolation was limited to 3 stations each.

Table 2.4 Prevalence of *V. parahaemolyticus* in sediment from different harvesting areas of Cochin estuary during various seasons

Stations	Pre-monsoon	Monsoon	Post-monsoon
1	-	+	-
2	-	+	-
3	-	-	-
4	-	-	-
5	+	+	-
6	+	+	+
7	-	+	+
8	+	+	+

2.5.3.3 Prevalence of *V. parahaemolyticus* in water during various seasons

In pre-monsoon season, *V. parahaemolyticus* could be detected in water samples from only 3 stations; stations 1, 3 and 8. Water samples from stations 2, 4, 5, 6 and 7 did not show the presence of *V. parahaemolyticus*.

In monsoon season, water samples from stations 1, 3 and 6 showed the presence of *V. parahaemolyticus*. However, *V. parahaemolyticus* could not be detected in water samples from stations 2, 4, 5, 7 and 8.

In post-monsoon season, water samples from only stations 1 and 6 showed the presence of *V. parahaemolyticus* while *V. parahaemolyticus* could not be isolated from all the other 6 stations .

Water samples exhibited no significant seasonal variation in prevalence of *V. parahaemolyticus*. During pre-monsoon and monsoon seasons,

V. parahaemolyticus could be isolated from 3 out of 8 stations, whereas during post-monsoon season only 2 stations exhibited the presence of *V. parahaemolyticus*.

Table 2.5 Prevalence of *V. parahaemolyticus* in water samples from various harvesting areas of Cochin estuary during various seasons

Stations	Pre-monsoon	Monsoon	Post-monsoon
1	+	+	+
2	-	-	-
3	+	+	-
4	-	-	-
5	-	-	-
6	-	+	+
7	-	-	-
8	+	+	+

2.5.3.4 Relative prevalence of *V. parahaemolyticus* in shellfish, sediment and water from different shellfish harvesting areas in Cochin estuary during pre-monsoon, monsoon and post-monsoon seasons

V. parahaemolyticus exhibited variation in the prevalence in shellfish, sediment and water samples isolated from the same station, during all the 3 seasons. Shellfish samples exhibited increased prevalence compared to sediment and water samples during all the seasons.

In pre-monsoon season, shellfish samples exhibited maximum prevalence of *V. parahaemolyticus* compared to sediment and water samples as shown in Table 2.6. Shellfish from 7 out of 8 stations showed the presence of *V. parahaemolyticus*, whereas sediment and water samples from only 3 out of 8 stations showed the presence of *V. parahaemolyticus.* Except station 1, shellfish from all the stations showed the presence of *V. parahaemolyticus.* *V. parahaemolyticus* was detected in sediment samples from stations 5, 6 and 8 and in water samples from stations 1, 3 and 8. Only station 8 exhibited the presence of *V. parahaemolyticus* in all the three samples.

Table 2.6 Relative prevalence of *V. parahaemolyticus* in shellfish, sediment and water from different shellfish harvesting areas of Cochin estuary during pre-monsoon season

Stations	Shellfish	Sediments	Harvesting waters
1	-	-	+
2	-	-	-
3	+	-	+
4	+	-	-
5	+	+	-
6	+	+	-
7	+	-	-
8	+	+	+

In monsoon season, shellfish and sediment samples from 6 out of 8 stations exhibited the presence of *V. parahaemolyticus*, whereas water samples from only 3 out of 8 stations showed the presence of *V. parahaemolyticus* (Table 2.7). Shellfish samples from all stations except stations 2 and 3 exhibited the presence of *V. parahaemolyticus*, whereas sediment samples from all stations except stations 3 and 4 showed the presence of *V. parahaemolyticus*. Water samples exhibited the lowest prevalence with only stations 1, 3 and 6 showing the presence of *V. parahaemolyticus.*

Table 2.7 Relative prevalence of *V. parahaemolyticus* in shellfish, sediment and water from different shellfish harvesting areas of Cochin estuary during monsoon season.

Stations	Shellfish	Sediments	Harvesting waters
1	+	+	+
2	-	+	-
3	+	-	+
4	+	-	-
5	+	+	-
6	+	+	+
7	+	+	-
8	+	+	-

V. parahaemolyticus could be detected in shellfish samples from 7 out of 8 stations during post-monsoon season, while sediment samples from only 3 stations out of 8 showed the presence of *V. parahaemolyticus* (Table 2.8). Water samples showed the least prevalence; only stations 1 and 6 were detected positive for *V. parahaemolyticus*. Shellfish samples from all stations except those from station 1 showed the presence of *V. parahaemolyticus* while sediment samples from stations 6, 7 and 8 showed the presence of *V. parahaemolyticus.*

Table 2.8 Relative prevalence of *V. parahaemolyticus* in shellfish, sediment and water from different shellfish harvesting areas of Cochin estuary during post-monsoon season

Stations	Shellfish	Sediments	Harvesting waters
1	-	-	+
2	+	-	-
3	+	-	-
4	+	-	-
5	+	-	-
6	+	+	+
7	-	+	+
8	-	+	+

2.5.3.5 Percentage incidence of *V. parahaemolyticus* in shellfish, sediment and water from shellfish harvesting areas of Cochin estuary

Out of the total 72 samples tested (shellfish (n=24), sediment (n=24) and water (n=24), shellfish samples showed the maximum incidence of *V. parahaemolyticus* (83.33%), followed by sediment samples (50%) and water samples recorded the least with 33.33% incidence (Table 2.9). Out of the total 317 presumptive *Vibrio* strains isolated from the 3 types of samples, 69 (21.72 %) strains were confirmed to be *V. parahaemolyticus* (Fig. 2.44). Maximum number of *V. parahaemolyticus* strains were contributed by shellfish

(50.7%), followed by sediment (33.3%) and water contributed the least (15.9%).

Table 2.9 Percentage incidence of *V. parahaemolyticus* in shellfish, sediment and water from shellfish harvesting areas of Cochin estuary

	No. of samples tested	**No. of samples positive**	**% of incidence**
Shellfish	24	20	83.33%
Sediment	24	12	50%
Water	24	8	33.33%

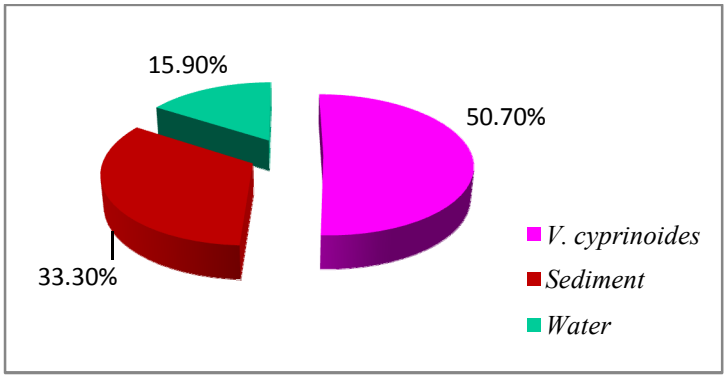

Figure 2.44 Percentage distribution of *V. parahaemolyticus* among *Villorita cyprinoides*, sediment and water samples from shellfish harvesting areas

2.5.4 Prevalence of *Salmonella* spp. in shellfish harvesting areas of Cochin estuary

The present study revealed the very low prevalence of *Salmonella* spp. in shellfish, sediment and water samples from shellfish harvesting areas of Cochin estuary. *Salmonella* could not be detected in shellfish and sediment samples from all the 8 stations, during all the three seasons *viz*. pre-monsoon, monsoon and post-monsoon seasons. *Salmonella* could be detected only in water sample from station 1, collected during the pre-monsoon season as given in Table 2.10.

Table 2.10 Percentage incidence of *Salmonella* in shellfish, sediment and water from Cochin estuary during the study period

	No. of samples tested	No. of samples positive	% of incidence
Shellfish	24	0	0%
Sediment	24	0	0%
Water	24	1	4.16 %

2.6 Discussion

2.6.1 Prevalence of faecal coliforms in shellfish, sediments and harvesting waters of Cochin estuary

Faecal contamination of shellfish growing areas poses significant threat to food safety associated with bivalve shellfish consumption. In shellfish, sediments and harvesting waters of Cochin estuary, high levels of faecal indicators such as faecal coliforms and enterococci were detected, which is indicative of poor sanitary quality as well as the probable presence of other enteric pathogens such as *Salmonella*.

Shellfish, sediment and water samples from shellfish harvesting areas of Cochin estuary exhibited seasonal variations in FC levels; comparatively higher FC levels were recorded during monsoon and post-monsoon seasons when compared to pre-monsoon season. This is in agreement with the findings of several researchers who have reported significant negative effect of rainfall events on the sanitary quality of the shellfish harvesting areas as indicated by higher faecal coliforms in shellfish, sediment and harvesting waters during monsoon season (Sasikumar and Krishnamoorthy, 2010; Raveendran *et al*., 1990). Increased surface run off during monsoon season increases the contamination of water bodies, which ultimately results in increased microbial contamination in shellfish and rainfall associated

disease outbreaks (Lipp *et al*., 2001). Storm water runoff is an important factor contributing to a significant proportion of *E. coli* accumulated by bivalves (Campos *et al*., 2011). Increased water temperatures as well as other inactivating physical factors such as UV light may also be responsible for reduced microbial concentrations during pre-monsoon season compared to the cooler monsoon, post-monsoon seasons (Chigbu *et al*., 2005; Lipp *et al*., 2001). An inverse relationship between rainfall and pollution of coastal waters from nonpoint-sources has been documented by several similar studies (Mok *et al*., 2016; Chigbu *et al*., 2005).

There are a few reports contradictory to our findings, where improvement of overall microbial status of some bivalve harvesting areas following rainfall have been reported (Lee and Morgan, 2003), possibly due to the dilution effects of rainfall in estuaries (Younger *et al*., 2003).

Significant variation in sample-wise prevalence of faecal coliforms was also noted. In pre-monsoon season, shellfish as well as sediment samples exhibited remarkably high FC levels when compared to water samples. Parallel findings have been reported by several researchers who observed elevated levels of faecal indicators in shellfish and sediments compared to overlying waters (Drummond *et al*., 2014; Burkhardt and Calci, 2000; Dore and Lees, 1995).

Shellfish non-specifically concentrates the contaminants in the surrounding waters; thus, in agreement to our present findings several researchers have reported bio-accumulation of bacteria in bivalve tissue to levels much higher than in the surrounding water. However, the accumulation rates differed among various filter-feeders; from a few fold to more than

hundreds-fold fold (Sasikumar and Krishnamoorthy, 2010; Burkhardt and Calci, 2000; Dore and Lees, 1995).

Based on the faecal coliform levels, 8 different shellfish growing areas under study were classified as follows. As per the European Union criteria, stations 1, 3, 4, 5, 6 and 7 were classified under class 'C' of shellfish growing areas. Stations 2 and 8 were classified at 'B' level during pre-monsoon season and 'C' during monsoon and post-monsoon seasons. As per the NSSP (US FDA) criteria all the stations were classified under prohibited areas during all the 3 seasons. These preliminary observations invite attention of the regulatory agencies to conduct further studies in this regard and implement stringent measures to improve the overall sanitary quality of the growing areas.

2.6.1.1 Presence of pathogenic *E. coli* groups in shellfish, sediment and harvesting waters of Cochin estuary

Present study revealed high prevalence of pathogenic *E. coli* groups in shellfish, sediment and water samples collected from Cochin estuary, which is indicative of the potential health hazard involved in consumption of shellfish harvested from the estuary. Pathogenic serogroups constituted 80.77%, 100% and 90.91% of the serotyped strains of shellfish (*V. cyprinoides*), sediment and harvesting water samples respectively.

Enterohaemorrhagic and enterotoxigenic strains were present in shellfish, sediments and water samples from Cochin estuary. In addition, enteropathogenic strains in shellfish, uropathogenic strains in sediment samples and enteroinvasive strains in shellfish samples were also detected.

The overall prevalence of enterohaemorrhagic O157:H7 (7.07%) *E. coli* in shellfish harvesting areas under study needs special mention. In shellfish

and sediment samples the prevalence of O157:H7 was 9.09% and 11.10% respectively, while water samples tested O157:H7 negative.

Though there are many reports on the isolation of *E. coli* O157:H7 from various other sources such as fresh vegetables and sprouts, very less literature is available on their isolation from shellfish harvesting areas. Our findings are in agreement with some previous reports on isolation of O157 *E. coli* from French oysters (Gourmelon *et al*., 2006; Guyon *et al*., 2000). However, Gourmelon *et al*. 2006) reported relatively higher prevalence (27.8%) whereas Guyon *et al*. (2000) reported lesser prevalence of O157 *E. coli*, compared to our present results. Baliere *et al*. (2015) reported higher prevalence of shigatoxigenic *E. coli* in oyster, sediment and water samples compared to present study. Bennani *et al*. (2011) reported the presence of shigatoxin-producing O157:H7 and non-O157 *E. coli* in shellfish and marine environments in the Mediterranean coast of Morocco. However, the incidence of O157:H7 (STEC) was very less (1.9%) compared to our results. In agreement with our findings, there is a recent report on isolation of enteropathogenic and enterohemorrhagic *E. coli* strains in oysters harvested from urban estuaries (Brandao *et al*., 2017). Though there are no previous reports of O157:H7 isolations from shellfish growing areas of India, its presence in shrimp from India was first reported by Surendraraj *et al*. (2010). To our knowledge, this is the first report of *E. coli* O157:H7 from shellfish growing areas in India.

2.6.2 Prevalence of faecal streptococci in shellfish harvesting areas.

Present study revealed high faecal streptococci (enterococcal subgroup) levels in shellfish growing areas of Cochin estuary, which is indicative of the extent of faecal contamination the estuary has undergone. The enterococcal

sub group of faecal streptococci have been considered as secondary indicators of potable water contamination while assessing the pollution status of both marine and freshwaters (Leclerc *et al*., 1996**;** Murray, 1990; US EPA, 1986). Even though it was considered earlier as a good index to determine the source of faecal contamination (human *vs* animal) (Winslow and Palmer, 1910), it is no longer considered so, as different faecal streptococcal species are proved to have differential survival rates (APHA, 1992; US EPA, 1986).

Though a preliminary study by Sobsey (1989) have suggested a correlation between illness and enterococcal counts in shellfish flesh at harvest, it is not considered for the assessment of growing areas for bivalve cultivation, as per the current EC or NSSP regulations. Faecal streptococci levels were high in shellfish and sediments during all the three seasons ie. pre-monsoon, monsoon and post-monsoon seasons alike. However, seasonal variations in FS levels were observed in water samples. Lowest prevalence was observed in water samples during pre-monsoon season while comparatively higher FS levels were observed during monsoon as well as post-monsoon seasons.

The FS levels exhibited sample-wise variation also. During all the 3 seasons, highest incidence was observed in shellfish and sediment samples, compared to that of water samples. This is in agreement with the findings of Slanetz *et al*. (1968) where 10-20 folds higher concentrations of coliforms, faecal coliforms, and faecal streptococci were found in oyster tissue than in the surrounding sea water. Similar findings were made by Lalitha and Surendran (2005), who reported high levels of *E. coli* and enterococci in black clam *Villorita cyprinoides* var. *cochinensis* and water samples collected from three clam harvesting areas in Vembanad Lake which did not conform to legal standards. Chinnadurai *et al*. (2016) also studied the bioaccumulation of

bacteria in oysters from Ashtamudi Lake, Kerala, India. Maximum level of FS was found during monsoon season in oyster tissue, while lower maximum count was observed in harvesting water during the pre-monsoon season.

Apart from their role as faecal indicators, many researchers have reviewed that enterococci as opportunistic or rather emerging pathogens responsible for many community-acquired, hospital-acquired (nosocomial) super infections such as endocarditis, bacteraemia etc (Morrison *et al*., 1997; Murray, 1990). Another matter of serious concern is the exceptional degree of multiple drug resistance (intrinsic as well as acquired) exhibited by the enterococcal species which poses serious challenge to chemotherapy. The incidence of these multidrug resistant enterococci in food sources indicate the possibility of pathogen recycling between food and environmental sources (Murray, 1990).

2.6.3 Prevalence of *V. parahaemolyticus* in shellfish harvesting areas

V. parahaemolyticus could be isolated from all the 8 shellfish harvesting areas selected for the study. *V. parahaemolyticus* is a halophilic bacterium native to estuarine, marine and coastal surroundings and hence its presence in shellfish growing areas have been widely reported (Letchumanan *et al*., 2014; Yu *et al*., 2013; Ristori *et al*., 2007). Though majority of the environmental strains of *V. parahaemolyticus* are found to be non-pathogenic (Ceccarelli *et al*., 2013; DePaola *et al*., 2000), a number of seafood related disease outbreaks due to pathogenic strains have been reported (Li *et al*., 2014; Hara-Kudo *et al*., 2012). A number of shellfish associated disease outbreaks due to consumption of raw oysters, clams, cockles etc. have been widely reported from various geographical locations (Fuenzalida *et al*., 2007; Deepanjali *et al*., 2005; Potasman *et al*., 2002).

Seasonal variation was not observed much, in prevalence of *V. parahaemolyticus* in shellfish, sediment and water samples. However, sediment samples demonstrated slightly increased *V. parahaemolyticus* isolations during monsoon season compared to pre-monsoon and post-monsoon seasons. Contradictory to our findings many researchers have reported seasonal variation in *V. parahaemolyticus* isolations (Urquhart *et al*., 2015; Johnson *et al*., 2010). Increased concentrations of *V. parahaemolyticus* in water samples and their corresponding isolations from shellfish has been found associated with warmer temperatures (Schets *et al*., 2010). Similar observations were made by some researchers where increased detection of *V. parahaemolyticus* was noted in oysters harvested in the spring and summer rather than in the winter (Su and Liu *et al*., 2007; DePaola *et al*., 2000). This influence probably, may be more prominent in temperate regions, where favourable warmer temperatures are reached only during summer seasons, which thus results in enhanced bacterial multiplication and improved isolations during these warmer months. Such temperature variations between seasons are not so prominent in tropical regions, where warmer water temperatures exist year round. Precipitation and salinity differences also were not found to influence *V. parahaemolyticus* incidence during our study, except for the slight increase in isolation from sediment during monsoon season. This was not in agreement with the findings of Yu *et al*., (2013) where *V. parahaemolyticus* density in water samples was found to be significantly associated with precipitation while that in the sediment or shellfish samples depended significantly on the salinity of the seawater. However, in agreement with our results, some other researchers could not find any correlation between temperature, salinity and *V. parahaemolyticus* prevalence (Ristori *et al*., 2007).

Shellfish samples exhibited maximum prevalence of *V. parahaemolyticus* (83.33%) followed by sediment (50%) and water samples (33.33%) showed the least prevalence during all the seasons. Similar observation was made by Yu *et al*. (2013) in Taiwan, where increased prevalence was observed in oyster and clam samples followed by sediment samples and water samples exhibited the least prevalence. Several similar studies have reported the isolation of *V. parahaemolyticus* from shellfish growing areas (Mannas *et al*., 2014; Schets *et al*., 2010).

2.6.4 Prevalence of *Salmonella* in shellfish harvesting areas

Present study revealed that *Salmonella* is probably not a serious threat to shellfish food safety in the study area. *Salmonella* could be detected only in one water sample from station 1, collected during the pre-monsoon season and the prevalence of *Salmonella* in water was found to be very less (4.16 %). This is in agreement with the findings of Mok *et al*. (2016) where pathogens such as *Salmonella* or *Shigella* could not be detected in any of the oyster samples collected from Hansan-Geojeman area, a prominent shellfish growing area in Korea. Parallel findings have been made by several other researchers. Lunestad *et al*. (2016) investigated the prevalence of *Salmonella* in blue mussels harvested from the coast of Norway during the six-year period from 2007 to 2012 and found a very low prevalence of 0.3%. Similarly, Martinez-Urtaza *et al*. (2003) reported *Salmonella* in only1.8% of shellfish samples harvested from coast of Spain.

This was not in agreement with the results of several other researchers who reported increased prevalence of *Salmonella* in shellfish. Kumar *et al*. (2008) studied the presence of *Salmonella* in seafood samples from the harbours and retail fish markets of Cochin. Using cultural, ELISA and PCR

methods, *Salmonella* incidences of 21.4%, 16.6% and 35.7% were reported in shellfish including clam, mussel and oyster. Bujjamma *et al*. (2015) also reported slightly higher levels of *Salmonella* (9.72%) in shellfish from a domestic fish market, Guntur city, Andhra Pradesh, India collected during a one year study (2010-11).

2.6.5 Prevalence and significance of bacteria in sediments

Present study revealed higher FC and FS levels in sediments compared to overlying waters, which is in agreement with the findings of several other researchers (Drummond *et al*., 2014; Anderson *et al*., 2005; Davies *et al*., 1995). Similar findings were made by Martinez-Manzanares *et al*. (1992) in Spain, who reported maximum FS levels in sediments, followed by shellfish samples whereas, water samples demonstrated the least. Legnani *et al*. (1998) studied the microbiological quality of shellfish growing area in Italy and found higher FS levels in sediment samples (59%) compared to water samples (39%). However, mussels and clams recorded the maximum of FS levels of 80% and 100% respectively. On the contrary, Bennani *et al*. (2012) reported maximum enterococci concentrations in sediments of shellfish growing areas in Meditteranean coast of Morocco followed by shellfish and least was recorded in sea water.

Several factors such as increased nutrient content (Alkan *et al*., 1995; Anderson *et al*., 1983), presence of particles for attachment, protection from inactivating factors such as sunlight (Chandran and Hatha, 2003) have been responsible for enhanced bacterial numbers and its extended survival in sediments compared to surrounding waters. Binding to particle surfaces increases the persistence of bacteria in environment by providing protection from abiotic and biotic stresses (Davies *et al*., 1995). Another probable

reason for increased bacterial incidence in sediments is the anaerobic condition that prevails (instead, aerobic condition prevails in water column and hence more predatory activity), which does not provide a suitable environment for predatory protozoa that prey on FC (Crabill *et al*., 1999).

The re-suspension of the sediment bound bacteria by anthropogenic activities such as dredging and natural phenomenon like storms, may lead to increased microbial levels in the upper water column (Yamahara *et al*., 2007; Anderson *et al*., 2005; Davies *et al*., 1995). Long term survival of bacteria in the viable-but-non-culturable (VBNC) state have also been demonstrated in sediments, which on return of favourable conditions may transform into viable form (Rozen and Belkin, 2001).

Sediment samples exhibited remarkable seasonal variations in FC levels; comparatively higher MPN indices were recorded during monsoon and post-monsoon seasons when compared to pre-monsoon season. This is in agreement with the reports of several researchers stating a direct positive correlation between temperature and predation rates (Barcina *et al*., 1997; Sherr *et al*., 1988; Anderson *et al*., 1983; McCambridge and McMeekin, 1980). Thus increased zooplankton activity in summer may be partly responsible for decreased bacterial concentration during pre-monsoon season (Mezrioui *et al*., 1995).

Prevalence of *V. parahaemolyticus* in sediment samples also slightly showed, seasonal variation as shown in Table 2.2. Highest incidence was observed during monsoon season, compared to pre-monsoon and post-monsoon seasons. In present study, *V. parahaemolyticus* could be isolated from 50% of sediment samples whereas only 33.33% of the water samples

were positive for *V. parahaemolyticus*. This is in agreement with the findings of Chandran *et al*. (2011) who observed enhanced survival of *V. parahaemolyticus* in sediments collected from Vembanad Lake along south west coast of India compared to overlying water, which can be attributed to the increased organic content present in it.

In summary, present study reveals the high level of faecal contamination that resulted in the poor sanitary quality of the shellfish growing areas located along Cochin estuary. This was reflected in the sanitary quality of the shellfish harvested also, which did not conform to the legal standards during most of the seasons. Moreover, 89% of the serotyped *E. coli* strains from the shellfish growing areas belonged to pathogenic serogroups. Another matter of serious concern is the detection of enterohaemorrhagic *E. coli* O157:H7 from sediments as well as shellfish from this estuary. Presence of pathogenic strains in sediments has to be seriously dealt with because, even if the overall water quality is improved the sediment may act as permanent repository, contaminating the overlying water column if upwelling happens as in events such as storms or dredging. *V. parahaemolyticus* is a bacterium native to estuaries and marine environments, hence its presence is quite natural in such environments. However, due to filter-feeding, there are possibilities of accumulation of cells in shellfish meat compared to surrounding water. Hence, even though the infectious dose of *V. parahaemolyticus* is relatively high, sometimes in bivalves the number sufficient for an infectious dose may be attained, causing problems to the consumers if the strains are potentially pathogenic. Another remarkable finding is that, present study does not identify *Salmonella* as a potential threat to shellfish food safety in the area. Present study emphasises the necessity of regulatory

interventions in improving the sanitary quality of the shellfish harvesting areas by implementing measures such as monitoring and certification, and above all generating an awareness among the public in this regard.

Chapter 3

RISK ASSESSMENT OF FAECAL INDICATOR AND PATHOGENIC BACTERIA ISOLATED FROM SHELLFISH GROWING AREAS OF COCHIN ESTUARY

Contents

3.1 Introduction

Antibiotic resistance among microorganisms is one of the most serious problems encountered during disease management. Emergence of more and more resistant bacteria to newer classes of antibiotics is a real challenge in chemotherapy. The worst consequence of antibiotic resistance is that the once commonly used life saving drugs become no longer useful and even the smallest infections sometimes prove lethal. Evidence from various parts of the world indicates an overall decline in the total stock of antibiotic effectiveness: resistance to all first-line and last resort antibiotics have been found to be increasing at an alarming rate (CDDEP, 2015a; WHO, 2014). Eventhough drug resistance has often been considered primarily as a clinical problem, the increased role of non-clinical environments in dissemination of antibiotic resistance genes (ARG) has been identified recently (Berglund, 2015). As a result of indiscriminate use of antibiotics in aquaculture, animal husbandry, agriculture and careless disposal of hospital wastes, there is an

increase in antibiotic residues discharged into natural environments for which the aquatic environments act as the ultimate sink. Evidences suggest that prolonged exposure to these antibiotic residues, results in the selection of drug resistant mutants. (Berglund, 2015; Chattopadhyay *et al*., 2015). Once developed resistance, there is high risk of horizontal as well as vertical transmission of resistance rendering more and more organisms resistant.

3.2 Review of Literature

3.2.1 Antibiotic resistance

Antibiotic resistance is nowadays considered as one of the paramount public health challenges globally (WHO, 2014; CDC, 2013a; Levy, 2002). The extensive use and misuse of antibiotics have resulted in development of antibiotic resistance among several human pathogens, reducing the possibilities for treatments of infections and jeopardizing medical procedures, such as organ transplantations or implants, where infective complications are common and antibiotic therapy is inevitable to prevent those infections (Davies *et al*., 2010; WHO, 2000). Antibiotic resistance has long been considered as a clinical problem, however recently there are increasing evidences for the role played by environments in dissemination of antibiotic resistance (Martinez, 2012).

Monitoring programs for antibiotic resistance have been established in Europe, North America and Latin America to regulate the use of antibiotics (Boerlin *et al*., 2005). Antibiotics are released into the environment from various sources. Nowadays antibiotics are widely used for therapy of infected humans and animals as well as for the prophylaxis and growth promotion of food-producing animals. Many studies suggest that inadequate selection and abuse of antibiotics might have lead to resistance in various bacteria making the treatment of bacterial infections much more difficult (Kolar *et al*., 2001).

Multidrug-resistant bacterial strains are prevalent in human and animal isolates all over the world (Swartz *et al*., 2002).

Antibiotics are released into the environment from various sources. After use in human beings, antibiotics and their metabolites will be discharged to the sewerage system (Daughton and Ternes, 1999) and depending on their chemical nature and persistence the compounds might be degraded or associate with sewage sludge (Anukool *et al*., 2004). Antibiotics applied for human beings may also reach agricultural soils directly through irrigation with wastewaters and surface waters (Uyttendaele *et al*., 2016; Allende, 2015; Kinney *et al*., 2006). Veterinary pharmaceuticals or their metabolites are released into the environment either directly, from use in aquaculture and the treatment of animals, or indirectly during the application of manure and slurry from the livestock facilities to land (Daghrir and Drogui, 2013; Boxall *et al*., 2004). Compounds that reach the soil system will be eventually transported to surface water or groundwater and be cycled and re-cycled within the environment (Topp *et al*., 2008). The several routes through which human beings get exposed to antibiotics, antibiotic-resistant bacteria or antibiotic resistance genes (ARGs) include livestock (Novick, 1981) that have accumulated veterinary drugs and resistant flora through the food chain; fish exposed to pharmaceuticals released to surface waters either intentionally (aquaculture treatments) or unintentionally; groundwater and surface water containing residues of pharmaceuticals which is used for drinking purpose; and coastal waters which are used for recreation or shellfish production (Wellington *et al*., 2013). Usage of antibiotics in aquaculture remains a potential source of antibiotic residues to aquatic environments (Inglis *et al*., 1993). It has been viewed that indiscriminate antimicrobial use in humans

may act as the major selective force for multi-drug resistant clones in Enterobacteriaceae in many developing countries (Okoli *et al*., 2002; Kariuki *et al*., 1996). On the contrary, in developed countries antimicrobial use in agriculture is considered to be the major driving force in the selection and dissemination of bacterial resistance (Witte, 1998). It is observed that in most countries, about 20 percent of antibiotics are used in hospitals and other healthcare facilities, and the remaining 80 percent are used in the community, either prescribed by healthcare providers or purchased directly by consumers or caregivers without prescription (Kotwani and Holloway, 2011). Further, antibacterial antibiotics are often used for treating viral infections such as cough and colds which are not only ineffective, but also lead to widespread antibiotic resistance.

Sewage microflora is often considered as potential habitat of resistance genes present on mobile genetic elements, which are readily transferred to other bacteria in close proximity (Knapp *et al*., 2010). These bacteria carrying mobile genetic elements such as integrons might transfer antibiotic resistance genes from animals to animals as well as to humans (Aarestrup and Wegener, 1999; Recchia *et al*., 1995).

3.2.1.1 Antibiotic resistance among *E. coli* isolates from environmental sources

The significant role of natural environments as reservoirs of antibiotic resistant bacteria has been emphasized in several studies (Suzuki, 2011; Henriques *et al*., 2006). *E. coli* is a natural inhabitant of the enteric tract of humans and other warm-blooded animals. It is the widely accepted faecal indicator, since its presence does not occur in other niches, unless and until there is faecal contamination. It is easily detectable and its presence in food,

water and other sources is indicative of recent faecal contamination and possible presence of other pathogens (Costa, 2013; Edberg *et al*., 2000; Krumperman, 1983). Even though most *E. coli* are non-pathogenic, there are several potentially pathogenic strains with virulent factors capable of causing diarrheal disease in humans (BAM, 2011; Kaper *et al*., 2004).The wide use and abuse of antibiotics in human therapy as well as for other uses in aquaculture, animal husbandry etc has produced multiple antibiotic resistant (MAR) *E. coli*. *E. coli* contamination of food originating from these high-risk environments has been identified by MAR indexing of *E. coli* isolates obtained from food (Krumperman, 1983).

Several reports have been published on antibiotic resistance among *E. coli* isolated from diverse environments such as rivers (Amos *et al*., 2014; Korzeniewska *et al*., 2013; Tacao *et al*., 2013), drinking water (Coleman *et al*., 2013; Talukdar *et al*., 2013; De Boeck *et al*., 2012) sewage water (Koczura *et al*., 2012), surface water (Kamruzzaman *et al*., 2013; Dolejska *et al*., 2009) and estuarine water (Sukumaran and Hatha, 2015; Sukumaran *et al*., 2012; Abhirosh *et al*., 2011; Chandran *et al*., 2008). It has been viewed that nutrient-rich environments such as wastewater offer optimal conditions for horizontal gene transfer, which frequently involves the passage of plasmids and transposons responsible for antibiotic resistance (Kelly *et al*., 2009).

E. coli resistant to third generation cephalosporins have been reported world wide. More and more *E. coli* and related bacteria are becoming resistant to newer third-generation cephalosporins through the production of Extended-Spectrum Beta-Lactamase (ESBL) enzymes, which make treatment of such infections extremely difficult (CDDEP, 2015a; Amos *et al*., 2014; Dhanji *et al*., 2011). An alarming increase in the number of ESBL positive

E.coli has been reported from many European countries (EARS-Net, 2014; Dhanji *et al*., 2011). Increased incidence of ESBL positive *E.coli* from several Asian countries also has been reported which showed 26% to 50% resistance to third and fourth generation cephalosporins (Lu *et al*., 2012). Lohiya *et al*. (2015) has reported 20% of the *E. coli* from rural settings of New Delhi exhibiting ESBL type antibiotic resistance.

Carbapenem-resistant Enterobacteriaceae (CRE) which are resistant even to last-resort carbapenems is another big in challenge in chemotherapy (Gaibani *et al*., 2011; Papp-Wallace *et al*., 2011). There are reports of carbapenem resistant *E. coli* from Europe (10%) (EARS-Net, 2014) and US (2%) (CDC, 2013a). Carbapenem resistant *E. coli* has been reported from a tertiary care hospital in India (Gopalakrishnan and Sureshkumar, 2010). It is reported that 13% of *E. coli* in India in 2013, were resistant to carbapenems (CDDEP, 2015b).

Cochin estuary, located toward the Southwest coast of India, is a tropical estuary which has undergone considerable pollution during recent periods, mainly due to development of satellite townships along the banks of this estuary as well as the rivers which joins this estuary. There are several recent reports stating prevalence of drug resistance *E. coli* and other bacteria in the study area (Sukumaran and Hatha, 2015; Sukumaran *et al*., 2012; Abhirosh *et al*., 2011; Chandran *et al*., 2008; Hatha *et al*., 2004). Sukumaran and Hatha (2015) reported significant resistance to ampicillin (23.07%), tetracycline (19.23%), co-trimoxazole (15.38%), and cefotaxime (15.38%), in extraintestinal pathogenic *E. coli* (ExPEC) isolated from the study area. Chandran *et al*. (2008) has isolated *E. coli* belonging to various pathogenic sero groups with high MAR index values indicating contamination

from high risk sources. About 95% of the isolates were found to be multidrug resistant and resistance greater than 80% was observed against antibiotics such as vancomycin, novobiocin, kanamycin, oxytetracycline, tetracycline and streptomycin.

3.2.1.2 Antibiotic resistance among *E. coli* isolates from food sources

Contamination of foods with antibiotic resistant bacteria could be a significant threat to public health as there is the distinct possibility of horizontal transmission of antibiotic resistance to other bacteria of human clinical significance through mobile genetic elements. *E. coli* is an ideal candidate vehicle for such transfers because it is a common flora in the gastrointestinal tracts of both humans and animals and survives well in other environments also. Contamination of aquatic environments is a matter of great concern, since it may act as potential source of antibiotic resistant bacteria in humans, acquired directly or indirectly through food chain (Henriques *et al*., 2006). Once acquired, the antibiotic resistant genes may exist in the environmental bacteria for a long time, even after the selection pressure ceases (Tamminen *et al*., 2011; Bean *et al*., 2005). Several previous reports have been published on antibiotic resistance among *E. coli* isolated from food producing animals (Filioussis *et al*., 2013; Jones-Dias *et al*., 2013; Jouini *et al*., 2009), meat (Abdel-Rhman *et al*., 2015; Laury *et al*., 2009; Selvan *et al*., 2007). Several studies have reported that *E. coli* isolates from animals and food products can exhibit resistance to many classes of antibiotic agents (Veldman *et al*., 2011; Lu *et al*., 2010; Seputiene *et al*., 2010). Multidrug resistant *E. coli* has been reported in chicken (Momtaz *et al*., 2012; Ferens and Hovde, 2011). Previous studies from India have revealed antibiotic resistant bacteria associated with seafood (Gangurde *et al*., 2014; Kumar *et al*., 2005).

With increased contamination of aquatic environments worldwide, increased contamination of the aquaculture products harvested have been reported. The food safety concern associated with bivalve shellfish (Prato *et al*., 2013) is mainly due to the filter feeding nature, which results in the accumulation of all the contaminants present in the growing area. Increased bacterial contamination and presence of antibiotic resistance in shellfish has been observed in a number of previous studies. The contamination of the harvesting areas due to sewage discharges may result in contamination of shellfish with drug resistant bacteria (Watkinson, 2007). Van *et al*. (2007) reported 61.6% of multidrug resistant *E. coli* in several food sources including shellfish. Fifty-seven percent of *E. coli* contained mobile genetic elements such as integrons which harboured gene cassettes responsible for resistance against aminoglycosides, trimethoprim, ampicillin and chloramphenicol. It was also demonstrated that the drug resistant phenotypes could be transferred to organisms of same or different species by conjugative plasmid transfer. Van *et al*. (2007) reported comparatively lesser incidence of multiple drug resistance in shellfish samples (35%) compared to other meat such as pork and chicken.

3.2.1.3 Antibiotic resistance among *V. parahaemolyticus* strains from environmental sources

Vibrio parahaemolyticus is a marine micro-organism native to estuarine waters throughout the world. It is widely distributed in marine waters, sediments, and is a part of the natural microflora of bivalve shellfish (Baker-Austin *et al*., 2008; Yeung and Boor, 2004).*V. parahaemolyticus* is recognized as the leading cause of human gastroenteritis associated with seafood consumption in the United States (Daniels *et al*., 2000a). *V. parahaemolyticus* is a common cause of foodborne disease in Asia (Liu *et al*., 2004; Alam *et al*.,

2002). Consumption of raw or undercooked seafood is one of the primary causes of gastroenteritis characterised by diarrhoea, vomiting, abdominal cramps, nausea etc (DePaola *et al.*, 2003). Resistance to penicillin and ampicillin have been widely reported in *V. parahaemolyticus* from a number of sources such as coastal waters (Zanetti *et al*., 2001), wastewater (Okoh and Igbinosa, 2010), fish farms (Labella *et al*., 2013), shrimp farms (Reboucas *et al*., 2011; Tendencia and De la Pena, 2001) and shellfish samples (Ottaviani *et al*., 2013). Shaw *et al*. (2014) carried out a study in Chesapeake Bay and Coastal Bays surface water and found that *V. parahaemolyticus* expressed 96% of intermediate resistance to chloramphenicol and 68% resistance to penicillin. Baker-Austin *et al*. (2008) reported high levels of antibiotic resistance in *V. parahaemolyticus* isolates from sediment and waters of Atlantic coast of Georgia and South Carolina and found that 24% isolates demonstrated resistance to 10 or more agents including beta lactams, aminoglycosides, and other classes of antibiotics. Virulence genes were detected in some isolates; however no apparent correlation was found between virulence and site, sample type, or season of isolation. Slightly reduced antibiotic resistance was reported among the virulent strains.

Okoh and Igbpinosa (2010) detected antibiotic resistance genes in several isolates and viewed the possibility of genetic exchange between clinical and environmental *Vibrio* spp. Environmental *V. parahaemolyticus* isolates have exhibited resistance to wide range of antibiotics such as penicillin, sulfamethoxazole (Okoh and Igbinosa, 2010), furazolidone, neomycin B and penicillin G (Vaseeharan *et al*., 2005), tetracycline, oxytetracycline and trimethoprim/sulfamethoxazole (Labella *et al*., 2013) and lincomycin (Ottaviani *et al*., 2001).

A few researchers have demonstrated drug resistance in *V. parahaemolyticus* from India. Vaseeharan *et al*. (2005) reported 100% resistance to ampicillin and 80% resistance to furazolidone, neomycin B and penicillin G in *V. parahaemolyticus* isolates from shrimp culture hatcheries and ponds in India. Silvester *et al*. (2015) reported high prevalence of multiple antibiotic resistant bacteria from Cochin estuary and an adjoining shrimp farm. The strains were reported to have high multiple antibiotic resistance index (MAR) values.

3.2.1.4 Antibiotic resistance among *V. parahaemolyticus* strains from food sources

V. parahaemolyticus is a causative of serious human seafood borne gastroenteritis which is occasionally fatal. Being a natural flora of marine and brackish environments, multidrug resistant *V. parahaemolyticus* has been isolated from seafood worldwide (Elmahdi *et al*., 2016; Hu and Chen, 2016; Wang *et al*., 2015; Haendiges *et al*., 2014; Iwamoto *et al*., 2010). Many antibiotic resistant bacterial genera including *Vibrio* have emerged during the past few decades (WHO, 2014; Cabello, 2006). There are several reports about antibiotic resistance of *V. parahaemolyticus* isolated from seafood from various geographical locations; Gulf of Mexico (Panicker *et al*., 2004), Malaysia (Al-Othrubi *et al*., 2014), Brazil (Costa *et al*., 2013), China (Hu and Chen, 2016; Xu *et al*., 2016), India (Silvester *et al*., 2015; Reyhanath and Kutty, 2014; Sudha *et al*., 2014; Manjusha *et al*., 2005), USA (Baker-Austin *et al*., 2008; Han *et al*., 2007; McLaughlin *et al*., 2005) and Bangladesh (Hossain *et al*., 2013). In an important study carried out in United States, *V.parahaemolyticus* was found to be susceptible to most of the antibiotics used except ampicillin (57% ampicillin-resistant and 24% ampicillin-intermediate resistant) (Han *et al*., 2007). Several other studies also reported ampicillin resistance of *V. parahaemolyticus* (Shaw *et al*., 2014; Zanetti *et al*., 2001).

Sudha *et al*. (2014) reported incidence of *V. parahaemolyticus* isolates showing maximum resistance against ampicillin and colistin followed by amoxycillin, carbenicillin, ceftazidime and cephalothin from clams, shrimp and crabs from retail markets of Cochin. Manjusha *et al*. (2005) have investigated antibiotic resistance of *Vibrio* spp. from brackish and marine environments from south west coast of India and found that 54% have shown multiple antibiotics resistance (MAR). *Vibrio* strains from shrimp, mussel and sepia also showed high resistances. Out of the 83.19% antibiotic resistant *Vibrio* strains, 30.3% were resistant to three antibiotics, 55.5% were resistant to 4-10 antibiotics and 14.14% were resistant to more than 10 antibiotics. Isolates from water samples of aquaculture farms exhibited comparatively lower antibiotic resistance than that observed in coastal areas. Interestingly *Vibrio* spp. collected from fish and tissue samples were more resistant than those from water samples. Irrespective of the sampling sites highest incidence of antibiotic resistance was observed against antibiotics such as amoxycillin, ampicillin, carbencillin and cefuroxime followed by rifampicin and streptomycin.

3.2.1.5 Antibiotic resistance among enterococci from environmental sources

Enterococcus spp. are part of the normal gut microbiota of both humans and animals, and can survive in diverse harsh conditions (Soheili *et al*., 2014; Cesar and Arias, 2013). Eventhough most *Enterococcus* species are commensal organisms, some species are opportunistic human pathogens or rather emerging pathogens, responsible for many community-acquired, hospital-acquired (nosocomial) super infections such as endocarditis (Munita *et al*., 2013), bacteraemia etc. (Moellering, 1992; Murray, 1990).

E. faecalis and *E. faecium* are the most prevalent enterococcal species cultured from humans, accounting for more than 90% of the clinical isolates

(De Perio, 2006). Though enterococci do not cause severe systemic infections, they pose potential therapeutic challenge because of their inherent as well as acquired resistance to a number of antibiotics. Of particular concern is the intrinsic antibiotic resistance demonstrated against amino glycosides and cephalosporins. Enterococci intrinsically, resist beta-lactam antibiotics due to the production of penicillin-binding proteins (PBPs) which enable them to synthesize some cell-wall components. They also demonstrate intrinsic resistance towards penicillinase-resistant penicillins, cephalosporins, nalidixic acid, macrolides, aztreonam, trimethoprim-sulfamethoxazole and low levels of clindamycin and amino glycosides (Tendolkar *et al*., 2003; Morrison *et al*., 1997).

In addition to intrinsic resistance, enterococci also have acquired resistance to penicillin by beta-lactamases, chloramphenicol, tetracyclines, rifampin, fluoroquinolones, aminoglycosides (high levels) and vancomycin. The emergence of vancomycin resistant enterococci (VRE), particularly *E. faecium* is a growing challenge in chemotherapy, as it leaves the clinicians treating VRE infections, with limited therapeutic options (Chaje and Zadernowska, 2016; De Perio, 2006). *E. faecium* has often displayed acquired resistance to higher concentrations of penicillin, than is possible by intrinsic resistance due to production of low affinity penicillin binding proteins (Rybkine *et al*., 1998). Acquired resistance against High-level aminoglycoside (Jones *et al*., 1995), fluoroquinolones (Kanematsu *et al*., 1998) macrolides, tetracycline, and chloramphenicol have also been observed in enterococci. Chromosomal mutations may contribute to resistance to rifampin and fusidic acid among others (Gold, 2001).

Acquired resistance is usually transposon or plasmid encoded and is transmissible whereas intrinsic resistance is based in chromosomal genes, and typically are non-transferrable (Huycke *et al*., 1998; Murray., 1998). Acquired antibiotic resistance is exchanged through resistance-encoding genes which are carried on conjugative transposons, pheromone-responsive plasmids, and other broadhost-range plasmids (Rice *et al*., 1995).

Newer antibiotics such as quinupristin-dalfopristin, linezolid, daptomycin and tigecycline with activity against many VRE strains are quite promising, but resistance to these agents also has been already reported (Tsai *et al*., 2012). A mutation (G2576U) in the domain V of the 23S rRNA is responsible for linezolid resistance, whereas resistance to quinupristin-dalfopristin may be due to several mechanisms such as modification of enzymes, active efflux, and target modification. Resistance of *E. faecalis* and *E. faecium* to daptomycin, a newer cyclic lipopeptide antibiotic that acts on the bacterial cell membrane, has also been reported. The emergence of antibiotic resistant strains may be due to the indiscriminate use of these antibiotics in animal husbandry, veterinary medicine and for human use (Klare *et al*., 2003).

In order to combat infections caused by these highly resistant *E. faecalis* and *E. faecium* various synergistic antibiotic combinations have been attempted; ceftaroline and daptomycin has appeared to be the most effective combination (Smith *et al*., 2015).

Multidrug resistant (MDR) enterococci have been reported from various environmental sources worldwide. Environmental waters such as agricultural wells (Tansuphasiri *et al*., 2006) wells in animal farms, rivers, coastal waters (Al-Gheethi *et al*., 2013; Dada *et al*., 2013), sea water (Bennani *et al*., 2012), canals, sewage effluents (Al-Gheethi *et al*., 2013),

sediments (Bennani *et al*., 2012; Dang *et al*., 2011) and other eutrophic environments (Ali *et al*., 2016) have shown the presence of drug resistant enterococci. The species mainly considered were *E. faecalis* and *E. faecium*, but other species were also recovered (Daniel *et al*., 2015; Gilmore, 2002). Several studies from Malaysia, Thailand, Vietnam, Indonesia, and other Southeast Asian countries reported the isolation of MDR enterococci isolated from livestock and animal-related products (Chaje and Zadernowska, 2016; Usui *et al*., 2014; Getachew*et al*., 2013).

Bennani *et al*. (2012) reported higher degrees of antibiotic resistance in enterococci isolated from seawater, sediment and shellfish from coastal Mediterranean environments of Morocco. In seawater samples, the highest resistance was obtained against rifampicin (33%), whereas in shellfish and sediment, the highest resistance (28% and 24% respectively) was detected against erythromycin.

As enterococci are normal inhabitants of the intestinal tract, they are exposed to antimicrobial selection every time; since animals are often subjected to antimicrobial therapy or antibiotics given for growth promotion (Hammerum *et al*., 2010). Interaction between the different reservoirs may result in wide spread of MDR enterococci. This finding was supported by a study conducted in Vietnam where similar *E. faecium*, *E. faecalis*, andother *Enterococcus* spp. were isolated from water and sediment of ponds and manure samples of pigs; which suggested that *Enterococcus* spp. isolated from the ponds might have originated from pig manure (Dang *et al*., 2011). It has been viewed that the misuse of antibiotics in livestock production may stimulate an environmental reservoir of antibiotic resistance. The finding was confirmed by a study conducted by Miller (2000) where increased resistance

towards 7 antibiotics which were used for animal growth promotion was observed.

There have been a few reports regarding aminoglycoside resistance genes in enterococci from hospitals in India (Gangurde *et al*., 2014; Agarwal *et al*., 2009; Mendiratta *et al*., 2008). VRE have been reported from several hospitals, tertiary care hospitals (Gangurde *et al*., 2014; Praharaj *et al*., 2013; Shafiyabi *et al*., 2013) and clinical specimens (Gangurde *et al*., 2014) from India. Praharaj *et al*. (2013) studied vancomycin resistance in enterococci from a tertiary hospital in India. A considerable level of vancomycin resistance was reported and vanA phenotype and genotype was the most common type observed. Co-existance of *vanA* and *vanC1* gene clusters was also identified in an *E. gallinarum* strain which conferred it a high level glycopeptide resistance. Singh (2009) studied enterococci of equine origin in North India and found that 99.6% were multiple-drug resistant with 80% strains showing resistance to vancomycin.

3.2.1.6 Antibiotic resistance among enterococci from food sources

Several studies have investigated the antibiotic resistance among enterococci from food sources (Ben Said *et al*., 2017; Chaje and Zadernowska, 2016; Tansuphasiri *et al*., 2006; Aarestrup *et al*., 2002; Mac *et al*., 2002; Franz *et al*., 2001). Some of these studies revealed that majority of the isolates from foods were resistant to one or more antibiotics (Obeng *et al*., 2014; Aarestrup *et al*., 2002; Franz *et al*., 2001). Reports about occurrence of vancomycin-resistant enterococcal (VRE) strains and strains with multiple antibiotic resistances from food sources are a matter of great concern (Ben Said *et al*., 2017; Franz *et al*., 2001).One important possibility for the development of VRE in food animals is the selective pressure exerted by the use of avoparcin

(a growth promoter in animal husbandry) which exhibits cross resistance to vancomycin. These VRE has every chance of spreading through food chain and may contaminate various natural environments also (Ben Said *et al*., 2017; Pavia *et al*., 2000; Klein *et al*., 1998; Van Den Braak *et al*., 1998).

Antibiotic resistance of Enterococcal species such as *E. faecalis* and *E. faecium* isolated from various foods from diverse geographic areas have been analysed in a number of studies (Ben Said *et al*., 2017; Mathur and Singh, 2005; Robrido *et al*., 2000; Davies and Roberts, 1999; Quednau *et al*., 1998). MDR enterococci have been isolated from various food sources and food animals such as raw meat (Chaje and Zadernowska, 2016; Robrido *et al*., 2000; Klein *et al*., 1998), fermented milk and meat products (Giraffa, 2002; Franz *et al*., 2001; Giraffa and Sisto, 1997) chicken meat (Obeng *et al*., 2014; Yurdakul *et al*., 2013), sea food (Ben Said *et al*., 2017; Boss *et al*., 2016) including shellfish (Bennani *et al*., 2012). The studies suggest high prevalence of MDR enterococci in foods; however most of the strains were sensitive towards antibiotics of clinical relevance such as ampicillin and vancomycin. Sultana *et al*. (2014) studied the prevalence of drug resistant bacteria in frozen foods from Bangladesh in which enterococcal group contributed least. Obeng *et al*. (2014) evaluated the phenotypic drug resistance and occurrence of resistance genes in order to study the phase of the production cycle during which the meat chickens and egg layers are being colonized with drug resistant bacteria. Phenotypically resistant enterococcal isolates isolated from 3-5 days old chickens itself carried genetic determinants for resistance to tetracycline, bacitracin and tylosin; which indicated colonization took place at very early phases. Previous studies have also reported prevalence of MDR enterococci in seafoods including shellfish (Ben Said *et al*., 2017; Bennani *et al*., 2012; Moore *et al*., 2008; Wilson and

McAfee, 2003). Comparison of antibiotic resistances of enterococci from different sources of coastal Mediterranean environments of Morocco revealed that isolates from shellfish and sediment samples showed highest resistance against erythromycin, whereas seawater samples highest resistance against rifampicin (Bennani *et al*., 2012). Wilson and McAfee (2003) examined presence of VRE in shellfish and found that 1.6-2.7% of enterococci from shellfish exhibited resistance to high levels of vancomycin. It was suggested that rather than acting as sources for widespread human infection they may represent an ultimate sink and reservoir for these persistent organisms.

Although intrinsic drug resistance is not horizontally transmitted, there are reports stating horizontal transfer of acquired resistance in enterococci (Palmer *et al*., 2010; Cocconcelli *et al*., 2003; Teuber *et al*., 1999; Doucet-Populaire *et al*., 1991). Transfer of erythromycin and tetracycline resistance genes among *E. faecalis* isolates have been demonstrated during the fermentation process of cheese and sausages (Cocconcelli *et al*., 2003). Doucet-Populaire *et al*. (1991) also demonstrated transfer of erythromycin and tetracycline resistance genes from *E. faecalis* to *Listeria monocytogenes in vitro* and *in vivo* conditions

3.2.2 Presence of antibiotic resistance genes

3.2.2.1 Antibiotic resistance genes in *E. coli*

Antibiotic resistant bacteria pose a major threat to public health (WHO, 2014; Levy, 2002). This is due to the possibility that the genes encoding the resistance determinants, if present on mobile genetic elements may be transferred to other commensal bacteria or bacteria of clinical significance (Schuurmans *et al*., 2014).

E. coli is an ideal candidate vehicle for such transfers as, it is a flora in the gastrointestinal tracts of both humans and animals and also has the ability to survive in the environment under diverse conditions. Further if the environment is contaminated with antibiotic resistant bacteria carrying virulence genes there is distinct possibility that it may enter into food chain and turn into potential human pathogens or transfer the genes to other potential human pathogens. Thus a cycling of resistance genes between environment, food sources and humans may occur (Schroeder *et al*., 2004; Van den Bogaard *et al*., 2000; Aarestrup and Wegener, 1999).

3.2.2.1.1 Extended spectrum beta lactamase genes in *E. coli*

Extended spectrum beta lactamases (ESBLs) are beta lactamases that have the following features: they are transferable; can hydrolyze penicillins, first, second and third-generation cephalosporins, and aztreonam (but not the cephamycins); and they can be blocked (*in vitro*) by beta-lactamase inhibitors such as clavulanic acid (Tham *et al*., 2010). The term extended-spectrum beta-lactamase (ESBL) was coined by Philippon in 1989 (Philippon *et al*., 1989). Many attempts have been made to classify beta lactamases. Based on primary structure (Ambler, 1980), enzymatic properties and biochemical attributes (Bush *et al*., 1995), and the increasingly available amino acid sequences (Bush and Jacoby, 2010), four major classes (A, B, C, D) have been described. Serine beta lactamases which belong to class A are the most abundant (Philippon *et al*., 2016), with more than 500 enzymes. The most clinically significant extended spectrum beta lactamases (ESBL) are temoneira (TEM), sulfhydryl variable (SHV) and Cefotaximase-München (CTX-M) type enzymes (Bush and Fisher, 2011). TEM-1 was the first plasmid mediated beta-lactamase described in gram-negative organisms (Datta and

Kontomichalou, 1965). Although, TEM and SHV variants were the most predominant ESBLs produced by Enterobacteriaceae, recently CTX-M has replaced TEM and SHV as the most common type of ESBLs.

ESBL-producing *E. coli* strains are prevalent in hospitals as well as community-acquired bacterial infections (Arpin *et al*., 2005; Pitout *et al*., 2005). Studies from different parts of the world have showed incidence of ESBL-producing *E. coli* in food or contaminated water (Imirzalioglu *et al*., 2016; Xi *et al*., 2015). A few studies have reported the probable horizontal transfer of resistance genes from either human sewage or clinical sources to fish in rivers or lakes in which drainage of wastewater from treatment plants occurs (Abgottspon *et al*., 2014; Blaak *et al*., 2014; Jiang *et al*., 2012). Moremi *et al*. (2016) confirmed that the ESBL-producing genotypes observed in humans and animals were also present in fish from Lake Victoria and enrironmental samples indicating the sources of contamination.

There are many recent reports regarding transmission of ESBL producing Enterobacteriaceae from farm animals to humans especially through food chain (Lazarus *et al*., 2015; Valentin *et al*., 2014; Kluytmans *et al*., 2013; Leverstein-van Hall *et al*., 2011; Overdevest *et al*., 2011). The Panel on Biologic Hazards of the European Food Safety Authority (EFSA, 2011) concluded that a risk exists for transmission of extended-spectrum beta-lactamase (ESBL) and plasmid-encoded AmpC (pAmpC) producing Enterobacteriaceae from farm animals, especially from poultry, to humans through the food chain. This finding is a matter of serious concern because ESBL and pAmpC enzymes hydrolyze extended-spectrum cephalosporins, which are one of the most widely used antimicrobial drug classes and also listed as critically useful antimicrobial drugs in human medicine by the World

Health Organization (WHO, 2007). High frequency of ESBL/pAmpC-producing *E. coli* has been reported from farm animals, particularly broilers, in Europe (Seiffert *et al*., 2013). Transmission of ESBL *E.coli* between poultry and extraintestinal human infections were identified in a few studies (Kluytmans *et al*., 2013; Overdevest *et al*., 2011). In Sweden the overall frequency of ESBL/pAmpC-producing *E. coli* is reported to be low, compared to that in other countries of Europe (Swedres-Svarm, 2014). This is probably due to the practice of reduced use of antimicrobial drugs in animals and humans in Sweden. However, as an exception large frequency of pAmpC-producing *E. coli* has been reported in poultry and domestic chicken meat in Sweden (Egervärn *et al*., 2014; Borjesson *et al*., 2013).

The spread of CTX-M-type ESBLs has dramatically increased in the most parts of world. This poses serious threat to the clinical use of third generation cephalosporins for the treatment of severe infections and also limits the choice of effective antimicrobial drugs to carbapenems or colistin. Thus the emergence of ESBLs, as an important cause of transferable multidrug resistance in bacteria especially *E. coli*, is now a serious threat for public health.

3.2.2.1.2 Occurence of *Mcr-1* gene in *E. coli*

Mcr-1 is the first reported plasmid-mediated resistance gene which confers resistance to the antibiotic colistin (Liu *et al*., 2016b). Colistin belongs to the family of polymyxins, cationic polypeptides, with broad-spectrum activity against Gram negative bacteria, including most species of the family Enterobacteriaceae. It has been often used as a last line drug of choice used to treat serious infections caused by carbapenem resistant Enterobacteriaceae either singularly or in combination with other antibiotics.

Although chromosomal mediated colistin resistance have been reported earlier (Breazeale *et al*., 2005; Tran *et al*., 2005), the first report of plasmid-mediated colistin resistance (*mcr-1* gene) was from food animals, food and humans in China (Liu *et al*., 2016b). Since then, there have been a number of reports about the detection of *mcr-1* gene from different sources from diverse geographical locations. Recent studies have revealed the spread of *mcr-1* gene across most of the continents (Battisti, 2016; Falgenhauer *et al*., 2016; Grami *et al*., 2016; Kluytmans-van den Bergh *et al*., 2016; Malhotra-Kumar *et al*., 2016b; Suzuki *et al*., 2016).

Several recents studies have reported the isolation of *mcr-1* gene from various food animals such as swine (Falgenhauer *et al*., 2016; Liu *et al*., 2016b; Suzuki *et al*., 2016), chicken (Perrin-Guyomard *et al*., 2016; Petrillo *et al*., 2016; Shen *et al*., 2016), turkey (Battisti, 2016; Perrin-Guyomard *et al*., 2016), beef (Mulvey *et al*., 2016), pork meat and sausage (Webb *et al*., 2016; Liu *et al*., 2016b); vegetables (Zurfuh *et al*., 2016); environmental sources such as river water (Zurfuh *et al*., 2016), water (Petrillo *et al*., 2016) etc.

There are a number of reports of isolation of *mcr-1* gene from humans either patients (Du *et al*., 2016; Falgenhauer *et al*., 2016; Hasman *et al*., 2015; Liu *et al*., 2016b; Thanh *et al*., 2016;) or asymptomatic human faecal carriers (Olaitan *et al*., 2016; Stoesser *et al*., 2016) or travellers (Arcilla *et al*., 2016).

There are reports of *mcr-1* gene from various sources from several Asian countries; China (Du *et al*., 2016; Liu *et al*., 2016b); Malaysia (Petrillo *et al*., 2016); Japan (Suzuki *et al*., 2016); Thailand (Olaitan *et al*., 2016) and Hong Kong (Wong *et al*., 2016).

The first report of *mcr-1* gene from India was made by Kumar *et al*. (2016b); from *E. coli* isolated from a urine sample collected from an

inpatient at a university hospital in India. The isolate showed multiple resistance to β-lactam antibiotics such as amoxycillin, amoxycillin-clavulanic acid, aztreonam, cefepime and cefotaxime.

Although *mcr-1 gene* has been identified very recently, it has to be believed that it has existed since long time back. The oldest *mcr-1* gene ever islolated as of now is that from *E. coli* isolated from chickens in China dating back to 1980s (Shen *et al*., 2016) and the oldest reported from Europe was isolated from a diarrhoeic veal calf dating back to 2005 (Haenni *et al*., 2016). It has also been reported in other Enterobacteriaceae members such as *Salmonella* (Public health England, 2015).

The increased colistin resistance may be due to its use and abuse in veterinary and human applications. Colistin is widely used in veterinary medicine especially for controlling diarrhoeal diseases in pig and poultry production. It is widely used in China where it was first reported (Liu *et al*., 2016b) and also in many European countries (Kempf *et al*., 2013) for more than 50 years. However, in humans it is parenterally used as a last resort drug to treat serious carbapenemase resistant bacteria.

The ever increasing and rapid dissemination of colistin resistance is believed to be plasmid-mediated rather than chromosomal. The rate of colistin resistance transmission to another competent *E. coli* was found to be at a fairly high frequency of 10^1 to 10^3 cells per recipient cell by conjugation which itself accounts for its rapid dissemination (Liu *et al*., 2016b).

3.2.2.2 Occurrence of vancomycin resistance genes in enterococci

Multiple drug resistance is a common phenomenon in the genus *Enterococcus* with some species such as *E. faecium* intrinsically more

resistant than others (Arias and Murray, 2012; Gold and Moellering, 1996). Intrinsic resistance has been reported against beta-lactams particularly penicillinase-resistant penicillins and cephalosporins, low concentrations of aminoglycosides, clindamycin, fluoroquinolones and Trimethoprim-sulfamethoxazole combinations. Acquired resistance has been reported against high concentrations of beta-lactams (via penicillin-binding proteins or beta-lactamase), high concentrations of aminoglycosides, glycopeptides such as vancomycin and teicoplanin, tetracycline, erythromycin, fluoroquinolones, rifampin, chloramphenicol, fusidic acid and nitrofurantoin (Gold, 2001; Gold and Moellering, 1996).

Six different types of vancomycin resistance have been characterized in enterococci based on both phenotypic and genotypic aspects (Shlaes *et al*., 1991). Out of these five types i.e.VanA, B, D, E, and G) correspond to acquired resistance whereas one type (VanC) is an intrinsic property of *E. gallinarum* and *E. casseliflavus-flavescens*.VanA and VanB are the most common and clinically important phenotypes. The *vanA* and *vanB* operons are present in transposons and located on plasmidsor in the chromosome whereas the *vanD*, *vanC*, *vanE*, and *vanG* operons have, been found only in the chromosome (Gold, 2001). The best known *vanA* transposon is Tn1546 (Arthur *et al*., 1992) and *vanB* has been found in 2 different transposons, Tn1547 and Tn5382 (Quintiliani and Courvalin, 1996).

The first report of enterococcal resistance to high concentrations of glycopeptide antibiotics such as vancomycin and teicoplanin was published during an outbreak of vancomycin-resistant *E. faecium* in a hospital (Uttley *et al*., 1988). VanA enterococci exhibit resistance to high levels of vancomycin (MIC≥64 µg/mL) and teicoplanin (MIC≥8 µg/mL) and presence of either

drug can induce resistance (Arthur *et al*., 1992). VanB enterococci are resistant to vancomycin concentrations ranging from 4 µg/mL - 1024 µg/mL but are susceptible to teicoplanin (Evers and Courvalin, 1996). *VanA* and *vanB* clusters are found primarily in *E. faecalis* and *E. faecium*. There are evidences of horizontal transmission of these genes beyond the genus *Enterococcus*; several Gram-positive species carrying *vanA* and *Streptococcus bovis* carrying *vanB* have been identified (Poyart *et al*., 1997). The mechanism of glycopeptide resistance in VanA and VanB enterococci was found to be a cluster of genes which encodes an alternate biosynthetic pathway for the production of cell wall precursors that bind vancomycin poorly and thus reduces its action (Leclercq and Courvalin, 1997) VanC resistance phenotype was first described in *E. gallinarum* responsible for low levels of resistance to vancomycin and susceptible to teicoplanin (Leclercq *et al*., 1992). VanD resistance phenotype is constitutive and is not transferable by conjugation to other enterococci. They have slightly diminished susceptibility to teicoplanin (MIC≥ 4 µg/mL) (Depardieu *et al*., 2003). Other phenotypes such as VanE (Abadia *et al*., 2002) and VanG (McKessar *et al*., 2000) responsible for low level resistance to vancomycin and susceptibility to teicoplanin have also been reported in enterococci.

There are a number of reports from various parts of the world, regarding the detection of enterococci with various Van phenotypes from various sources. Vancomycin resistant enterococci have been detected from sewers and waste waters frequently (Borhani *et al*., 2014). Novais *et al*. (2005) detected *vanA*, *vanB* and *vanC* harbouring enterococci from waste waters of sewers from hospitals. Hassan and Belal (2016) found sixty-seven per cent of MDR enterococci from a hospital in Saudi Arabia showing

resistance against gentamicin, vancomycin, erythromycin, amoxicillin, cefazolin and tetracycline. Different variants of VanC also could be detected. MDR enterococci showing the presence of *vanA* gene was detected from mastitis milk (Goksel *et al*., 2016) and cheese (Furlaneto-Maia *et al*., 2014). In contrast a few studies which screened VRE in poultry, cattle farms (Ngbede *et al*., 2016) and ready to eat meat products (Chajecka-Wierzchowska *et al*., 2016) reported vancomycin sensitive enterococci eventhough resistance was observed against other antibiotics such as tetracycline, erythromycin, ampicillin, gentamicin and teicoplanin. There are reports of VRE from meat samples of diverse origin and environmental samples such as superficial waters and air (Messi *et al*., 2006).

VRE have been reported from several hospitals, tertiary care hospitals (Gangurde *et al*., 2014; Praharaj *et al*., 2013; Shafiyabi *et al*., 2013) and clinical specimens (Gangurde *et al*., 2014) in India. Praharaj *et al*. (2013) studied the vancomycin resistance in enterococci from a tertiary hospital in India. A considerable level of vancomycin resistance was reported and VanA phenotype and genotype was the most common type. Co-existance of *vanA* and *vanC1* gene clusters was also identified in an *E. gallinarum* isolate which conferred high level glycopeptide resistance to the isolate. Singh (2009) studied enterococci of equine origin in North India and found that 99.6% were multiple-drug resistant with 80% strains showing resistance to vancomycin.

The increased resistance to glycopeptides may be due to cross resistance to avoparcin (another glycopeptide drug) which was used as a growth promoter in food animals. Use of this glycopeptide at sub therapeutic concentrations in animals might have played a role in the development of acquired vancomycin resistance in enterococci (Wegener *et al*., 1999).

3.2.3 Occurrence of Toxigenic/Virulence genes

3.2.3.1 Occurrence of Toxigenic genes in *E. coli*

Shiga-like toxin-producing *E. coli* (STEC) or Verotoxin-producing *E. coli* (VTEC) is a known cause of serious illness in humans and a major public health concern (Yates, 2011; Paton and Paton, 1998b).The nature of illness range from mild diarrhoea to more severe forms such as severe bloody diarrhoea (hemorrhagic colitis) and life-threatening hemolytic uremic syndrome (HUS). *E. coli* O157:H7 was first recognized as a human pathogen in 1982, and was associated with two outbreaks due to consumption of contaminated hamburgers in the United States (Riley *et al*., 1983).

Eventhough STEC O157:H7 is the serotype that is most often associated with outbreaks and severe forms of diarrhoea, recently a number of many non-O157:H7 STEC serotypes causing similar illnesses have been reported (Tseng *et al*., 2014; Gould *et al*., 2013). Shiga toxins are considered to be the most important virulence factor of STEC. Shiga toxins are of two major types; stx_1 and stx_2 which are encoded by stx_1 and stx_2 genes, respectively. Each of stx_1 can be further divided into subtypes (stx_{1a}, 1c, 1d) and stx_2 into 7 subtypes (stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, and stx_{2g}), respectively (Scheutz *et al*., 2012).

The ability to produce shiga toxins stx_1 and stx_2 in *E. coli* is conferred by toxin-converting lysogenic bacteriophages (O'Brien *et al*., 1984). Since most of the virulence factors are encoded on mobile elements horizontal transfer between organisms is possible which could explain the production of Shiga toxins in more than 150 different serotypes of *E. coli* (Schmidt, 2001) and also some organisms can have characteristics of more than one pathotype. Although pathotype stx_{1a} has been linked to human illness, STEC

that produce subtypes stx_{2a}, stx_{2c}, and stx_{2d} are more frequently associated with the development of HC and HUS (Melton-Celsa, 2014; Friedrich *et al*., 2002).

In addition to *stx,* other virulence factors such as genes involved in cell adhesion, proteases, and toxins, as well as other putative virulence factors also contribute to human illness. These virulence factors include intimin, product of the *eaeA* gene found on the locus of enterocyte effacement (LEE)/pathogenicity island involved in the enterocyte attaching and effacing phenotype (A/E lesion) of bacteria and an enterohemolysin EHEC-*hlyA* gene (Baranzoni *et al*., 2016; Schmidt and Karch, 1996; Schmidt *et al*., 1995). LEE-positive STEC are expected to cause HUS more frequently than LEE-negative STEC (Luna-Gierke *et al*., 2014; Ethelberg *et al*., 2004; Toma *et al*., 2004). However, HUS caused by LEE-negative STEC has also been reported (Bielaszewska *et al*., 2009; Paton *et al*., 1999; Karmali *et al*., 1985).

Cattle and other ruminants such as sheep and goats are the natural, asymptomatic carriers of STEC. STEC has been isolated from swine faeces also and they persist and contaminate the environment thereafter (Tseng *et al*., 2014). Contamination of water bodies with STEC poses significant health risk for human health through consumption of contaminated water while bathing (Olsen *et al*., 2002), and may also contaminate the aquaculture products like shellfish (Fach *et al*., 2015; Bennani *et al*., 2011; Gourmelon *et al*., 2006).

STEC with various seropathotypes have been reported from various parts of the world from diverse sources ranging from environmental to food and clinical samples. Several studies have also revealed prevalence of STEC harbouring *stx* and *eaeA* genes in waste water, animal sewage, waste water from slaughter houses etc. Various studies reported prevalence of Shiga toxin-producing *E. coli* strains (STECs) in fresh produce by Multiplex PCR (Khalil

and Gomaa, 2016; Loukiadis *et al*., 2006). STEC O157:H7 was detected in 2.3% of produce types, and the maximum prevalent virulence markers was stx_2 (59%) followed by stx_1 (54.5%), *eaeA* (45.45%) and *hlyA*; 18.18% (4/22). The most prevalent toxin genotype observed was that of STECs possessing *stx1- eaeA* (27.3%), *stx2-eaeA* (22.7%), and *stx1-stx2* (13.6%).

STEC harbouring virulence genes have been reported from shellfish, sea water and sediments from shellfish growing environment (Fach *et al*., 2015; Bennani *et al*., 2011; Gourmelon *et al*., 2006). Bennani *et al*. (2011) detected shiga toxin $stx_1$and stx_2 in shellfish and stx_1 in sediment from Coastal Environments of Morocco; out of which 1.9% was detected in serotype O157:H7. Gourmelon *et al*. (2006) detected *stx* genes from mussels, oysters or cockles harvested from shellfish growing areas of France. Five strains carried stx_1 or stx_{1d} genes and one strain was only *eaeA* and *hlyA* positive *E. coli* O157:H7. El-Gamal *et al*. (2016) studied the prevalence and characterization of STEC O157 and non-O157 in commercial ground beef and instruments samples. Virulence genes such as *stx1*, stx_2, *eaeA* and multiple pathotypes were also detected.

Kumar *et al*. (2004) reported STEC isolations from seafood, beef and one clinical case in Mangalore, India. The seafood strains produced either stx_2alone or both stx_1 and stx_2, while the beef isolates produced stx_1 alone. All STEC strains and one non-STEC strain isolated from clam harboured EHEC-*hlyA*. Interestingly all the STEC isolates were *eaeA* negative.

3.2.3.2 Prevalence of virulence genes in *V. parahaemolyticus*

Tdh and *trh* were the virulent genes first identified in V. parahaemolyticus associated with disease outbreaks. They were identified in the 1980s

and were found to cause hemolytic reaction on Wagatsuma's blood agar (Shinoda, 2011).

Both thermostable direct hemolysin gene, *tdh* and its homolog, the thermostable direct hemolysin-related hemolysin gene *trh*, code for proteins that aggregate and insert into cell membranes, which result in nonspecific efflux of divalent cations and influx of water (Yanagihara *et al*., 2010; Raimondi *et al*., 2000).

Varying levels of occurrence of pathogenic genes have been reported in clinical as well as environmental isolates. Strains producing virulence genes, *tdh* and *trh*are generally considered pathogenic to man. The first multiplex PCR for the detection of *tlh*, *tdh*, and *trh* were described by Bej *et al*. (1999).

Majority of clinical strains produce virulence gene, thermostable direct haemolysin (*tdh*), while very less environmental strains produce this haemolysin (Ceccarelli *et al*., 2013; Sakazaki *et al*., 1968). Due to low detection frequencies of *tdh* and *trh* genes most environmental *V. parahaemolyticus* strains are considered to be non-pathogenic (Vieira *et al*., 2011; Nishibuchi and Kaper, 1995). It is demonstrated by low detection of *tdh* and *trh* genes in only 4.3% and 0.3% of environmental strains respectively from highly populated areas of South Carolina and Georgia coasts (Baker-Austin *et al*., 2008).

However the possible low recovery of virulence genes by Bej *et al*. (1999) is also doubted as a probable reason by some researchers (Gutierrez West *et al*., 2013), since usage of new PCR primers has drastically improved amplification of *tdh* and *trh* genes in environmental *V. parahaemolyticus* strains. Using their newly developed primers Gutierrez West *et al*. (2013)

reported relatively high prevalence of *tdh* in 48% of isolates when compared with the low detection of 23% positives by primers of Bej *et al*. (1999) from water and sediment in a relatively pristine southeastern North American Spartina marsh. Similarly comparatively high prevalence of *trh* (8%) was also reported using the new primers, while *trh* primers of Bej *et al*. (1999) enabled detection of *trh* only in the positive control and none of the test strains.

Klein and Lovell (2017) detected virulence genes in 50% environmental *V. parahaemolyticus* strains isolated from oysters. Velazquez-Roman *et al*. (2012) detected *tdh* and or *trh* in 52% of *V. parahaemolyticus* strains isolated from a heavily impacted area of shrimp mariculture using the Bej *et al*. (1999).

Some *tdh* negative strains from clinical cases were found to produce *trh* haemolysin (Honda *et al*., 1988). DePaola *et al*. (2003) reported presence of 21.8% of pathogenic strains and found that pathogenic strains were urease positive and possessed *tdh*- related hemolysin (*trh*) gene. Robert-Pillot *et al*. (2004) investigated the presence of *tdh* and *trh* genes in clinical, seafood and environmental isolates and found majority of the clinical isolates (91%) possessed the hemolysin genes. Only 1.5% of the seafood isolates possessed hemolysin genes, whereas two groups of environmental strains carried 3% and 15% percent hemolysin genes respectively.

Several researchers have detected the presence of virulent genes in seafood (Kumar *et al*., 2016a; Mala *et al*., 2015; Paydar *et al*., 2013), estuarine water (Alam *et al*., 2017; Akther *et al*., 2016) and molluscan shellfish (Thongchan *et al*., 2013). Nakaguchi (2013) detected varying frequencies of *tdh* and *trh* in molluscan shellfish from various Southeast Asian countries; the detection levels *tdh* and *trh* were as follows: Vietnam

(17.9 and 8.0%), Malaysia (11.1 and 16.7%) and Indonesia (9.1% and 13.6%) respectively. Kumar *et al*. (2016a) detected and characterised virulence factors other than classical *tdh* and/or *trh* such as T3SS2 operon in a *V. parahaemolyticus* isolate from the seafood harvested along southwest coast of India.

Even though it is widely accepted that virulence genes are associated with pathogenic *V. parahaemolyticus*, nontoxigenic *V. parahaemolyticus* strains causing acute gastroenteritis have been reported (Ottaviani *et al*., 2012). Absence of virulence genes *tdh*, *trh*, and even T3SS genes from some clinical strains (Ronholm *et al*., 2016; Ottaviani *et al*., 2012), as well as their occurence in numerous environmental isolates that are apparently not acquired from infected humans (Klein *et al*., 2014; Gutierrez West *et al*., 2013), suggests the possibility that *tdh* and *trh* may serve some environmental function and has nothing to do with human disease (Raghunath, 2015; Gutierrez West *et al*., 2013), though these functions are unknown. The virulence properties of four *V. parahaemolyticus* strains causing acute gastroenteritis following consumption of indigenous mussels in Italy were investigated. The isolated strains were cytotoxic and adhesive but, lacked *tdh*, *trh*, and type three secretion system 2 (T3SS2) genes which are found to be associated with pathogenic strains. Another possibility is that probably some other virulence factors which are yet to be identified may be be associated with disease outbreaks. Virulent genes study by Vongxay *et al*. (2008) suggested that hemolysins *tdh* and/or *trh* may not be necessarily the only virulence factors of pathogenic *V. parahaemolyticus* isolates.

3.3 Objectives

- To study the prevalence of antibiotic resistance among *E.coli,* enterococci, and *V. parahaemolyticus* isolated from shellfish, sediment and harvesting waters of Cochin estuary.
- To determine the extent of prevalence of selected antibiotic resistance genes in *E. coli* ($bla_{\text{CTX-M}}$, bla_{TEM} and *mcr-1*) and enterococci (*vanA*, *vanB* and *vanC)*.
- To determine the prevalence of toxigenic genes (stx_1, stx_2, *eaeA* and *hlyA*) in *E. coli* isolated from shellfish, sediment and harvesting waters of Cochin estuary.
- To determine the prevalence of specific virulence genes (*tdh* and *trh*) in *V. parahaemolyticus* isolated from shellfish, sediment and harvesting waters of Cochin estuary.

3.4 Materials and Methods

3.4.1 Source of the isolates

A total of 125 *E. coli* isolates (50 from shellfish, 38 from sediment, and 37 from harvesting water), 88 isolates of Enterococci (46 from shellfish, 28 from sediment, and 14 from harvesting water), and 62 isolates of V. *parahaemolyticus* (22 from shellfish, 26 from sediment, and 14 from harvesting water), were tested for their antibiotic sensitivity by agar disc diffusion method.

3.4.2 Antibiotic susceptibility testing

The antibiotic sensitivity of the selected bacterial strains was determined byagar disc diffusion method (Bauer *et al*., 1966) on Mueller-Hinton agar

(Hi-Media, India). The antibiotics tested and their concentrations were listed in Table 3.1.

Table 3.1 List of antibiotics used, class of the drug, abbreviation and their concentration

Sl. No.	Antibiotic used	Class of the drug	Abbreviation	Concentration (mcg)
1	Amikacin	Aminoglycoside	Ak	30
2	Gentamicin	Aminoglycoside	Gen	10
3	High level Gentamicin	Aminoglycoside	HLG	120
4	High level Streptomycin	Aminoglycoside	HLS	300
5	Streptomycin	Aminoglycoside	S	10
6	Rifampicin	Ansamycin	Rif	5
7	Imipenem	Carbapenem	Ipm	10
8	Meropenem	Carbapenem	Mrp	10
9	Cephalothin	Cephalosporin	Cep	30
10	Cephotaxime	Cephalosporin	Ctx	30
11	Cefoxitin	Cephalosporin	Cx	30
12	Cefpodoxime	Cephalosporin	Cpd	10
13	Teicoplanin	Glycopeptide	Tei	30
14	Vancomycin	Glycopeptide	Va	30
15	Erythromycin	Macrolide	E	15
16	Nitrofurantoin	Nitrofuran	Nit	100
17	Linazolid	Oxazolidinone	Lz	30
18	Ampicillin	Penicillin	A	10
19	Penicillin	Penicillin	Amc	30
20	Amoxyclav	Penicillin-clavulinic acid combination	P	10
21	Chloramphenicol	Phenicol	C	30
22	Colistin	Polypeptide	Cl	10
23	Trimethoprim	Pyrimidine derivative	Tr	5
24	Nalidixic acid	Quinolone	Na	30
25	Ciprofloxacin	Fluoroquinolone	Cip	5
26	Tetracycline	Tetracycline	Te	30
27	Doxycycline	Tetracycline	Do	30
28	Co-trimoxazole	Trimethoprim-Sulfamethoxazole	Cot	30

Pure cultures ofall the test organisms were inoculated into nutrient broth and incubated at 37 °C for 6-8 h. The cultures were then streaked onto surface dried sterile Mueller-Hinton agar plates using a sterile cotton swab. After 5 min of drying, antibiotic discs were aseptically placed over the seeded Mueller-Hinton agar plates, sufficiently separated from each other so as to avoid overlapping of inhibition zones. After overnight incubation of the inoculated plates at 37 °C, diameter of the inhibition zone was measured and the isolates were classified as sensitive, intermediate or resistant, according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2015).

3.4.3 MAR indexing of the test organisms

Multiple antibiotic resistance (MAR) indices of the test organisms were calculated according to Krumperman (1983). MAR index of an individual isolate was calculated by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics to which the isolate was exposed. The strains with MAR index greater than 0.2 is considered to have originated from high risk sources of contamination such as human, poultry orcattle where antibiotics are frequently used.

3.4.4 Extraction of genomic DNA from the bacterial isolates

Genomic DNA was extracted from the isolates by the boiling method (Devi *et al*., 2009). Refer section **2.4.3.1.2.1.**

3.4.5 Detection of antibiotic resistant genes by PCR

The primers used for the detection of antibiotic resistant genes are listed in Table 3.2.

Table 3.2 List of primers used in the detection of various antibiotic resistance/toxigenic/virulence genes

Genes investigated	Organism	Primer sequence (5′ - 3′)		PCR product	Reference
		Forward	Reverse		
Antibiotic resistance genes					
Beta-lactams					
Bla$_{TEM}$	*E. coli*	GAGTATTCAACATTTTCGT	ACCAATGCTTAATCAGTGA	857 bp	Marynard *et al*. (2003)
Bla$_{CTX-M}$	*E. coli*	CGATGTGCAGTACCAGTA A	TTAGTGACCAGAATCAGCGG	585 bp	Batchelor *et al*. (2005)
Colistin					
Mcr-1	*E. coli*	CGGTCAGTCCGTTTGTTC	CTTGGTCGGTCTGTAGGG	309 bp	Liu *et al*. (2016b)
Vancomycin					
Van A	Enterococci	GGGAAAACGACAATTGC	GTACAATGCGGCCGTTA	647 bp	Puneet Bhatt *et al*. (2015)
Van B	Enterococci	ACGGAATGGGAAGCCGA	TGCACCCGATTTCGTTC	732 bp	Puneet Bhatt *et al*. (2015)
Van C	Enterococci	ATGGATTGGTAYTKGTAT	TAGCGGGAGTGMCYMGTAA	815/827 bp	Puneet Bhatt *et al*. (2015)
Toxigenic genes					
Stx$_1$	*E. coli*	ATAAATCGCCATTCGTTGACTAC	AGAACGCCCACTGAGATCATC	180 bp	Paton and Paton (1998)
Stx$_2$	*E. coli*	GGCACTGTCTGAAACTGCTCC	TCGCCAGTTATCTGACATTCTG	255 bp	Paton and Paton (1998)
Eae	*E. coli*	GACCCGGCACAAGCATAAGC	CCACCTGCAGCAACAAGAGG	384 bp	Paton and Paton (1998)
HlyA	*E. coli*	GCATCATCAAGCGTACGTTCC	AATGAGCCAAGCTGGTTAAGCT	534 bp	Paton and Paton (1998)
Virulence genes					
Tdh	*V. parahaemolyticus*	GTAAAGGTCTCTGACTTTTGGAC	TGGAATAGAACCTTCATCTTCACC	270 bp	Bej *et al*. (1999)
Trh	*V. parahaemolyticus*	TTGGCTTCGATATTTTCAGTATCT	CATAACAAACATATGCCCATTTCCG	500 bp	Bej *et al*. (1999)

3.4.5.1 Detection of *bla*$_{TEM}$ gene in *E. coli*

The presence of *bla*$_{TEM}$ gene was detected using the PCR method as described by Marynard *et al*., (2003). The optimized protocol was carried out with a PCR mix total reaction volume of 25 µL consisting of sterile Milli Q water (15.5 µL), 10x PCRbuffer (2 µL), primer (1 µL each), dNTP mix (1 µL, 200 mM), template (4 µL), and Taq DNA polymerase (0.5 µL). Amplification was performed with a Mini Thermal cycler (BioRad, USA) with the following thermal cycling profile: 1 cycle of 5 min at 94 °C; 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C, 1.5 min at 72 °C; 1 cycle of 5 min at 72 °C.

3.4.5.2 Detection of *bla*$_{CTX-M}$ gene in *E. coli*

The presence of *bla*$_{CTX-M}$ gene was detected using the PCR method as described by Batchelor *et al*. (2005). The optimized protocol was carried out with a PCR mix total reaction volume of 25 µL consisting of sterile Milli Q water (15.5 µL), 10x PCR buffer (2 µL), primer (1 µL each), dNTP mix (1 µL, 200 mM), template (4 µL), and Taq DNA polymerase (0.5 µL). Amplification was performed with a Mini Thermal cycler (BioRad, USA) with the following thermal cycling profile: 1 cycle of 5 min at 94 °C; 35 cycles of 30 sec at 94 °C, 30 sec at 60 °C, 1.5 min at 72 °C; 1 cycle of 5 min at 72 °C.

3.4.5.3 Detection of *mcr-1* gene in *E. coli*

The presence of *mcr-1*gene was detected using the primers designed by Liu *et al.* (2016b). The optimized protocol was carried out with a PCR mix total reaction volume of 25 µL consisting of sterile Milli Q water (15.5 µL), 10x PCR buffer (2 µL), primer (1 µL each), dNTP mix (1 µL, 200 mM),

template (4 µL), and Taq DNA polymerase (0.5 µL). Amplification was performed with a Mini Thermal cycler (BioRad, USA) with the following thermal cycling profile: 1 cycle of 15 min at 94 °C; 25 cycles of 30 sec at 94 °C, 90 sec at 58 °C, 60sec at 72 °C; 1 cycle of 10 min at 72 °C.

3.4.5.4 Detection of glycopeptide resistance genes in enterococci

The presence of glycopeptide resistance genes, *vanA, vanB* and *vanC* was detected using the PCR method as described by Puneet Bhatt *et al.* (2015). The primers used in the detection of glycopeptide resistance genes in enterococci are listed in Table 3.2. The optimized protocol was carried out with a PCR mix total reaction volume of 25 µL consisting of sterile Milli Q water (15.5 µL), 10x PCR buffer (2 µL), primer (1 µL each), dNTP mix (1 µL, 200 mM), template (4 µL), and Taq DNA polymerase (0.5 µL). Amplification was performed with a Mini Thermal cycler (BioRad, USA) with the following thermal cycling profile: 1 cycle of 5 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 54 °C, 1 min at 72 °C; 1 cycle of 7 min at 72 °C for the final extension.

3.4.6 Detection of toxigenic/virulence genes

3.4.6.1 Detection of toxigenic/virulence genes in *E. coli* by multiplex PCR

The presence of shigatoxin genes such as stx_1, $stx_2$and virulence genes such as intimin gene (*eae*) and enterohemolysin gene (*hlyA)* was detected using multiplex PCR as described by Paton and Paton (1998a). The primers used in the detection of toxigenic/ virulence genes in *E. coli* are listed in Table 3.2. The optimized protocol was carried out with a PCR mix total reaction volume of 25 µL consisting of sterile Milli Q water (7.5 µL), 10x PCR buffer (2 µL), primer (1 µL each of each primer; thus a total of 8 µL

primer mix), dNTP mix (1 µL, 200 mM), template (4 µL), and Taq DNA polymerase (0.5 µL). Amplification was performed with a Mini Thermal cycler (BioRad, USA) with the following thermal cycling profile: 1 cycle of 5 min at 94 °C; 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C, 1.5 min at 72 °C; 1 cycle of 5 min at 72 °C.

3.4.6.2 Detection of virulence genes in *V. parahaemolyticus* by multiplex PCR

The primers used for the detection of virulence genes in *V. parahaemolyticus* are listed in Table 3.2. The presence of virulence genes thermostable direct haemolysin (*tdh*) and themostable haemolysin related haemolysin (*trh*) was detected using multiplex PCR (Bej *et al*., 1999) as described in section **2.4.3.3.2.1**.

3.4.7 Gel documentation and image analysis

The PCR products were then electrophoresed on a 1.5% agarose gel (Hi-Media, India) in 1x TBE Buffer (Himedia, India) containing 0.5 µg/mL of ethidium bromide (Himedia, India). The amplicon sizes were compared with a suitable DNA ladder and visualized by Gel Documentation system (GelDoc EZ Imager, BioRad, USA).

3.4.8 Statistical analysis

Statistical analysis of the results was performed using SPSS software 20 (Statistical Package for Social Science). Chi-square test was applied to test the difference in the antibiotic resistance of bacterial strains from different sources and also among those from different stations. Significance level was set at $\alpha = 0.05$.

3.5 Results

3.5.1 Overall antibiotic resistance of *E. coli* strains isolated from shellfish harvesting areas of Cochin estuary

The prevalence of antibiotic resistance was assessed in a total of 125 *E. coli* strains isolated from shellfish (*Villorita cyprinoides*), sediment and harvesting waters from shellfish harvesting areas of Cochin estuary (Figure 3.1). *E. coli* collectively showed remarkable resistance towards all the antibiotics, though there were variations in individual resistances towards various antibiotics. Majority of the strains were resistant to cefpodoxime (97.6%), followed by imipenem (89.6%) and ampicillin (83.2%). Resistance to antibiotics such as amikacin (28.8%), gentamicin (26.4%) meropenem (24%), ciprofloxacin (18.4%), trimethoprim (17.6%), chloramphenicol (15.2%) and cotrimoxazole (14.4%) were low.

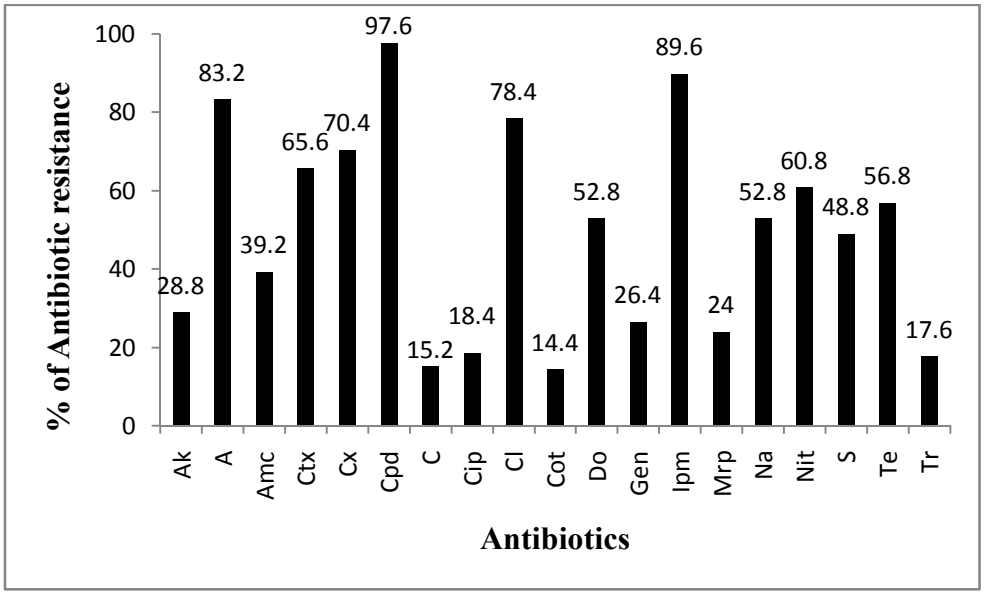

Figure 3.1 Overall antibiotic resistance of *E. coli* strains isolated from shellfish harvesting areas of Cochin estuary

3.5.1.1 Antibiotic resistance of *E. coli* strains isolated from shellfish (*Villorita cyprinoides*)

E. coli strains from shellfish showed resistance against all the antibioitics used; higher levels of resistance were shown against cefpodoxime (98%) followed by imipenem (92%) and colistin (90%) (Figure 3.2). Resistance to antibiotics such as trimethoprim (18%) and cotrimoxazole (14%) were low.

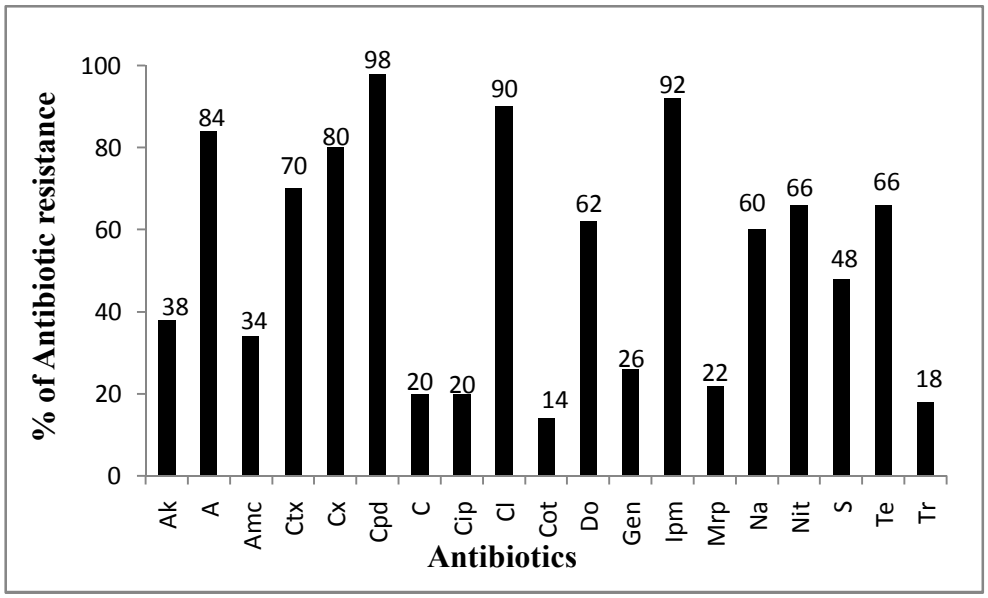

Figure 3.2 Antibiotic resistance of *E. coli* strains isolated from shellfish (*Villorita cyprinoides*)

3.5.1.2 Antibiotic resistance of *E. coli* strains isolated from sediments from various shellfish harvesting areas

Among *E. coli* strains from sediment, resistance was shown against all the antibiotics used; highest resistance being exhibited against cefpodoxime (97.3%) followed by imipenem (78.9%) as shown in Figure 3.3. Lesser degrees

of resistance were shown against chloramphenicol (13.1%), trimethoprim (13.1%) and cotrimoxazole (7.8%).

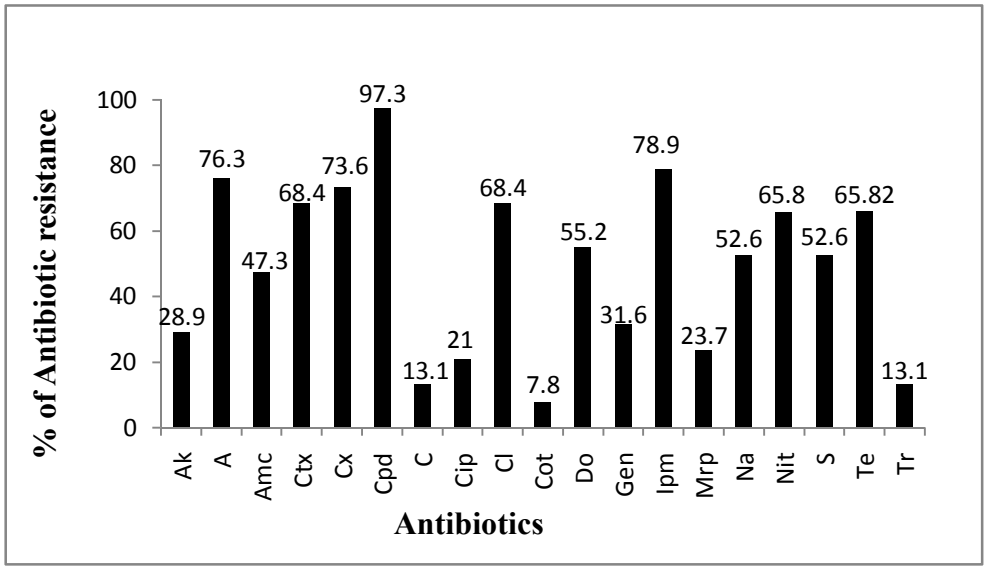

Figure 3.3 Antibiotic resistance of *E. coli* strains isolated from sediments from various shellfish harvesting areas

3.5.1.3 Antibiotic resistance of *E. coli* strains isolated from harvesting waters from various shellfish harvesting areas

Figure 3.4 shows the antibiotic resistance of *E. coli* strains from harvesting waters. *E. coli* strains from harvesting waters showed resistance against all the antibiotics; highest resistance was observed against cefpodoxime (97.3%) followed by ampicillin (89.2%), imipenem (83.7%) and colistin (73%). Lesser degrees of resistance was shown against antibiotics such as amikacin (16.2%), ciprofloxacin (13.5%) and chloramphenicol (10.8%).

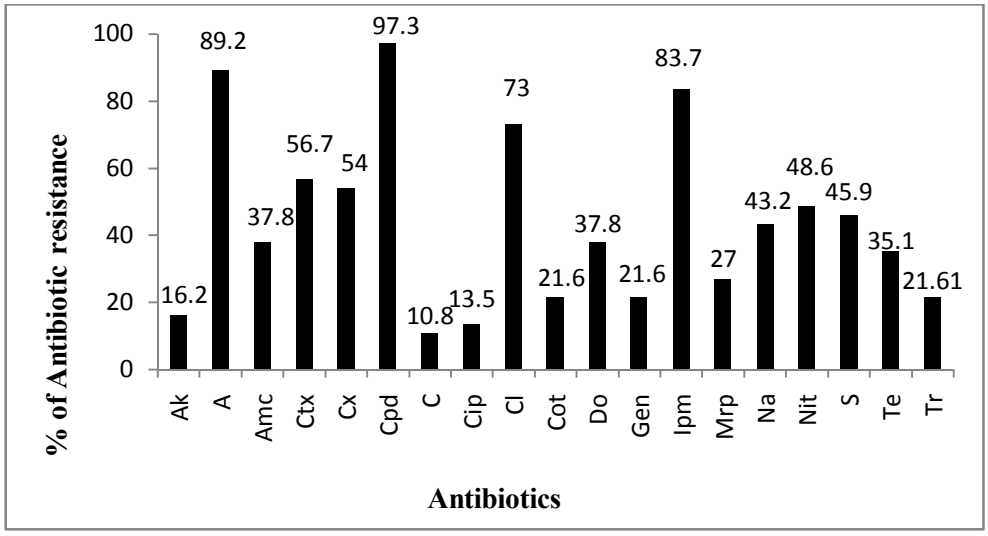

Figure 3.4 Antibiotic resistance of *E. coli* strains isolated from harvesting waters from various shellfish harvesting areas

3.5.1.4 Relative prevalence of antibiotic resistance among *E. coli* strains from shellfish, sediment and water from shellfish harvesting areas

The antibiotic resistance of *E. coli* strains from shellfish, sediment and harvesting waters is compared in Figure 3.5. Significant differences were observed in antibiotic resistance of *E. coli* strains from shellfish, sediment and harvesting waters. Among the three sources under study, *E. coli* strains from shellfish showed comparatively higher resistance against 10 antibiotics i.e. amikacin, cephotaxime, cefpodoxime, chloramphenicol, colistin, doxycycline, imipenem, nalidixic acid, nitrofurantoin and tetracycline ($p<0.05$). *E. coli* from sediment showed signigicantly higher resistance against amoxyclav, ciprofloxacin, gentamicin and streptomycin whereas those from harvesting waters showed highest resistance against ampicillin, cotrimoxazole, meropenem and trimethoprim ($p<0.05$). When the overall antibiotic resistance of *E. coli* strains from the the 3 samples were compared, strains from shellfish showed maximum resistance followed by those from sediments and harvesting waters ($p<0.001$).

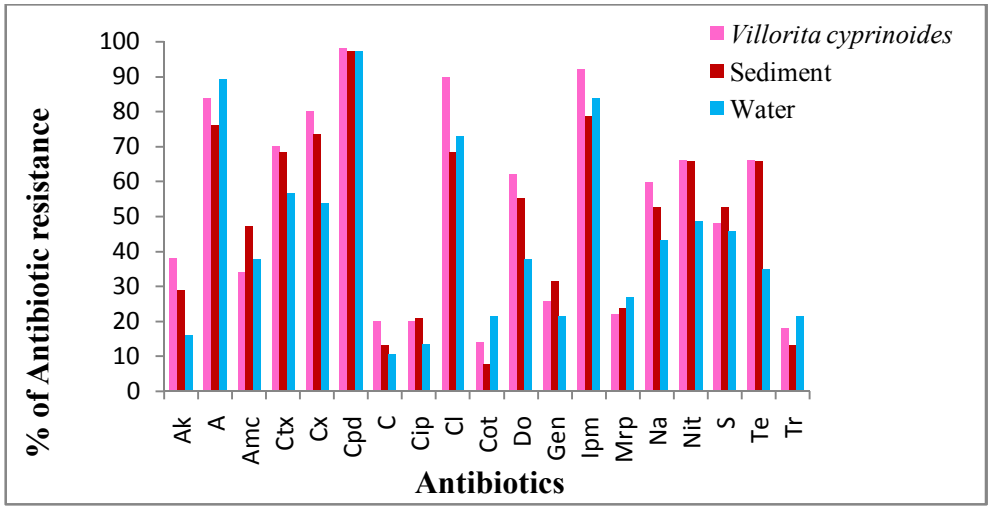

Figure 3.5 Comparison of antibiotic resistance profiles of *E. coli* strains isolated from shellfish, sediment and water from various shellfish harvesting areas

3.5.1.5 Spatial variation in antibiotic resistance among *E. coli* strains from various shellfish harvesting areas

Table 3.3 summarises the spatial variation in antibiotic resistance of *E. coli* isolated from the eight different stations under study, towards the 18 different antibiotics. For assessing antibiotic resistance at any particular station, resistance of strains from all the three sources collected from that station *viz.* shellfish, sediment and harvesting water was taken into account. Stations showed variations in the resistance towards various antibiotics. *E. coli* from 5 stations, stations 2, 3, 4, 7 and 8 showed 100% sensitivity towards cefpodoxime. Complete resistance was exhibited towards certain other antibiotics also: station 2 towards ampicillin; station 3 towards ampicillin and cefoxitin; station 8 towards cephotaxime, cefoxitin, cefpodoxime and colistin. *E. coli* from 3 stations, station 2, 3 and 8 showed complete sensitivity towards chloramphenicol. In addition to this, certain other stations also showed complete sensitivity towards various antibiotics: station 2 towards meropenem, station 3 towards gentamicin, station 7 towards amikacin and

gentamicin and station 8 towards ciprofloxacin. All the differences were found to be statistically significant ($p<0.001$). When the overall resistance towards all the 18 antibiotics were considered station 3 exhibited the maximum resistance, while station 2 exhibited the least ($p<0.001$).

Table 3.3 Spatial variation in antibiotic resistance profiles of *E. coli* strains isolated from various shellfish harvesting areas

	Prevalence of resistance among *E. coli* isolates							
Antibiotics	**Station 1 (n=14)**	**Station 2 (n=16)**	**Station 3 (n=16**	**Station 4 (n=17)**	**Station 5 (n=15)**	**Station 6 (n=15)**	**Station 7 (n=15)**	**Station 8 (n=17)**
Ak	19	14.3	42.9	27.6	38.5	21.7	0	60
Amc	61.9	28.6	42.9	13.8	23.1	56.5	30	40
Amp	80.1	100	100	89.7	92.3	73.9	70	40
Ctx	38.1	71.4	71.4	69	92.3	65.2	20	100
Cx	47.6	42.9	100	96.6	76.9	52.2	30	100
Cpd	95.2	100	100	100	92.3	95.7	100	100
C	9.5	0	0	13.8	7.7	34.8	30	0
Cip	38.1	14.3	42.9	13.8	30.8	21.8	10	0
Cl	66.6	42.9	100	86.2	92.3	82.6	70	100
Cot	4.8	14.3	28.6	13.8	7.7	17.4	30	20
Do	23.8	57.1	85.7	44.8	69.2	47.8	80	60
Gen	42.9	42.9	0	10.3	30.8	30.4	0	20
Ipm	85.7	71.4	100	86.2	84.6	87	90	80
Mrp	19	0	42.9	27.6	46.2	17.4	20	40
Na	33.3	28.5	57.1	55.17	38.5	69.6	70	60
Nit	38.1	71.4	85.7	65.5	61.5	34.8	90	80
S	33.3	28.5	85.7	62.1	61.5	30.4	70	40
Te	38.1	42.9	42.9	48.3	84.6	52.2	90	40
Tr	14.3	14.3	28.6	10.34	23.1	21.7	30	20

3.5.1.6 Multiple antibiotic resistance (MAR) index and antibiotic resistance pattern of *E. coli* strains isolated from shellfish (*Villorita cyprinoides*)

The incidence of antibiotic resistance patterns and multiple antibiotic resistance (MAR) indices of *E. coli* strains isolated from shellfish is shown in Table 3.4. It was observed that 98% of *E. coli* strains from shellfish were

multidrug resistant (MDR). The MAR indices ranged from 0.1-0.79 with an average of 0.53±0.15. Overall 50 different antibiotic resistance patterns were observed; all the patterns were exhibited in equal distributions.

Table 3.4 Multiple antibiotic resistance (MAR) of *E. coli* strains isolated from shellfish (*Villorita cyprinoides*)

MAR index	Resistance pattern	% isolates
0.1	Ipm, Cl	2
0.21	Cpd, Cx, Cl, Ipm	2
0.26	Amp, Cpd, Cl, Cot,Tr	2
0.31	Amp, Ctx, Cpd, Cl, Gen, Ipm	2
0.31	Amp, Ctx, Cpd, Cx, Cl, Te	2
0.31	Ak, Amp, Cpd, Cx, Ipm, Nit	2
0.37	Amp, Ctx, Cpd, Cx, , Cl, Do, Ipm	2
0.37	Amp, Cpd, Cl, Nit, Ipm, Mrp, Na	2
0.42	Amp, Cpd, C, Cl,Te, Na, Do, Ipm	2
0.42	Amp, Ctx, Cpd, Cx, Cip, Cl, Te, Ipm	2
0.42	Amc, Amp, Cpd, Cx, , Cl, Do, Nit, Ipm	2
0.42	Amp, Cpd, Cx, Cl, Do, Gen, S, Te	2
0.47	Ak, Amc, Amp, Ctx, Cpd, Cx, Gen, Ipm, Te	2
0.47	Amc, Ctx, Cpd, Cx, C, , Cl, Ipm, Na, Nit	2
0.47	Amp, Ctx, Cpd, Cx, , Cl, Do,Ipm, Na, Nit	2
0.47	Ctx, Cpd, Cx, C, Cl, Do, Ipm, Nit, S	2
0.47	Ctx, Cpd, Cx, Cl, Nit, Na, Ipm, S, Te	2
0.47	Ak, Ctx, Cpd, Cl, Do, Na, Nit, Ipm, S	2
0.53	Amp, Cpd, Cl, Cot, Do, Ipm, Nit, S, Te, Tr	2
0.53	Ak, Amc, Amp, Ctx, Cpd, Cx, Cip, Gen, Ipm, Na	2
0.53	Amp, Ctx, Cpd, C, Cl, Do, Ipm, Mrp, Na, Te	2
0.53	Ak, Amp, Ctx, Cpd, Cip, Gen, Te, Tr, Ipm	2
0.53	Ak, Amp, Ctx, Cpd, Cx, Cl, Do, Mrp, Na, Te	2
0.53	Ak, Amp, Ctx, Cpd, Cx, Cl, Do, Ipm, Mrp, Te	2
0.53	Amp, Cpd, Cx, Cip, Cl, Na, Nit, Ipm, Mrp, S	2
0.53	Amp, Cpd, Cx, Cl, Do, Gen, Ipm, Na, Nit, S	2
0.53	Ctx, Cpd, Ctx, Cl, Ipm, Na, Nit, Mrp, S, Te	2
0.58	Amp, Ctx, Cpd, Cx, Cip, Cl, Do, Na,Nit, Ipm,Te	2

Table 3.4 Continued….

0.58	Amp, Cpd, Cx, Cl, Cot, Do, Ipm, Nit, S, Te, Tr	2
0.58	Amp, Cpd, Cl, Cot, Do, Ipm, Na, Nit, S, Te, Tr	2
0.58	Ak, Amp, Ctx, Cpd, Cx, Cl, Ipm, Mrp, Nit, S, Te	2
0.58	Amc, Amp, Cpd, Cx, Cl, Do Na, Nit, Ipm, S, Te	2
0.58	Amc, Amp, Cpd, Cx, Ctx, Cl, Do, Ipm, S, Na, Nit	2
0.58	Ctx, Cpd, Cx, C, Cl, Do, Ipm, Na, Nit, S, Te	2
0.63	Ak, Amc, Amp, Ctx, Cpd, Cx, Cip, Do, Ipm, , Na, Nit, Te	2
0.63	Amp, Ctx, Amc, Cpd, Cx, C, Cl, Cot, Ipm, Na, Nit, Tr	2
0.63	Ak, Amp, Ctx, Cpd, Cx, Cl, Do, Gen, Ipm, Nit, S, Te	2
0.63	Ak, Amp, Ctx, Cpd, Cx, Cl, Do, Ipm, Na, Nit, S, Te	2
0.63	Amp, Ctx, Cpd, Cx, Cip, Cl, Do, Gen, Ipm, Na, S, Te	2
0.63	Amc, Amp, Ctx, Cpd, Cx, C, Cl, Ipm, Mrp, Na, S, Te	2
0.68	Amc, Amp, Ctx, Cpd, Cx, Cip, Cl, Do, Gen, Na, Nit, Ipm, Te	2
0.68	Ak, Amc, Amp, Ctx, Cpd, Cx, Cl, Ipm, Mrp, Na, Nit, S, Te	2
0.68	Ak, Amc, Amp, Ctx, Cpd, Cx, C, Cl, Do, Ipm, Na, Nit, Te	2
0.68	Ak, Amc, Amp, Cpd, Cx, Cl, Do, Gen, Ipm, Na, Nit, S, Te	2
0.68	Amc, Amp, Ctx, Cpd, Cx, Cl, Do, Ipm, Mrp, Nit, S, Te	2
0.74	Ak, Amc, Amp, Ctx, Cpd, Cx, C, Cl, Do, Gen, Ipm, Na, Nit, Te	2
0.74	Ak, Amc, Amp, Ctx, Cpd, Cx, Cl, Do, Gen, Ipm, Na, Nit, S, Te	2
0.74	Ak, Amc, Amp, Ctx, Cpd, Cx, Cip, Cl, Do, Gen, Ipm, Nit, Te, Tr	2
0.74	Amp, Ctx, Cpd, Cx, C, Cl, Cot, Do, Ipm, Na, Nit, S, Te, Tr	2
0.79	Ak, Amp, Ctx, Cpd, Cx, Cip, Cl, Cot, Ipm, Mrp, Na, Nit, S, Te, Tr	2

3.5.1.7 Multiple antibiotic resistance (MAR) index and antibiotic resistance pattern of *E. coli* strains isolated from sediments from shellfish harvesting areas

The prevalence of various antibiotic resistance patterns and multiple antibiotic resistance (MAR) indices of *E. coli* strains isolated from sediments from shellfish harvesting areas is shown in Table 3.5. It was observed that 98% of *E. coli* strains were multidrug resistant (MDR). The MAR indices ranged from 0.1-0.84 with an average of 0.5±0.18. Overall 37 different antibiotic resistance patterns were observed. The most repeated pattern of antibiotic resistance was found to be Amp, Cpd.

Table 3.5 Multiple antibiotic resistance (MAR) of *E. coli* strains isolated from sediments from shellfish harvesting areas

MAR index	Resistance pattern	% isolates
0.1	Amp, Cpd	5.3
0.21	Ctx, Cpd, Cx, Cl	2.63
0.21	Amc, Cpd, Ipm, Te	2.63
0.21	Amc, Cpd, Cx, Nit	2.63
0.26	Amc, Amp, Cpd, , Cl, Ipm	2.63
0.31	Ak, Amc, Cpd, Cx, Nit, Te	2.63
0.37	Ctx, Cpd, Cx, , Cl, Ipm, Na, Nit	2.63
0.37	Amp, C, Cpd, Cx, Cl, Ipm, Na	2.63
0.42	Amc, Amp, Cpd, Cl, Gen, Ipm, Mrp, Na	2.63
0.42	Amp, Ctx, Cpd, Cx, Cl, Do, Ipm, Te	2.63
0.42	Amp, Ctx, Cx, , Cl, Do, Nit, S, Te	2.63
0.42	Amp, Ctx, Cpd, Cx, Cl, , Ipm, , Nit, Te	2.63
0.42	Amp, S, Ctx, Cpd, Cx, Cip, Cl, Ipm	2.63
0.42	Cpd, , Cl, Do, Na, Nit, Ipm, S, Te	2.63
0.42	Cpd, Cl, Do, Ipm, , Na, Nit, S, Te	2.63
0.47	Amc, Amp, Ctx, Cpd, Cx, , Do, Ipm, S, Te	2.63
0.47	Amp, Ctx, Cpd, Cx, Cl, Gen, Ipm, Tr, S	2.63
0.47	Amp, Ctx, Cpd, Cx, Cl, S, , Ipm, , Nit, Te	2.63
0.53	Amc, Amp, Cpd, Do, Ipm, Mrp, Na, Nit, S, Te	2.63
0.53	Amp, Ctx, Cpd, Cx, Cl, Gen, Cip, Ipm, Na, S	2.63
0.53	Amp, Ctx, Cpd, Cx, Cl, Do, Ipm, Na, Nit, Te,	2.63
0.58	Amc, Amp, Cpd, Cl, C, Ipm, Na, Nit, Te	2.63
0.58	Amc, Amp, Ctx, Cpd, Cx, C, Ipm, Mrp, Na, Nit, Te	2.63
0.58	Ak, Amc, Amp, Ctx, Cpd, Cx, Do, Gen, Nit, S, Te	2.63
0.58	Amp, Ctx, Cpd, Cx, Do, Ipm, Mrp, Na, Nit, S, Te	2.63
0.58	Ak, Ctx, Cpd, Cx, , Cl, Cot, Do, Nit, Ipm, S, Tr	2.63
0.63	Ak, Amp, Ctx, Cpd, Cx, Cip, Cl, Do, Ipm, S, Te, Tr	2.63
0.63	Amp, Ctx, Cpd, Cx, Cl, Do, Ipm, Mrp, Na, Nit S, Te	2.63
0.68	Ak, Amc, Amp,Ctx, Cpd, Cx, , Cl, Do, Gen, Ipm, Mrp, Nit, S, Te	2.63
0.68	Ak, Amc, Amp, Cpd, Ctx, Cx, Cl, Gen, Ipm, Na, Nit, S, Tr	2.63
0.68	Ak, Amc, Amp, Ctx, Cpd, Cx, Cip, Do, Gen, Ipm, Na, Nit, Te	2.63
0.68	Amc, Cpd, Cx, Ctx, Cl, Do, Gen, Ipm, Mrp, Na, Nit, S, Te	2.63
0.74	Ak, Amc, Amp, Ctx, Cpd, Cx, Cip, Do, S, Gen, Ipm, Na, Nit, Te	2.63
0.74	Ak, Amc, Amp, , Ctx, Cpd, Cx, , Cl, Do, Gen, Ipm, Mrp, Na, Nit, Te	2.63
0.74	Amc, Amp, Ctx, Cpd, Cl, Cip, Do, Gen,Ipm, Mrp, Na, Nit, S, Te	2.63
0.79	Ak, Amp, Ctx, Cpd, Cx, Cl, C, Cot, Do, Ipm, Mrp, Na, Nit, S, Te	2.63
0.84	Ak, Amc, Amp, Ctx, Cpd, Cx, C, Cip, Cl, Cot Do, Gen, Na, Nit,Te, Tr	2.63

3.5.1.8 Multiple antibiotic resistance (MAR) index and antibiotic resistance pattern of *E. coli* strains isolated from harvesting water from shellfish harvesting areas

The prevalence of various antibiotic resistance patterns and multiple antibiotic resistance (MAR) indices of *E. coli* strains isolated from shellfish harvesting waters is shown in Table 3.6.

Table 3.6 Multiple antibiotic resistance (MAR) of *E. coli* strains isolated from shellfish harvesting waters.

MAR index	Resistant pattern	% isolates
0.1	Amp, Cpd	2.7
0.16	Amp, Amc, Cpd	5.4
0.16	Amp, Cpd, Ipm	2.7
0.21	Amp, Cpd, Cl, Ipm	8.1
0.26	Amp, Amc, Cpd, Cl, Ipm	5.4
0.26	Amp, Cx, Cpd, Ipm, S	2.7
0.26	Amp, Cx, Cpd, Cl, S	2.7
0.31	Amc, Amp, Ctx, Cl, Ipm, Nit	2.7
0.31	Amp, Cpd, Cx, Ipm, Na, Nit	2.7
0.31	Ctx, Cpd, Cx, Cl, Na, Nit	2.7
0.31	Na, Ctx, Cpd, Cx, Cl, Ipm	2.7
0.37	Amp, Cpd, Cl, Gen, Ipm, Mrp, Nit	2.7
0.37	Amp, Cpd, Ctx, Gen, Ipm, Nit, S	2.7
0.37	Ak, Amp, Cx, Cpd, Do, S, Ipm	2.7
0.42	Amp, C, Cl, S, Te, Cpd, Ipm, Mrp	2.7
0.42	Ak, Amp, Cx, Cpd, Ctx, Nit, S, Te	2.7
0.47	Amc, Amp, Cpd, Ctx, C, Cl, Gen, Ipm, Na	2.7
0.47	Amc, Amp, Cx, Ctx, Cpd ,Cl, Ipm, Na,Te	2.7
0.53	Amp, Amc, Gen,Ctx, Cpd,Cx, Do, Nit	2.7
0.53	Amc, Cpd, Cx, Cl, Cot, Do, Ipm, Na,Te, Tr	2.7
0.53	Amc, Ctx, Cpd, Cl, Cot, Do, Ipm, Na, Te, Tr	2.7
0.53	Amc, Amp, Ctx, Cx, Cpd, Cl, Ipm, Nit, S, Te	2.7
0.53	Amp, Ctx, Cpd, Cx, Cl, Do, Ipm, Mrp, Nit, S	2.7
0.58	Amp, Ctx, Cpd, Cl, Do, Ipm, Na, Nit, Te, Tr	2.7
0.58	Amp, Ctx, Cpd, Cx, C, Do, S, Gen, Ipm, Na, Te	2.7
0.58	Ak, Amp, Ctx, Cpd, Cx, Cl, Do, Ipm, Nit, S, Te	2.7
0.68	Ak, Amp, Ctx, Cpd, Cx, Cl, Cot, Ipm, Mrp, Na, Nit, S, Tr	2.7
0.74	Amp, Ctx, Cpd, Cx, Cip, Cl, Cot, Do, Ipm, Mrp, Na, Nit, S, Tr	2.7
0.74	Ak, Amc, Amp, Cpd, Cx, Ctx, Cl, Do, Ipm, Mrp, Na, Nit, S, Te	2.7
0.79	Ak, Amp, Ctx, Cpd, Cx, Cip, Cl, Cot, Do, Ipm, Na, Nit, Mrp, S, Tr	2.7
0.79	Amp, Ctx, Cpd, Cx, C, Cip, Cl, Do, Gen, Ipm, Mrp, Na, Nit, S, Te	2.7
0.84	Amc, Amp, Ctx, Cpd, Cip, Cl, Cot, Do, Gen, Ipm, Mrp, Na, Nit, S,Te, Tr	2.7
0.89	Amc, Amp, Ctx, Cpd, Cx, Cip, Cl, Cot, Do, Gen, Ipm, Mrp, Na, Nit, S, Te, Tr	2.7

It was observed that the 97.3% of *E. coli* strains were multidrug resistant (MDR). The MAR indices ranged from 0.1-0.89 with an average of 0.5±0.18. Overall 33 different antibiotic resistance patterns were observed. The most repeated antibiotic resistance pattern was found to be Amp, Cpd, Cl, Ipm.

3.5.*2* Overall antibiotic resistance of enterococci isolated from shellfish harvesting areas of Cochin estuary

The overall antibiotic resistance of enterococci isolated from shellfish harvesting areas of Cochin estuary against 18 antibioitcs is represented in Figure 3.6.

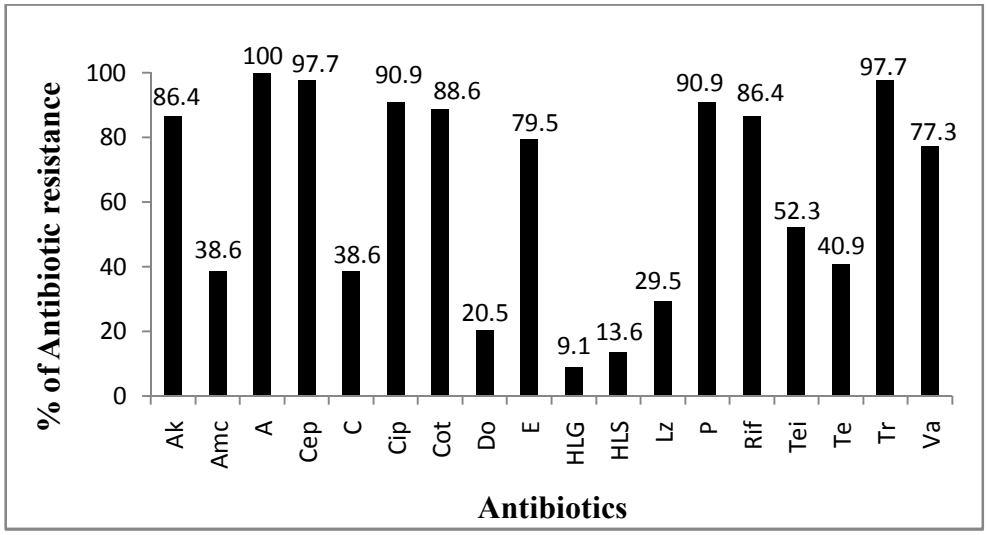

Figure 3.6 Overall antibiotic resistance of enterococci isolated from shellfish harvesting areas of Cochin estuary

Enterococci showed 100% resistance towards ampicillin. Higher degrees of resistance were shown against other antibiotics also such as cephalothin (97.7%), ciprofloxacin (90.9%), penicillin (90.9%) and trimethoprim (97.7%). Lesser degrees of resistance were shown against very few antibiotics such as high level streptomycin (13.6%) and high level gentamicin (9.1%).

3.5.2.1 Antibiotic resistance of enterococci isolated from shellfish (*Villorita cyprinoides*)

The antibiotic resistance of enterococci from shellfish is given in Figure 3.7. Enterococci strains from shellfish showed highest resistance against ampicillin (100%) followed by cephalothin and trimethoprim (95.7% each), penicillin (91.3%), amikacin, cotrimoxazole and rifampicin (86.9% each). Least resistances were shown against linazolid (17.4%) high level streptomycin (13%) and high level gentamicin (8.7%).

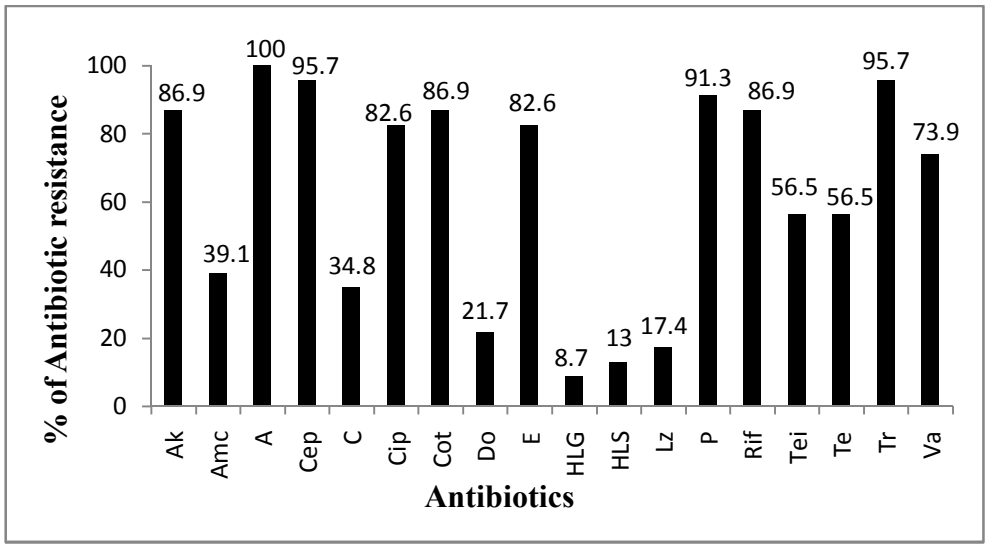

Figure 3.7 Antibiotic resistance of enterococci isolated from shellfish (*Villorita cyprinoides*)

3.5.2.2 Antibiotic resistance of enterococci isolated from sediments collected from various shellfish harvesting areas

Figure 3.8 shows the antibiotic resistance of enterococci from sediments of shellfish harvesting areas located along Cochin estuary. Enterococcal strains from sediments showed 100% resistance against antibiotics such as amikacin, ampicillin, cephalothin, ciprofloxacin, penicillin and trimethoprim.

Lesser resistance was shown against high level aminoglycosides such as high level streptomycin (21.4%) and high level gentamicin (14.3%) respectively.

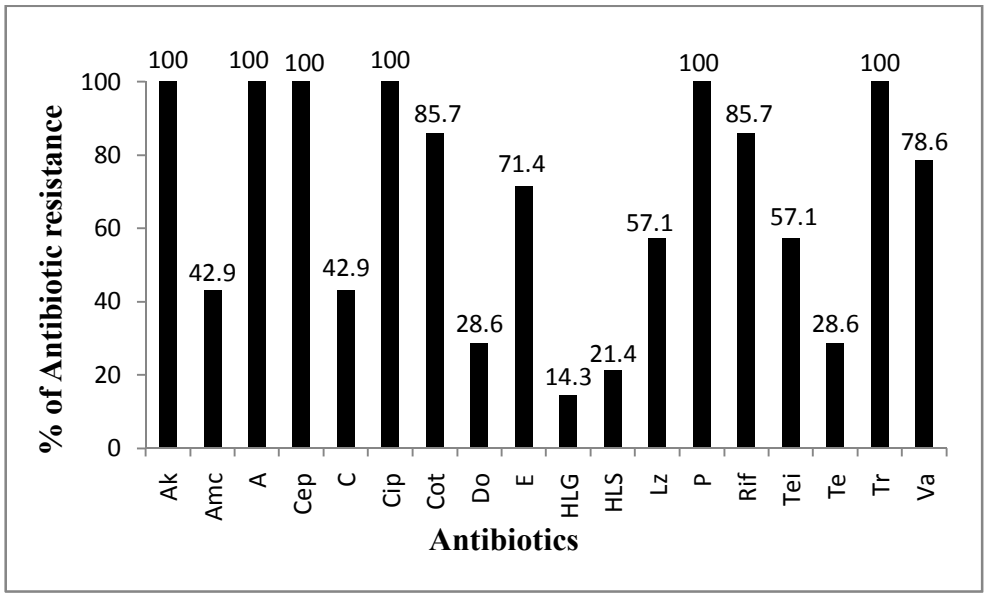

Figure 3.8 Antibiotic resistance of enterococci isolated from sediments collected from various shellfish harvesting areas

3.5.2.3 Antibiotic resistance of enterococci isolated from shellfish harvesting waters

The antibiotic resistance of enterococci isolated from shellfish harvesting waters is given in Figure 3.9. Enterococcal strains showed 100% resistance against ampicillin, cephalothin, ciprofloxacin, cotrimoxazole and trimethoprim. Low level of resistance was exhibited against tetracycline and linazolid (14.3%). All the strains from harvesting waters were sensitive towards doxycycline, high level streptomycin and high level gentamicin.

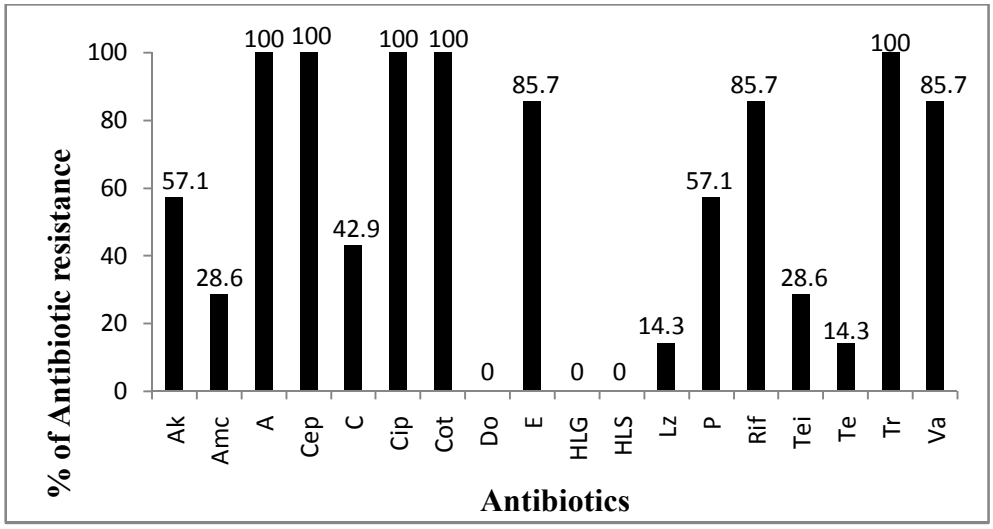

Figure 3.9 Antibiotic resistance of enterococci isolated from harvesting waters from various shellfish harvesting areas

3.5.2.4 Relative prevalence of antibiotic resistance among enterococci from shellfish, sediment and water from shellfish harvesting areas

The overall antibiotic resistance of enterococci strains from shellfish, sediment and harvesting waters against 18 antibioitcs is compared in figure 3.10. Enterococci from shellfish, sediment and water samples exhibited significant differences in their antibiotic resistance. Enterococci from all the three sources showed complete resistance towards ampicillin. Out of the three sources under study strains from shellfish samples showed maximum resistance against rifampicin, tetracycline and teicoplanin ($p<0.05$), whereas those from sediment showed maximum resistance against 10 antibiotics *viz*. amikacin, cephalothin, ciprofloxacin, doxycycline, high level gentamicin, high level streptomycin, linazolid, penicillin, teicoplanin and trimethoprim ($p<0.05$). Enterococci from harvesting water showed maximum resistance against cephalothin, ciprofloxacin, cotrimoxazole, erythromycin and trimethoprim ($p<0.05$) while complete sensitivity was exhibited towards doxycycline, high

level gentamicin and high level streptomycin. Enterococci strains from sediment showed maximum overall resistance followed by those from shellfish and harvesting waters ($p<0.001$).

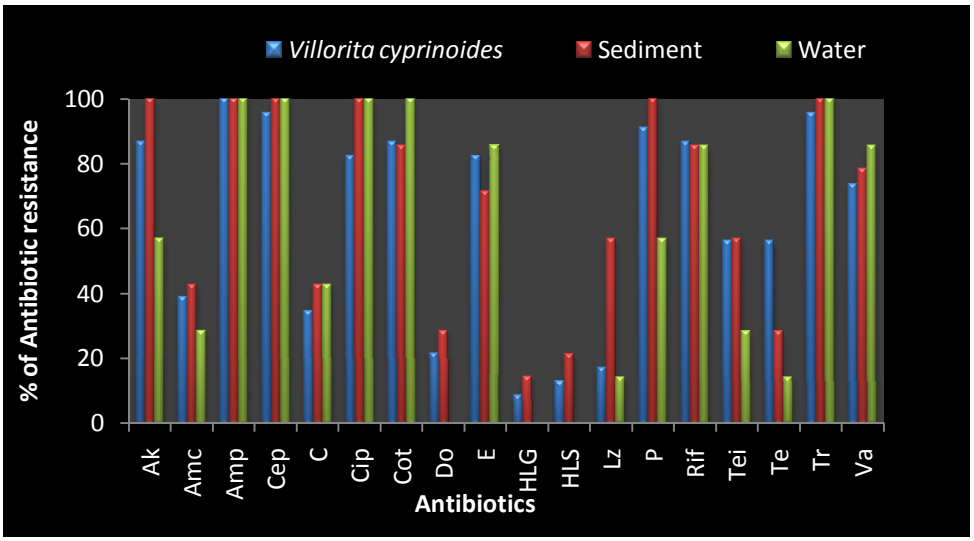

Figure 3.10 Comparison of antibiotic resistance profiles of enterococci isolated from shellfish, sediment and water from various shellfish harvesting areas

3.5.2.5 Spatial variation in antibiotic resistance among enterococci from various shellfish harvesting areas

The variation in antibiotic resistance of enterococci isolated from various stations is given in Table 3.7. Isolates from various stations showed 100% resistance towards variable number of antibiotics. Stations 1 and 2 showed 100% resistance against 7 antibiotics each; station 8 against 5 antibiotics; station 3, 4, 6 and 7 against 4 antibiotics each and station 5 against 2 antibiotics. Absolute sensitivity was shown by certain stations against variable number of antibiotics. Station 7 showed 100% sensitivity towards 6 antibiotics; stations 2, 5 and 8 against 3 antibiotics each and station 3 against one antibiotic. Enterococci from all the 8 stations showed significant

differences with respect to their antibiotic resistances ($p<0.001$). When overall resistance towards all the 18 antibiotics were considered station 1 exhibited the maximum resistance, while stations 5 and 7 exhibited the least ($p<0.001$).

Table 3.7 Spatial variation in antibiotic resistance profiles of enterococci isolated from various shellfish harvesting areas

	Prevalence of antibiotic resistance among enterococci							
Antibiotics	**Station 1 (n=10)**	**Station 2 (n=12)**	**Station 3 (n=11)**	**Station 4 (n=10)**	**Station 5 (n=12)**	**Station 6 (n=12)**	**Station 7 (n=9)**	**Station 8 (n=12)**
Ak	100	100	60	77.8	75	90	80	100
Amc	40	40	20	33.3	16.7	70	0	40
Amp	100	100	100	100	100	100	100	100
Cep	100	100	100	100	100	90	100	100
C	60	60	40	22.2	33.3	50	0	20
Cip	100	100	60	100	75	90	80	100
Cot	90	80	100	88.9	83.3	100	80	80
Do	20	0	20	33.3	25	10	0	20
E	100	80	60	66.7	58.3	90	60	80
HLG	10	0	0	22.2	0	10	0	0
HLS	20	0	20	22.2	0	20	0	0
Lz	40	20	40	11.1	0	60	20	0
P	100	100	80	77.8	75	100	100	80
Rif	90	80	80	88.9	83.3	90	80	80
Tei	80	80	40	55.6	16.7	80	0	80
Te	70	40	20	33.3	8.3	70	20	20
Tr	100	100	100	100	91.7	100	100	100
Va	90	100	40	77.8	58.3	80	80	80

3.5.2.6 Multiple antibiotic resistance (MAR) index and antibiotic resistance patterns of enterococci from shellfish (*Villorita cyprinoides*)

The prevalence of various antibiotic resistance patterns and multiple antibiotic resistance (MAR) indices of enterococcal strains from shellfish harvesting waters is shown in Table 3.8.

Table 3.8 Multiple antibiotic resistance (MAR) of enterococci isolated from shellfish (*Villorita cyprinoides*)

MAR index	Antibiotic resistance pattern	% of incidence
0.22	A, Cep, Cot, Tr	4.3
0.27	Ak, A, Cep, P, Tr	4.3
0.33	A, Cep, Cot, P, Rif, Tr	4.3
0.4	Ak, A, Cep, Cip, Cot, Do, Rif, Tr	4.3
0.5	Ak, Amc, A, Cep, Cip, E, P, Rif, Tei	4.3
0.5	Ak, Amc, A, Cep, Cot, E, P, Rif, Tr	4.3
0.5	Ak, A, Cep, Cip, E, P, Te, Tr, Va	4.3
0.56	Ak, A, Cep, Cip, Cot, E, P, Rif, Tr, Va	4.3
0.61	A, Cep, Cip, Cot, Do, E, P, Rif, Te, Tr, Va	4.3
0.61	Ak, A, Cep, Cip, Cot, Do, E, P, Rif, Te, Tr, Va	4.3
0.67	Ak, Amc, A, Cip, Cot, E, P, Rif, Tei, Te, Tr, Va	4.3
0.67	Ak, A, Cep, C, Cip, Cot, E, P, Rif, Tei, Tr, Va	4.3
0.67	Ak, Amc, A, Cep, Cip, Cot, E, P, Rif, Tei, Tr, Va	4.3
0.72	Ak, A, Cep, C, Cip, Cot, E, P, Rif, Tei, Te, Tr, Va	4.3
0.78	Ak, Amc, A, Cep, Cip, Cot, E, LZ, P, Rif, Tei, Te, Tr, Va	4.3
0.78	Ak, Amc, A, Cep, C, Cip, Cot, E, P, Rif, Tei, Te, Tr, Va	8.7
0.78	Ak, A, Cep, C, Cip, Cot, E, Lz, P, Rif, Tei, Te, Tr, Va	8.7
0.78	Ak, Amc, A, Cep, Cip, Cot, E, HLS, P, Rif, Tei, Te, Tr, Va	4.3
0.78	Ak, A, Cep, Cip, Cot, Do, E, HLG, HLS, P, Rif, Te, Tr, Va	4.3
0.83	Ak, Amc, A, Cep, C, Cip, Cot, Do, E, P, Rif, Tei, Te, Tr, Va	4.3
0.89	Ak, A, Cep, C, Cip, Cot, E, HLG, HLS, LZ, P, Rif, Tei, Te, Tr, Va	4.3

It was observed that 100% of enterococci strains from shellfish were multidrug resistant (MDR). The MAR indices ranged from 0.22-0.89 with an average of 0.63±0.19. Twenty one different antibiotic resistance patterns were observed. The most repeated antibiotic resistance patterns were found to be Ak, Amc, Amp, Cep, C, Cip, Cot, E, P, Rif, Tei, Te, Tr, Va and Ak, Amp, Cep, C, Cip, Cot, E, Lz, P, Rif, Tei, Te, Tr, Va.

3.5.2.7 Multiple antibiotic resistance (MAR) index and antibiotic resistance patterns among enterococci from sediments of shellfish harvesting areas

The prevalence of various antibiotic resistance patterns and multiple antibiotic resistance (MAR) indices of enterococci isolated from sediments

from shellfish harvesting areas is shown in Table 3.9. It was observed that 100% of enterococci were multidrug resistant (MDR). The MAR indices ranged from 0.44-1 with an average of 0.68±0.19. Thirteen different antibiotic resistance patterns were observed. The most repeated antibiotic resistance patterns were found to be Ak, Amc, Amp, Cep, C, Cip, Cot, E, P, Rif, Tei, Te, Tr, Va and Ak, Amp, Cep, C, Cip, Cot, E, Lz, P, Rif, Tei, Te, Tr, Va.

Table 3.9 Multiple antibiotic resistance (MAR) indices of enterococci isolated from sediments from shellfish harvesting areas

MAR Index	Ab resistance pattern	% of incidence
0.44	Ak, Amp, Cep, Cip, Cot, P, Rif, Tr	7.14
0.5	Ak, Amp, Cep, Cip, E, P, Tei, Tr, Va	7.14
0.5	Ak, Amp, Cep, Cip, Cot, P, Rif, Tr, Va	7.14
0.5	Ak, Amc, Amp, Cep, Cip, Lz, P, Tr, Va	7.14
0.56	Ak, Amp, Cep, Cip, Cot, E, Lz, P, Rif, Tr	7.14
0.56	Ak, Amc, Amp, Cep, Cip, Cot, Lz, P, Rif, Tr	7.14
0.61	Ak, Amp, Cep, Cip, Cot, E, P, Rif, Tei, Tr, Va	7.14
0.61	Ak, Amp, Cep, Cip, Cot, E, Lz, P, Rif, Tr, Va	7.14
0.67	Ak, Amp, Cep, C, Cip, Cot, E, P, Rif, Tei, Tr, Va	7.14
0.78	Ak, Amc, Amp, Cep, C Cip, Cot, Do, E, P, Rif, Tei, Tr, Va	7.14
0.78	Ak, Amp, Cep, C, Cip, Cot, E, Lz, P, Rif, Tei, Te, Tr, Va	7.14
0.94	Ak, Amc, Amp, Cep, C, Cip, Cot, Do, E, HLS, Lz, P, Rif, Tei, Te, Tr, Va	7.14
1	Ak, Amc, Amp, Cep, C, Cip, Cot, Do, E, HLG, HLS, Lz, P, Rif, Tei, Te, Tr, Va	14.28

3.5.2.8 Multiple antibiotic resistance (MAR) index and antibiotic resistance patterns among enterococci from harvesting waters from shellfish harvesting areas

The prevalence of various antibiotic resistance patterns and multiple antibiotic resistance (MAR) indices of strains isolated from shellfish harvesting waters is shown in Table 3.10. It was observed that 100% of enterococci were multidrug resistant (MDR). The MAR indices ranged from 0.4-0.61 with an average of 0.55±0.19. Seven different antibiotic resistance patterns were observed and all exhibited equal distribution.

Table 3.10 Multiple antibiotic resistance (MAR) indices of enterococci isolated from harvesting water

MAR Index	Ab resistance pattern	% of incidence
0.4	Amc, Amp, Cep, Cip, Cot, E, Rif, Tr	14.28
0.5	Amp, Cep, Cip, Cot, P, Rif, Tei, Tr, Va	14.28
0.56	Ak, Amp, Cep, C, Cip, Cot, E, Rif, Tr, Va	14.28
0.56	Ak, Amp, Cep, C, Cip, Cot, E, P, Rif, Tr, Va	14.28
0.61	Ak, Amp, Cep, Cip, Cot, E, P, Rif, Te, Tr, Va	14.28
0.61	Amc, Amp, Cep, Cip, Cot, E, Lz, P, Tei, Tr, Va	14.28
0.61	Ak, Amp, Cep, C, Cip, Cot, E, P, Rif, Tr, Va	14.28

3.5.3 Overall antibiotic resistance of *V. parahaemolyticus* isolated from shellfish harvesting areas of Cochin estuary

The overall antibiotic resistance of *V. parahaemolyticus* isolated from shellfish harvesting areas of Cochin estuary is given in Figure 3.11. Maximum resistance was shown against ampicillin and cefpodoxime (93.5% each). Lower resistance was shown against tetracycline (9.7%) and chloramphenicol (6.5%). All the *V. parahaemolyticus* strains were sensitive towards doxycycline.

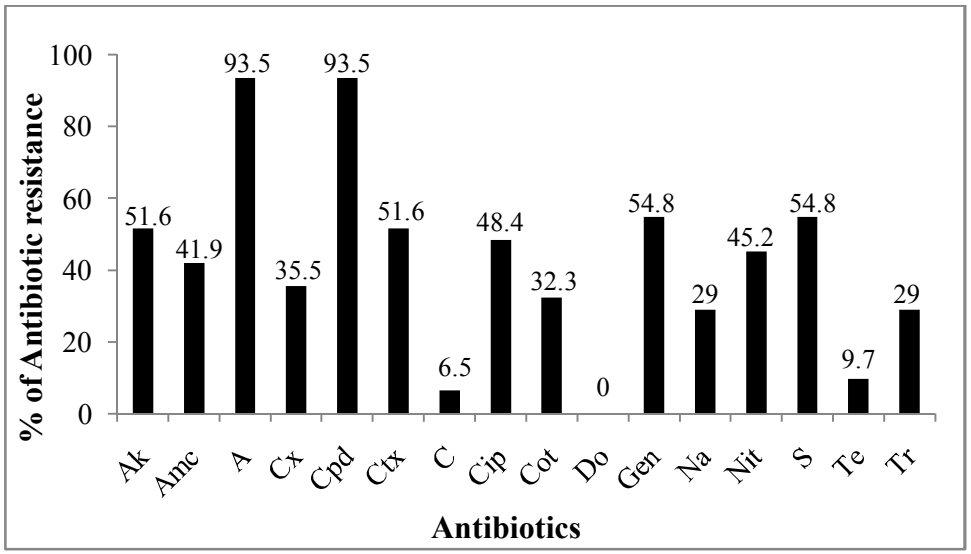

Figure 3.11 Overall antibiotic resistance of *V. parahaemolyticus* isolated from shellfish harvesting areas of Cochin estuary

3.5.3.1 Antibiotic resistance of *V. parahaemolyticus* isolated from shellfish (*Villorita cyprinoides*)

The antibiotic resistance of *V. parahaemolyticus* strains isolated from shellfish is represented in Figure 3.12. All the strains were resistant to ampicillin and cefpodoxime. Lower degrees of resistance were recorded against trimethoprim (18.2%), tetracycline (11.1%) and nalidixic acid (9.1%). All the *V. parahaemolyticus* strains from shellfish were sensitive towards chloramphenicol and doxycycline.

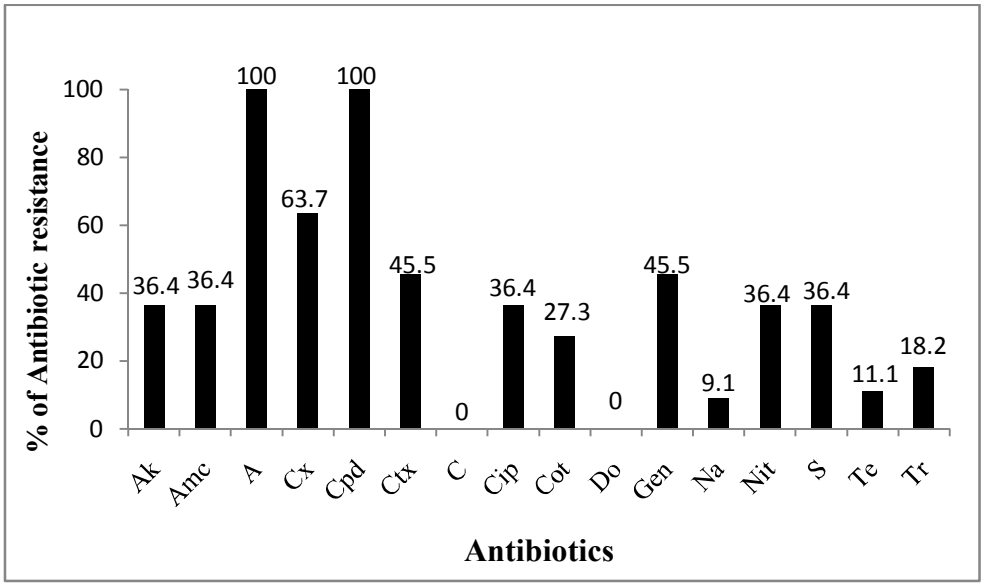

Figure 3.12 Antibiotic resistance of *V. parahaemolyticus* isolated from shellfish (*Villorita cyprinoides*)

3.5.3.2 Antibiotic resistance of *V. parahaemolyticus* from sediments from various shellfish harvesting areas

Figure 3.13 shows the antibiotic resistance of *V. parahaemolyticus* strains isolated from sediment samples. Maximum resistance was exhibited against ampicillin (100%) and cefpodoxime (92.3%) respectively. Lower degrees of

resistance were observed against cefoxitin (15.4%) and tetracycline (7.7%) respectively, while all the strains were sensitive towards chloramphenicol and doxycycline.

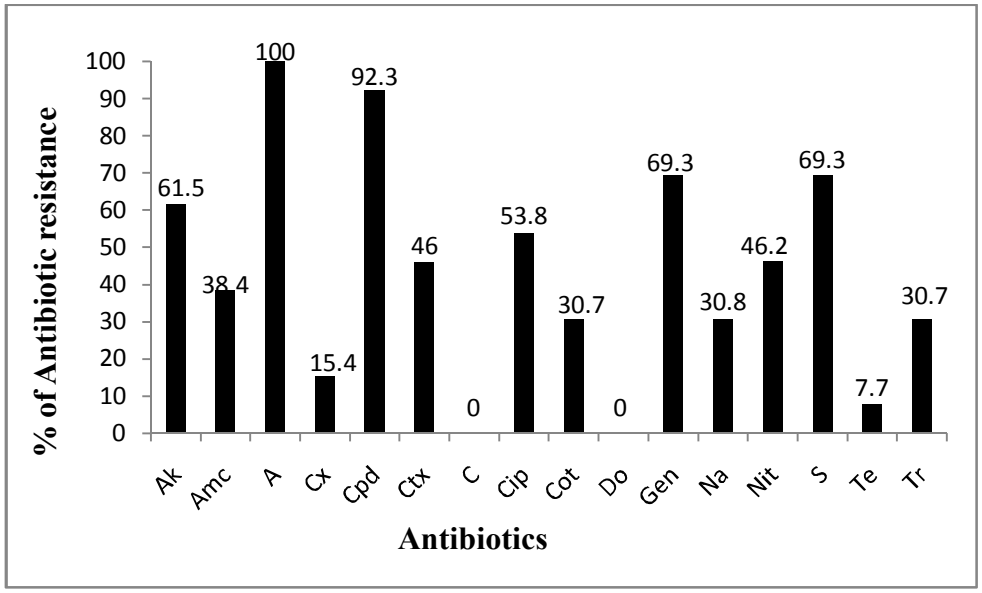

Figure 3.13 Antibiotic resistance of *V. parahaemolyticus* isolated from sediments collected from various shellfish harvesting areas

3.5.3.3 Antibiotic resistance of *V. parahaemolyticus* isolated from harvesting waters from various shellfish harvesting areas

The antibiotic resistance of *V. parahaemolyticus* strains from shellfish harvesting waters is given in Figure 3.14. Maximum resistance was shown against cefpodoxime (85.7%), followed by cephotaxime (71.5%) and ampicillin (71%). Lower degrees of resistance were shown against cefoxitin and chloramphenicol (28.6% each) and tetracycline (14.3%), while all the strains were sensitive towards doxycycline.

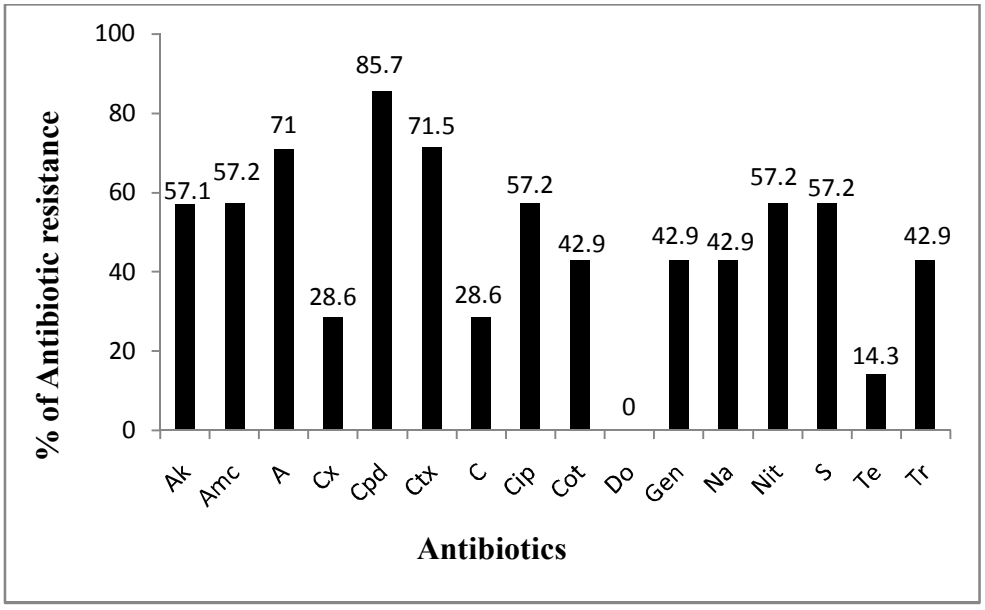

Figure 3.14 Antibiotic resistance of *V. parahaemolyticus* isolated from harvesting waters from various shellfish harvesting areas

3.5.3.4 Relative prevalence of antibiotic resistance among *V. parahaemolyticus* isolated from shellfish, sediment and harvesting water from shellfish harvesting areas

The antibiotic resistance of *V. parahaemolyticus* strains from shellfish, sediment and harvesting waters is compared in Figure 3.15. *V. parahaemolyticus* from shellfish, sediment and water samples showed significant differences in their antibiotic resistance. The strains from shellfish showed maximum relative resistance against 3 antibiotics ampicillin, cefoxitin and cefpodoxime ($p<0.05$), whereas those from sediment showed highest resistance against 4 antibiotics *viz*. amikacin, ampicillin, gentamicin and streptomycin ($p<0.05$). *V. parahaemolyticus* strains from harvesting waters showed maximum relative resistance against 9 antibiotics *viz*. amoxyclav, cephotaxime, choramphenicol, ciprofloxacin, cotrimoxazole, nalidixic acid, nitrofurantoin and trimethoprim ($p<0.05$). Resistance to chloramphenicol was

seen only among *V. parahaemolyticus* from harvesting waters, while those from all the 3 sources were sensitive towards doxycycline. *V. parahaemolyticus* strains from harvesting waters showed maximum overall antibiotic resistance followed by those from sediment and shellfish ($p<0.001$).

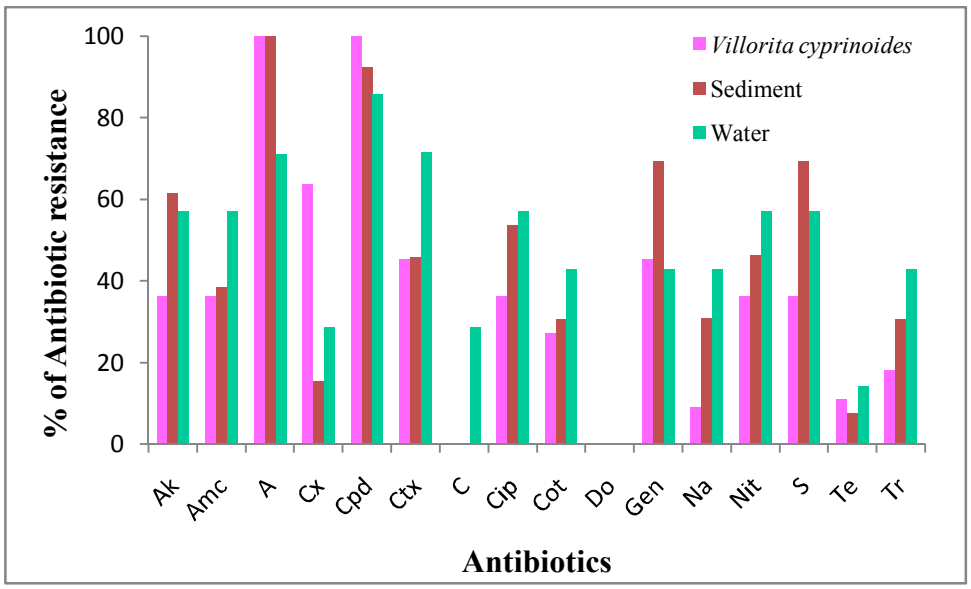

Figure 3.15 Comparison of antibiotic resistance profiles of *V. parahaemolyticus* isolated from shellfish, sediment and water from various shellfish harvesting areas

3.5.3.5 Spatial variation in antibiotic resistance among *V. parahaemolyticus* from various shellfish harvesting areas

V. parahaemolyticus strains from various stations showed differences in their resistance towards various antibiotics. The strains from station 3 showed 100% resistance against 12 out of 16 antibiotics. Stations 4 and 5 showed complete sensitivity towards 8 antibiotics; station 6 was totally sensitive to 5 antibiotics and stations 7 and 8 recorded complete sensitivity towards 9antibiotics as shown in Table 3.11.*V. parahaemolyticus* strains from all the 8 stations were completely sensitive to doxycycline. When the

overall resistance towards all the 16 antibiotics were considered station 3 exhibited the maximum resistance, while station 6 exhibited the lowest ($p<0.001$).

Table 3.11 Spatial variation in antibiotic resistance among *V. parahaemolyticus* from various shellfish harvesting areas

	Prevalence of antibiotic resistance among *V. parahaemolyticus*							
Antibiotic	**Station 1(n-=7)**	**Station 2 (n=8)**	**Station 3 (n=7)**	**Station 4 (n=8)**	**Station 5 (n=7)**	**Station 6 (n=8)**	**Station 7 (n=9)**	**Station 8 (n=8)**
Ak	28.6	66.7	100	33.3	50	22.2	66.7	66.7
Amc	48.9	66.7	100	0	0	22.2	66.7	66.7
Amp	85.7	100	100	100	100	88.9	66.7	66.7
Cx	57.1	66.7	100	0	0	44.4	0	0
Cpd	100	100	100	66.6	100	88.9	66.7	66.7
Ctx	48.9	66.7	100	0	0	0	0	0
C	14.3	0	50	0	0	0	0	0
Cip	28.6	66.7	100	0	25	22.2	66.7	66.7
Cot	48.9	33.3	100	33.3	0	11.1	0	0
Do	0	0	0	0	0	0	0	0
Gen	28.6	66.7	50	66.6	75	33.3	66.7	66.7
Na	14.3	66.7	100	0	25	11.1	0	0
Nit	28.6	66.7	100	33.3	25	33.3	0	0
S	28.6	66.7	100	66.6	75	22.2	66.7	66.7
Te	28.6	0	50	0	0	0	0	0
Tr	48.9	33.3	100	33.3	0	0	0	0

3.5.3.6 Multiple antibiotic resistance (MAR) index and antibiotic resistance patterns among *V. parahaemolyticus* from shellfish (*Villorita cyprinoides*)

Table 3.12 shows the various antibiotic resistance patterns and MAR indices of *V. parahaemolyticus* strains isolated from shellfish. It was observed that 90% of the strains were MDR and 8 different antibiotic resistance patterns were observed. The MAR index values ranged from 0.13-0.8 with an average of 0.37±0.22. The most repeated antibiotic resistance pattern was Amp, Cx, Cpd.

Table 3.12 Multiple antibiotic resistance (MAR) of *V. parahaemolyticus* isolated from shellfish (*Villorita cyprinoides*)

MAR index	Antibiotic resistance pattern	% of incidence
0.13	Amp, Cpd	9.1
0.19	Amp, Cx, Cpd	36.4
0.38	Amc, Amp, Cx, Cpd, Cot, Tr	9.1
0.44	Amc, Amp, Cx, Cpd, Ctx, Gen, Nit	9.1
0.5	Ak, Amc, Amp, Cpd, Ctx, Cip, Gen, S	9.1
0.5	Ak, Amp, Cpd, Ctx, Cip, Gen, Na, Nit, S	9.1
0.56	Ak, Amp, Cpd, Ctx, Cip, Cot, Gen, Nit, S	9.1
0.88	Ak, Amc, Amp, Cx, Cpd, Ctx, Cip, Cot, Gen, Na, Nit, S, Te, Tr	9.1

3.5.3.7 Multiple antibiotic resistance (MAR) index and antibiotic resistance patterns among *V. parahaemolyticus* isolated from sediments from shellfish harvesting areas

The MAR indices of *V. parahaemolyticus* strains from sediments and their antibiotic resistance patterns are presented in Table 3.13. It was observed that 84.6% of the strains were MDR and 11 different resistance patterns were observed. The MAR indices ranged from 0.13-0.88 with an average of 0.37±0.22. The most repeated antibiotic resistance patterns were Amp, Cpd and Ak, Amp, Cpd, Ctx, Cip, Cot, Gen, Na, Nit, S, Tr.

Table 3.13 Multiple antibiotic resistance (MAR) of *V. parahaemolyticus* from sediments from shellfish harvesting areas

MAR Index	Resistance pattern	% of incidence
0.13	Amp, Cpd	15.4
0.19	Amp, Cx, Cpd	7.7
0.25	Amp, Cpd, Gen, S	7.7
0.25	Amp, Cot, Nit, Tr	7.7
0.31	Ak, Amp, Cpd, Gen, S	7.7
0.44	Ak, Amc, Amp, Cpd, Cip, Gen, S	7.7
0.5	Ak, Amc, Amp, Cpd, Ctx, Cip, Gen, S	7.7
0.56	Ak, Amc, Amp, Cpd, Ctx, Cip, Gen, Nit, S	7.7
0.63	Ak, Amc, Amp, Cpd, Ctx, Cip, Gen, Na, Nit, S	7.7
0.69	Ak, Amp, Cpd, Ctx, Cip, Cot, Gen, Na, Nit, S, Tr	15.4
0.88	Ak, Amc, Amp, Cx, Cpd, Ctx, Cip, Cot, Gen, Na, Nit, S, Te, Tr	7.7

3.5.3.8 Multiple antibiotic resistance (MAR) of *V. parahaemolyticus* isolated from water from shellfish harvesting areas

The antibiotic resistance patterns and MAR indices of *V. parahaemolyticus* strains from shellfish harvesting waters is given in Table 3.14. It was observed that 85.7% of the total strains were MDR and 5 different antibiotic resistance patterns were observed. The MAR indices ranged from 0.13-0.88 with an average of 0.47±0.33. The most repeated antibiotic resistance pattern was found to be Ak, Amc, Amp, Cpd, Ctx, Cip, Gen, Na, Nit, S, Tr.

Table 3.14 Multiple antibiotic resistance (MAR) of *V. parahaemolyticus* isolated from water from shellfish harvesting areas

MAR index	Antibiotic resistance pattern	% of incidence
0.19	Amp, Cpd, Ctx	14.3
0.19	Amc, Cpd, Tr	14.3
0.69	Ak, Amc, Amp, Cpd, Ctx, Cip, Gen, Na, Nit, S, Tr	28.6
0.75	Ak, Amc, Amp, Cx, Cpd, Ctx, Cip, Cot, Gen, Na, Nit, S	14.3
0.81	Ak, Amc, Amp, Cx, Cpd, Ctx, C, Cip, Cot, Na, Nit, S, Tr	14.3

3.5.4 Prevalence of antibiotic resistance genes in *E. coli*

3.5.4.1 Prevalence of extended spectrum beta lactamase (ESBL) bla_{CTX-M} gene in *E. coli*

Thirty six *E. coli* strains which exhibited resistance to beta lactam antibiotics were screened for the presence of ESBL genes. $Bla_{\text{CTX-M}}$ gene was detected in 91.7% of the strains selected for the study. The gel image of the PCR product showing $bla_{\text{CTX-M}}$ gene of *E. coli* is presented in Figure 3.16. The prevalence of $bla_{\text{CTX-M}}$ gene frequent among *E. coli* isolates from shellfish, followed by those from sediment and water from harvesting areas. The percentage distribution of $bla_{\text{CTX-M}}$ gene in *E. coli* from various sources is given in Figure 3.17.

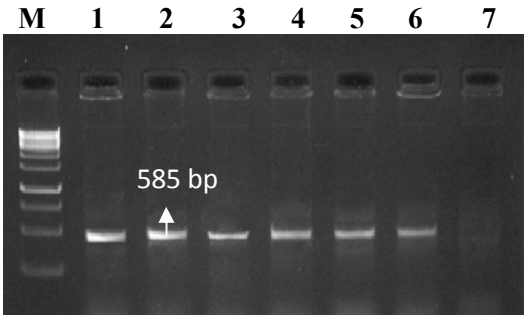

Figure 3.16 Gel image showing bla_{CTX-M} gene of *E. coli* from shellfish harvesting areas of Cochin estuary
Lane M: 250 bp ladder, lanes 1-6: bla_{CTX-M} gene, lane 7: negative control

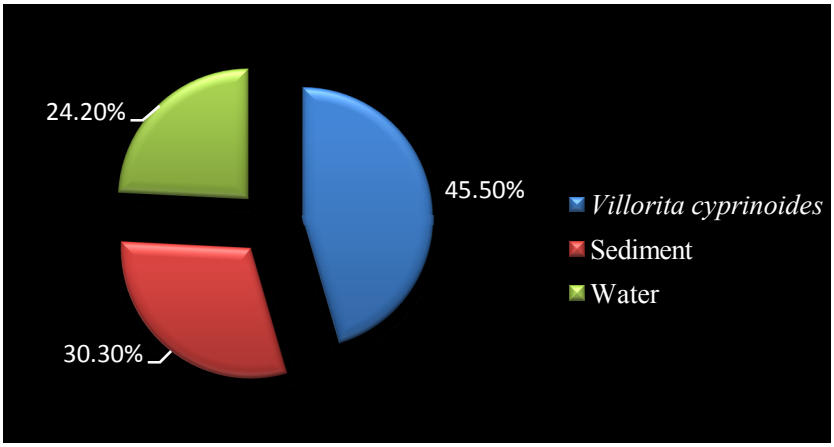

Figure 3.17 Prevalence of bla_{CTX-M} gene in *E. coli* from shellfish harvesting areas of Cochin estuary

3.5.4.1.1 Distribution of bla_{CTX-M} gene among various *E. coli* serogroups

The distribution of bla_{CTX-M} gene was found to vary with different *E. coli* serogroups (Figure 3.18). Among the serogrouped strains maximum prevalence was associated with O157 (18%) followed by O121 (15%), O8 (9.1%) and O126 (6%). Serogroups O8, O2, O84, O120, O141 contributed 3% each. However the untypable group of *E. coli* contributed the maximum of 33% to bla_{CTX-M} gene prevalence.

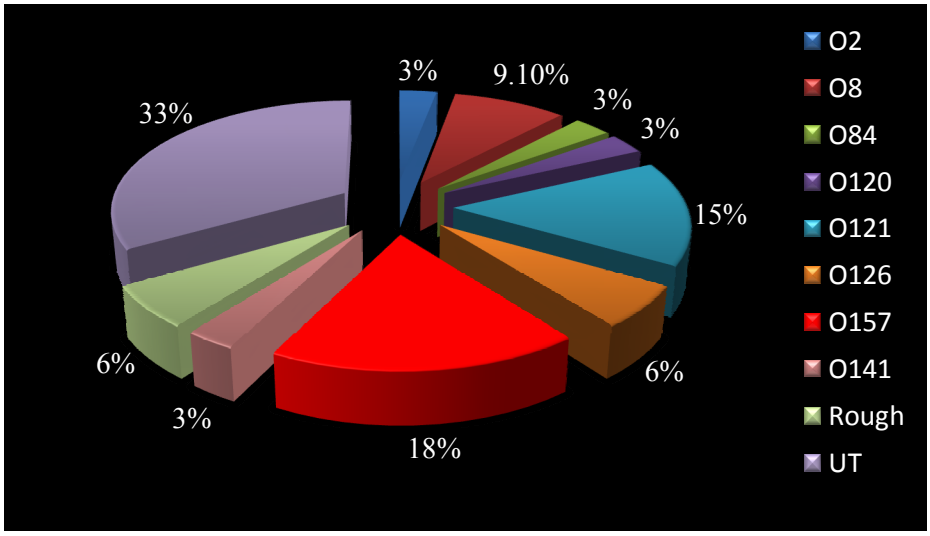

Figure 3.18 Distribution of *bla*$_{CTX-M}$ gene among various *E. coli* serogroups isolated from shellfish harvesting areas of Cochin estuary

3.5.4.1.2 Distribution of *bla*$_{CTX-M}$ gene among various pathogenic *E. coli* serogroups

Distribution of *bla*$_{CTX-M}$ genes varied among different pathogenic *E. coli* serogroups is given in figure 3.19.

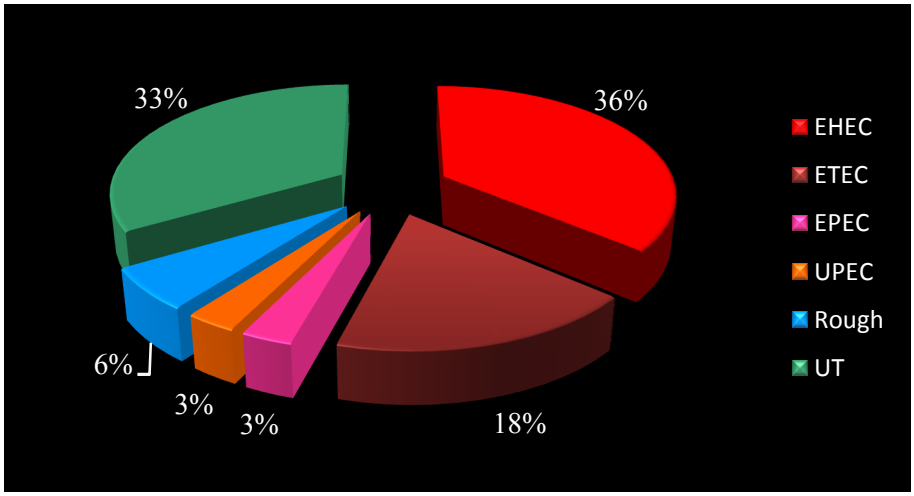

Figure 3.19 Distribution of *bla*$_{CTX-M}$ gene among various pathogenic *E. coli* serogroups isolated from shellfish harvesting areas of Cochin estuary

Approximately 33% of the strains which possessed the *bla*$_{CTX-M}$ genes were untypable. All the identified serogroups which showed the presence of *bla*$_{CTX-M}$ genes were pathogenic. Maximum prevalence of *bla*$_{CTX-M}$ gene was recorded in EHEC (36%), followed by ETEC (18%), UPEC and EPEC (3% each) serogroups.

3.5.4.1.3 Spatial variation in distribution of *bla*$_{CTX-M}$ genes in *E. coli*

The prevalence of *bla*$_{CTX-M}$ gene in *E. coli* varied with stations as shown in figure 3.20. Highest prevalence was observed at station 4 (39.4%) and lowest at station 8 (9.1%). *E. coli* from stations 2 and 7 lacked *bla*$_{CTX-M}$ gene.

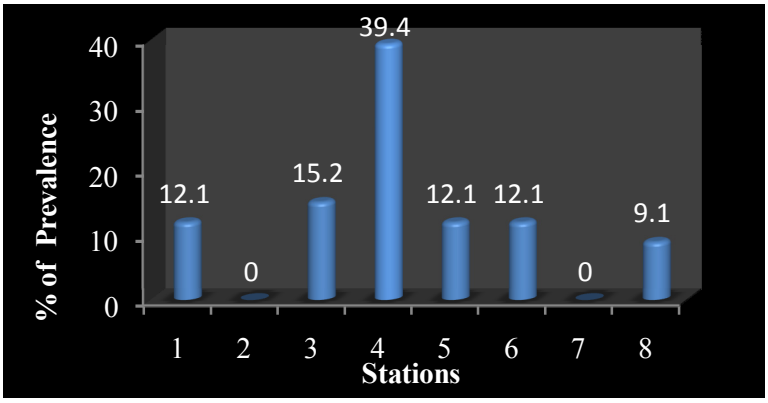

Figure 3.20 Spatial variationss in distribution of *bla*$_{CTX-M}$ genes

3.5.4.2 Prevalence of *bla*$_{TEM}$ gene in *E. coli*

Thirty six *E. coli* strains which exhibited complete resistance to beta lactam antibiotics were screened for the presence of *bla*$_{TEM}$ genes. *Bla*$_{TEM}$ gene was detected in 77.7% of the strains selected for the study. The gel image of the PCR product showing *bla*$_{TEM}$ gene of *E. coli* is presented in Figure 3.21. The prevalence of *bla*$_{TEM}$ gene was highest in *E. coli* from shellfish, followed by those from sediment and harvesting waters. Figure

3.22 represents the distribution of *bla*$_{TEM}$ gene in *E. coli* strains isolated from various sources.

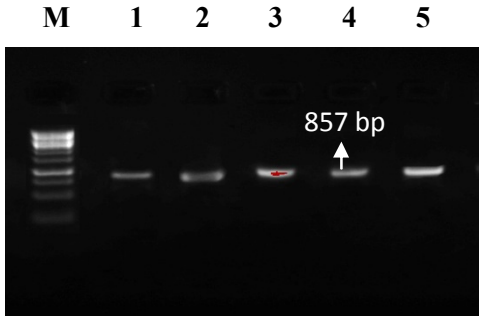

Figure 3.21 Gel image showing *bla*$_{TEM}$ gene of *E. coli* from shellfish harvesting areas of Cochin estuary
Lane M: 250 bp ladder, lanes 1-5: *bla*$_{TEM}$ gene

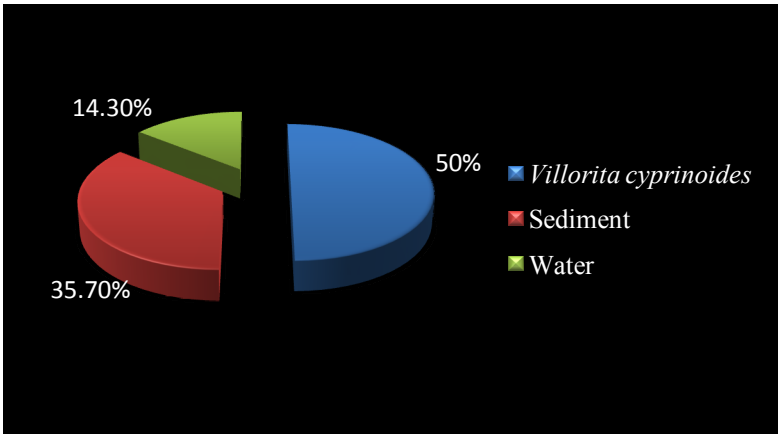

Figure 3.22 Relative prevalence of *bla*$_{TEM}$ gene in *E. coli* from shellfish harvesting areas of Cochin estuary

3.5.4.2.1 Distribution of *bla*$_{TEM}$ gene among various *E. coli* serogroups

The distribution of *bla*$_{TEM}$ gene was found to vary with diverse *E. coli* serogroups (Figure 3.23). Among the serogrouped strains maximum prevalence was associated with O157 (21%) followed by O121 (18%) and

O8 (10.7%). Serogroups O2, O126 and O141 contributed 4% each. However the untypable group of *E. coli* contributed the maximum of 32% to bla_{TEM} gene prevalence.

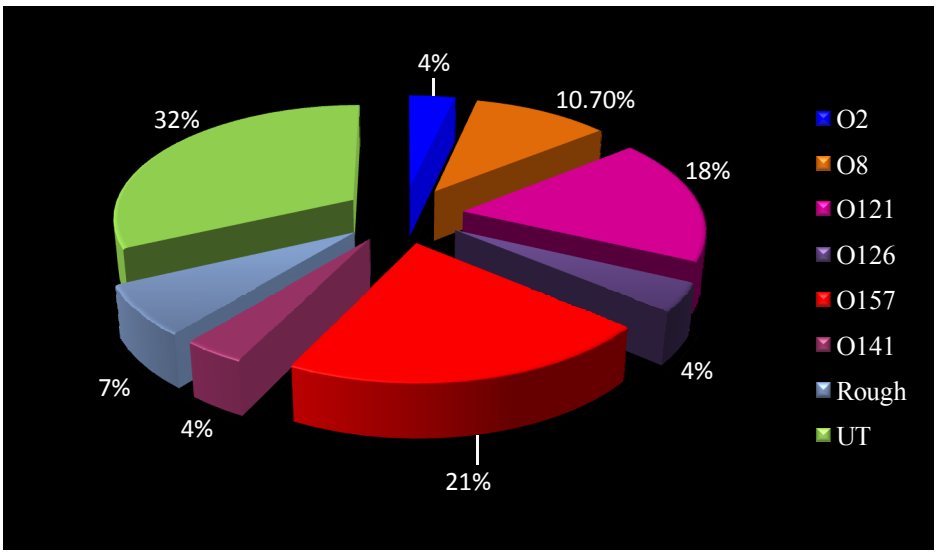

Figure 3.23 Distribution of bla_{TEM} gene among various *E. coli* serogroups isolated from shellfish harvesting areas of Cochin estuary

3.5.4.2.2 Distribution of bla_{TEM} gene among various pathogenic *E. coli* serogroups

The distribution of bla_{TEM} genes varied among different pathogenic *E. coli* serogroups (Figure 3.24). Approximately 32% of the strains which possessed the bla_{TEM} genes were untypable. All the identified serogroups which showed the presence of bla_{TEM} genes were pathogenic. EHEC recorded maximum prevalence of bla_{CTX-M} gene (39%), followed by ETEC (18.7%), and UPEC (4%) serogroups.

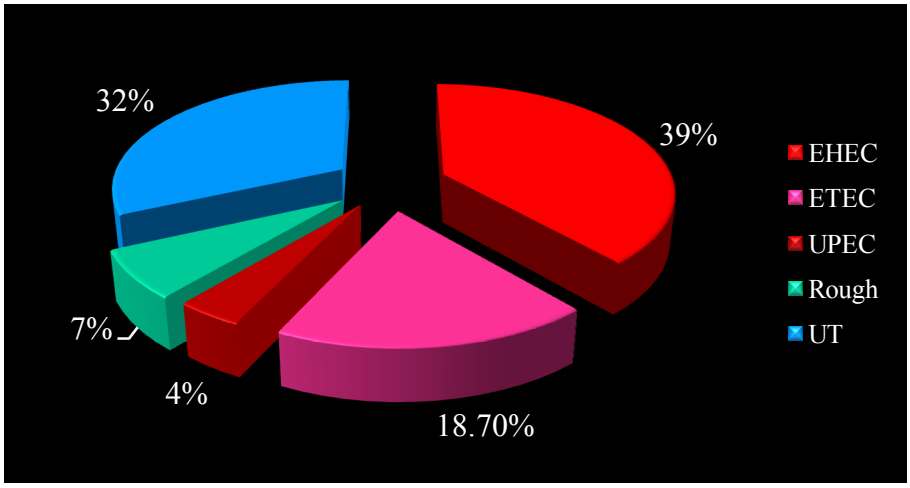

Figure 3.24 Distribution of *bla*$_{TEM}$ gene among various pathogenic *E. coli* serogroups isolated from shellfish harvesting areas of Cochin estuary

3.5.4.2.3 Spatial variation in distribution of *bla*$_{TEM}$genes in *E. coli*

The prevalence of *bla*$_{TEM}$ gene in *E. coli* varied with stations as shown in Figure 3.25. Highest prevalence was observed at station 4 (35.7%) and lowest at station 8 (7.14%). *E. coli* from stations 2 and 7 lacked *bla*$_{TEM}$ gene.

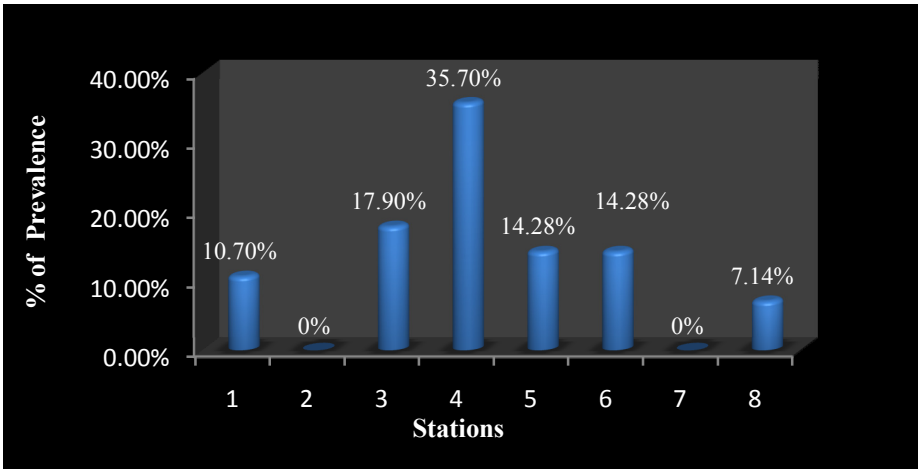

Figure 3.25 Spatial variations in distribution of *bla*$_{TEM}$ genes

3.5.4.3 Prevalence of *mcr-1* gene in *E. coli*

Eleven *E. coli* strains which exhibited complete absence of inhibition zone for colistin were screened for the presence of plasmid coded colistin resistance gene *mcr-1*. *Mcr-1* gene was detected in 3 out of 11 isolates selected for the study; one *E. coli*strain each isolated from shellfish and sediment from station 7 and one strain from shellfish harvested from station 5. The gel image of the PCR product showing *mcr-1*gene of *E. coli* is presented in Figure 3.26.

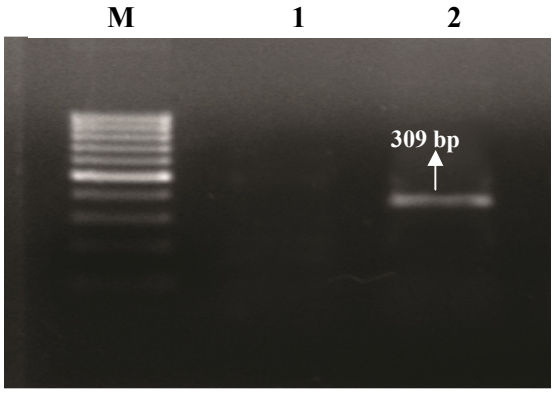

Figure 3.26 Gel image showing PCR product of *mcr-1*gene of *E. coli*
Lane M: 100 bp ladder,
lane 1: negative control, lane 2: *mcr-1*gene

3.5.5 Antibiotic resistance genes in Enterococci

Enterococci strains isolated from shellfish harvesting areas of Cochin estuary were screened for the presence of vanomycin resistance genes *vanA*, *vanB* and *vanC*. *VanA* gene was detected in one *E. faecalis strain*isolated from sediment from station 1, while*VanB* gene was detected in one *E. faecium* strain isolated from shellfish from station 6 (Figure 3.27).

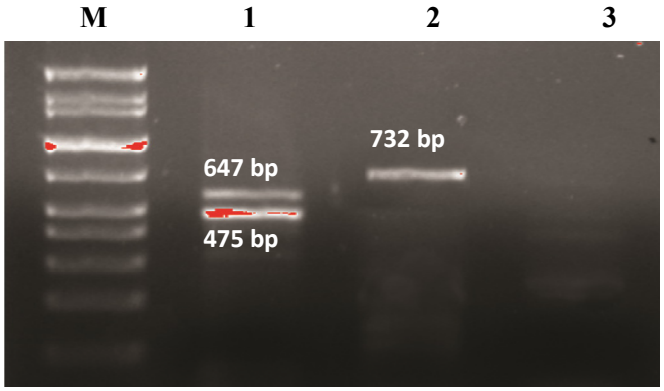

Figure 3.27 Gel image showing PCR products of vancomycin resistance genes of enterococci
Lane M: 100 bp ladder, lane 1: *VanA* gene (647 bp) and *E. faecalis* specific gene (475 bp), lane 2: *VanB* gene (732 bp), lane 3: negative control

3.5.6 Occurrence of toxigenic genes in *E. coli*

Multiplex PCR analysis of 125 *E. coli* strains from shellfish harvesting areas of Cochin estuary revealed the presence of shiga toxin genes (stx_1, stx_2), intimin (*eae*) gene and enterohemolysin gene (*hlyA)*. The results are summarised in Table 3.18. Out of the three samples selected for study, maximum prevalence was observed in *E. coli* from shellfish samples followed by those from sediment and least was recorded in strains from harvesting waters (Table 3.18).

Table 3.15 Prevalence of toxigenic genes in *E. coli* from shellfish harvesting areas of Cochin estuary.

Source	Prevalence of toxigenic genes in *E. coli* (%)
Overall shellfish harvesting area	78.1%
Shellfish	84%
Sediment	78.1%
Harvesting water	69%

3.5.6.1 Distribution of various toxigenic genes in *E. coli* from shellfish harvesting areas

The gel images of various toxigenic genes detected in *E. coli* during present study is shown in Figures 3.28 and 3.29. Among the four toxigenic genes screened shiga toxin gene, stx_2 showed the maximum overall prevalence (54.3%), followed by intimin (*eae*) gene (36.2%), shiga toxin gene, stx_1(20%), and enterohemolysin gene (*hlyA*) showed the least prevalence (6.7%) as shown in Figure 3.30.

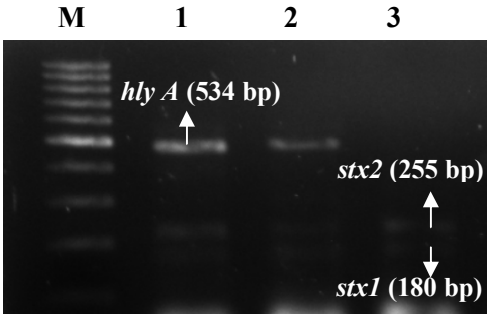

Figure 3.28 Gel image of PCR products with representative isolates carrying various toxigenic genes

Lane M: 100 bp ladder, lanes 1& 2: *hly A,* stx_2 *and* stx_1 genes of *E. coli*, lane 3: stx_2 *and* stx_1 genes of *E. coli*

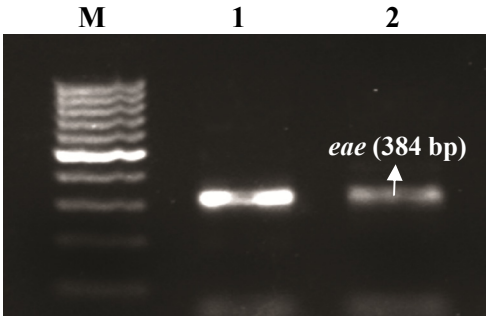

Figure 3.29 Gel image of PCR products with representative isolates carrying *eae* gene
Lane M: 100 bp ladder, lanes 1and 2: *eae* gene of *E. coli*

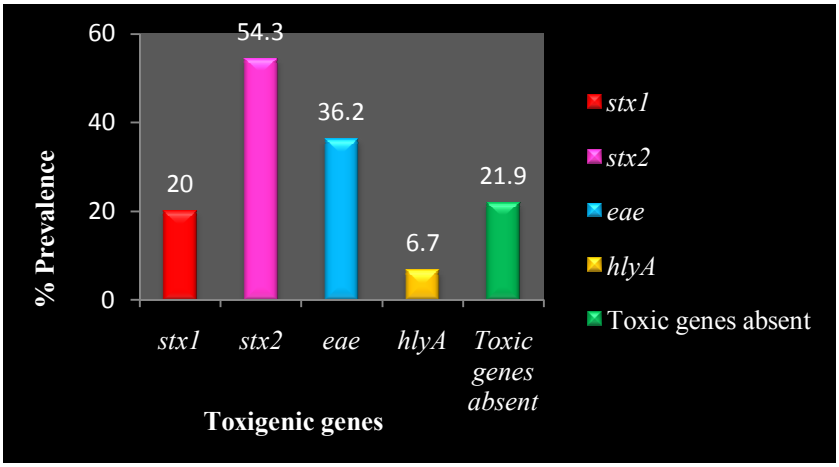

Figure 3.30 Overall prevalence of toxigenic genes in *E. coli* from shellfish growing areas

3.5.6.2 Relative prevalence of various toxigenic genotypes in *E. coli* from shellfish, sediment and harvesting waters

The relative prevalence of various toxigenic genotypes in *E. coli* from shellfish, sediment and harvesting waters is shown in Figure 3.31.

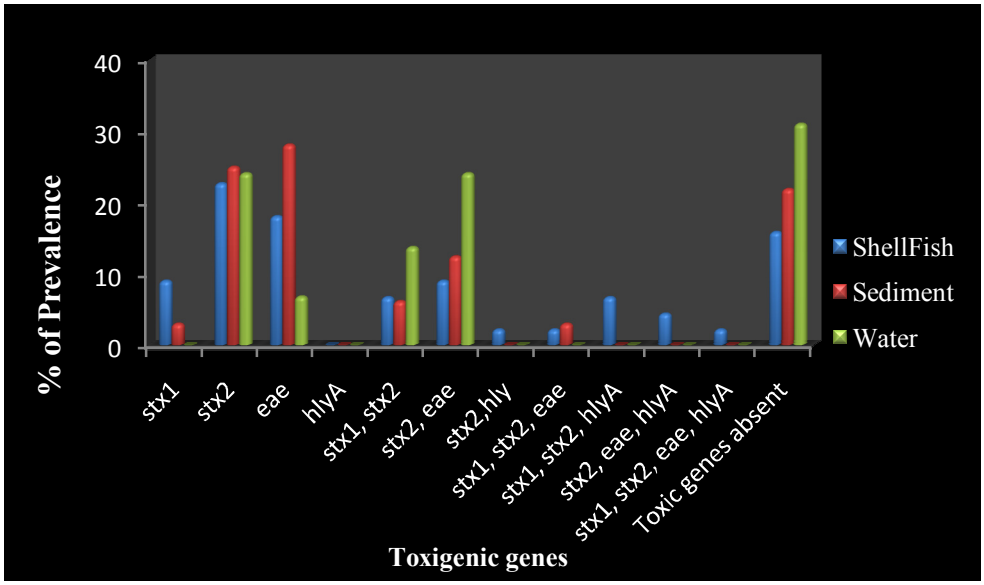

Figure 3.31 Relative prevalence of various toxigenic genotypes in *E. coli* from shellfish, sediment and harvesting waters

Maximum diverse toxigenic genotypes were exhibited by *E. coli* strains from shellfish; followed by sediment and least by harvesting waters. *E. coli* strains from shellfish exhibited 10 different genotypes; whereas those from sediment and harvesting waters exhibited 6 and 4 different genotypes respectively.

3.5.6.3 Co-existence of toxigenic genes in *E. coli*

The extent of co-existence of various toxigenic genes in *E. coli* are summarised in Figures 3.32-3.34. Shellfish samples exhibited the co-occurrence of maximum number of toxigenic genes followed by sediment samples and harvesting waters demonstrated the least. In shellfish samples co-existence of two, three and four genes were observed as shown in Figure 3.32. In sediment samples co-occurrence of only two and three toxigenic genes were observed (Figure 3.33), whereas inharvesting waters co-occurrence of only two genes was observed as shown in Figure 3.34.

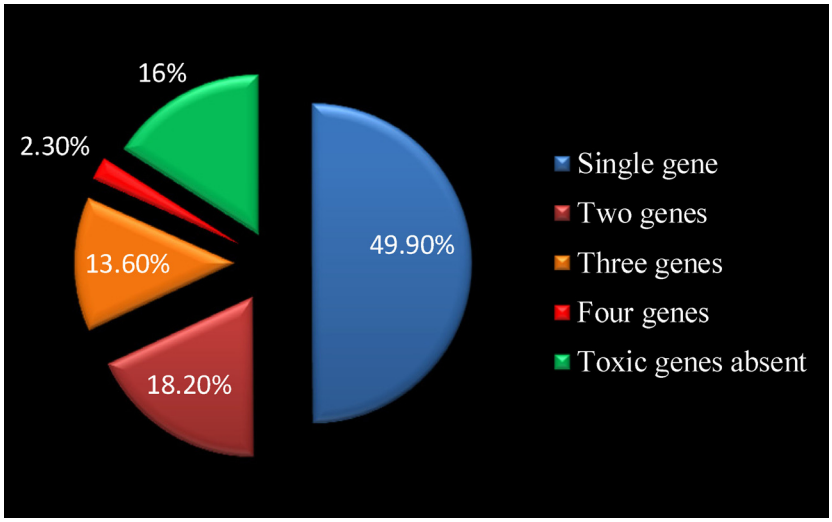

Figure 3.32 Co-existence of toxigenic genes in shellfish samples

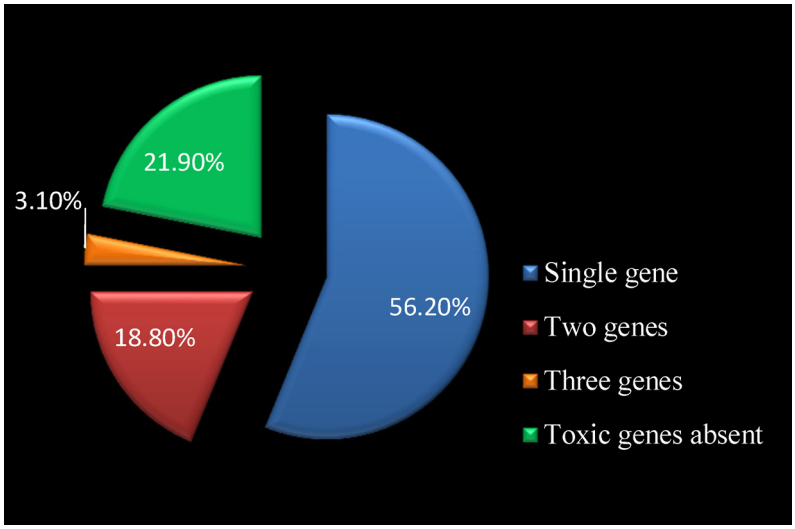

Figure 3.33 Co-existence of toxigenic genes in sediment samples

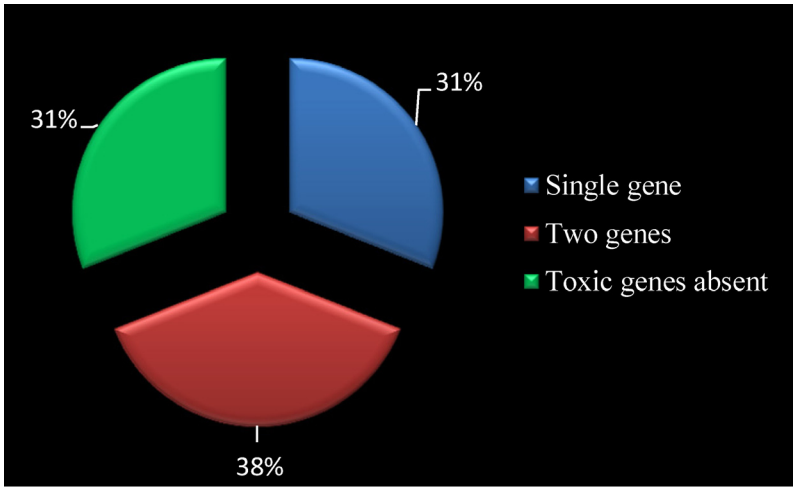

Figure 3.34 Co-existence of toxigenic genes in shellfish harvesting waters

3.5.7 Overall prevalence of virulence genes in *V. parahaemolyticus*

Virulence genes were detected in *V. parahaemolyticus* strains from shellfish, sediment and harvesting water samples from Cochin estuary. Figure 3.35 represents the gel image showing PCR products of virulence genes of *V. parahaemolyticus*.

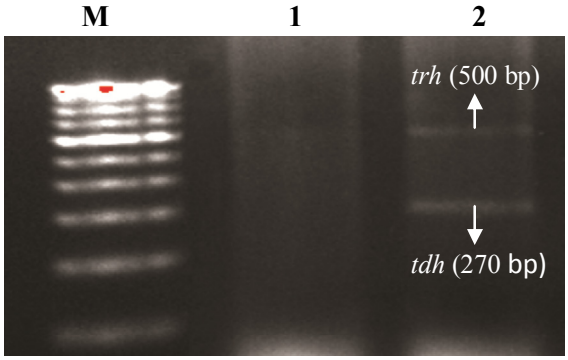

Figure 3.35 Gel image of PCR products with representative isolates carrying virulence genes of *V. parahaemolyticus*
Lane M: 100 bp ladder, lane 1: negative control, lane 2: *trh* and *tdh* genes of *V. parahaemolyticus*

The overall prevalence of virulence genes in *V. parahaemolyticus* is given in Figure 3.36. Virulence genes could be detected in 25.8% of the isolates. *Trh* was found in 19.35% of *V. parahaemolyticus* strains, while 3.20% were positive for *tdh* gene alone. Co-existence of both *trh* and *tdh* genes was found in 3.20% of the total strains.

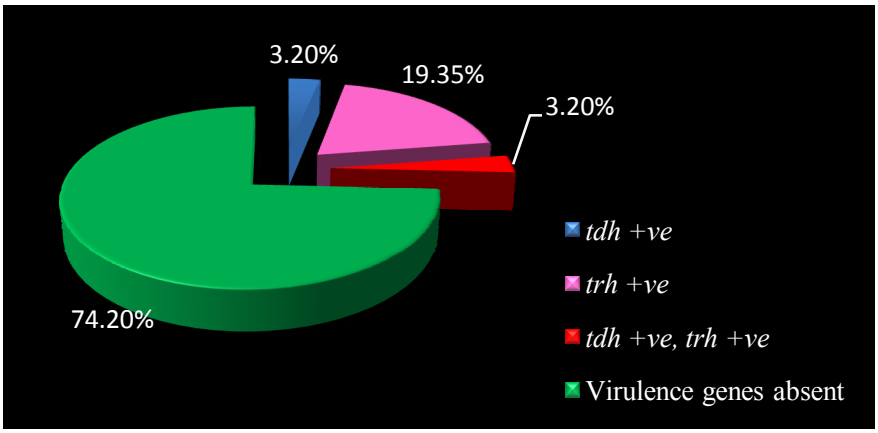

Figure 3.36 Overall prevalence of virulence genes in *V. parahaemolyticus*

3.5.7.1 Relative prevalence of virulence genes in *V. parahaemolyticus* from various sources

The relative prevalence of virulence genes in *V. parahaemolyticus* is shown in Figure 3.37. Sediment samples showed maximum prevalenc of *trh* gene (8 strains), followed by shellfish samples (4 strains). *Tdh* gene could be detected in 2 strains from shellfish. The co-occurrence of *tdh* and *trh* genes was detected in 2 strains from sediment samples. However *V. parahaemolyticus* strains from harvesting waters lacked both *tdh* and *trh* virulence genes.

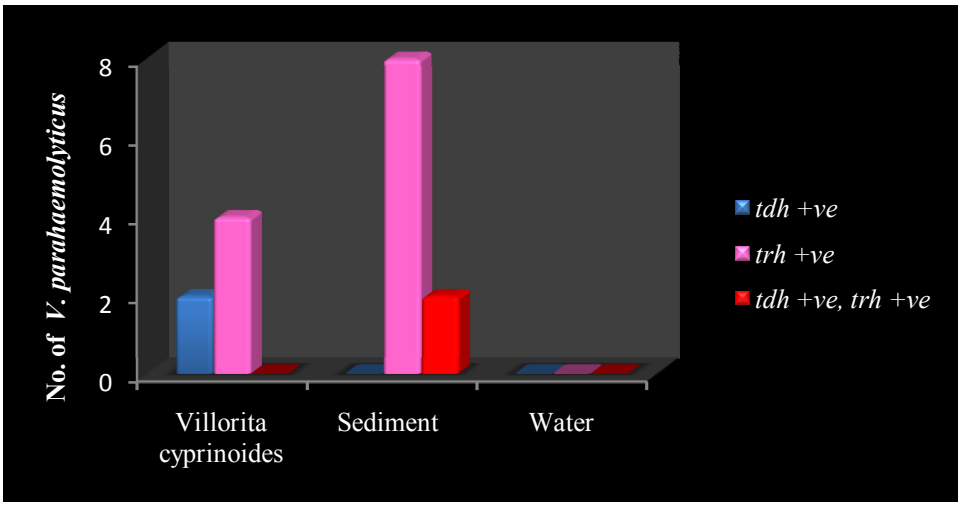

Figure 3.37 Relative prevalence of virulence genes in *V. parahaemolyticus* from shellfish, sediment and harvesting waters

3.6 Discussion

3.6.1 Antibiotic resistance of *E. coli* from shellfish harvesting areas of Cochin estuary

E. coli strains isolated from shellfish harvesting areas of Cochin estuary collectively showed resistance towards all the 19 antibiotics tested, though there was variation in resistance among individual strains towards various

antibiotics. Maximum resistance was shown against cefpodoxime, followed by imipenem and ampicillin. Remarkable levels of resistance were shown towards other antibiotics also, which included the potent carbapenem such as meropenem and the drug colistin usually used as a last line drug of choice.

Prevalence of drug resistant bacteria in Cochin estuary has been previously reported (Sukumaran and Hatha, 2015; Sukumaran *et al*., 2012; Hatha *et al*., 2004). The present study has revealed a drastic increase in the prevalence of multidrug resistant strains from the study area (97.6%) than previous reports (Sukumaran and Hatha, 2015; Sukumaran *et al*., 2012; Chandran *et al*., 2008). Our results were high, compared to those in previous studies, which reported 61.6% and 35% multidrug resistant *E. coli* in several food sources including shellfish (Van *et al*., 2008; Van *et al*., 2007). Sukumaran *et al*. (2012) also reported comparatively lesser prevalence of 53.33% of multidrug resistant *E. coli* strains from this study area.

In our study, greater incidence of MDR was observed among isolates from shellfish than those from sediment. Similarly strains from shellfish exhibited slightly higher MAR indices than those from sediment and harvesting water samples. MAR indices higher than 0.2 are often indicative of contamination from high-risk sources such as hospital waste, commercial poultry farm waste etc. that somehow found their way the estuarine environment (Krumperman, 1983). It was observed that 94.4% of the total strains from shellfish harvesting areas and 98%, 94.7% and 89.1% of strains from shellfish sediment and harvesting waters respectively showed MAR index higher than 0.2, which indicates that these areas are contaminated from high-risk sources.

Bacterial strains from shellfish exhibited more diverse antibiotic resistance patterns than those from sediment and harvesting water samples. These differences can be attributed to the prolonged exposure of the strains in these sedentary fauna, to the environmental pollutants including antibiotic residues accumulated in the less disturbed bottom waters. In present study, a remarkable increase in resistance towards ampicillin (83.2%) was observed compared to previous reports from the study area where relatively lower levels of ampicillin resistances of 65.33% (Sukumaran *et al*., 2012) and 23.07 % (Sukumaran and Hatha, 2015) were reported. In this study the strains showed 60.8% resistance towards nitrofurantoin and 52.8% against doxycycline, commonly prescribed drugs to treat urinary tract infections caused by *E. coli*.

Interestingly, tetracycline resistance followed a declining trend. Previous studies by our research group showed a gradually decreasing trend in tetracycline resistance from Cochin estuary estuary. A progressive decline in tetracycline resistance from 83% (Chandran *et al*., 2008) to 69.6% (Abhirosh *et al*., 2011), was observed in two related studies conducted within an interval of three years. The progressive decline from initial reports may be due to the reduced discharge of tetracycline residues into the estuary from various sources, ultimately reducing the selective pressure in the environment and resistance towards tetracycline. Decreasing tetracycline resistances of 33.3% and 19.23% were reported by two consecutive reports by Sukumaran *et al.* (Sukumaran *et al*., 2015, 2012). However in the present study higher tetracycline resistance of 56.8% was observed. As pointed out by Hsu *et al*. (1992), the pattern of bacterial antibiotic resistance exhibited may reflect the history of antibiotic application and hence the drug resistance pattern observed can be used as an indicator of antibiotic application.

In present study, increased resistance was shown towards streptomycin, gentamicin, chloramphenicol, amikacin ciprofloxacin and trimethoprim were observed, whereas slightly reduced resistance was shown towards cotrimoxazole when compared to the reports of Sukumaran *et al*. (2012) and Sukumaran and Hatha (2015). In contrary, higher streptomycin and amikacin resistances than present study were reported by Chandran *et al*. (2008) and Abhirosh *et al*. (2011) respectively. However the antibiotic resistance observed towards all the classes of antibiotics in our study were found to be higher than several previous reports (Abdel-Rhman *et al*., 2015; Ryu *et al*., 2012; Kumaran *et al*., 2010; Van *et al*., 2007).

Among the three sources under study, the strains from shellfish exhibited increased resistance at varying levels towards 10 antibiotics when compared with that of strains from sediment and harvesting waters. Greater resistance was shown by clam isolates towards amikacin, cephotaxime, cefpodoxime, chloramphenicol, colistin, doxycycline, imipenem, nalidixic acid, nitrofurantoin and tetracycline. The prevalence of multidrug resistant bacteria in seafoods of Tuticorin Coast, Southeastern India has been previously reported (Immaculate *et al*., 2012). Kumar *et al*. (2005) observed comparatively lesser resistance of *E. coli* in tropical seafood to penicillin (42%) and complete sensitivity to chloramphenicol, ciprofloxacin and gentamicin which was not in agreement with our present findings, indicating that bacteria from environmental sources is gradually acquiring resistance to almost all discovered classes of antibiotics. Another serious concern is the higher degree of resistance observed in *E. coli* from shellfish harvesting areas in general, and those from shellfish in particular, against potent third generation cephalosporins such as cephotaxime, carbapenems such as imipenem and

meropenem and lastline drug of choice colistin. Due to lack of previous reports related to resistance against these antibiotics from the study area, a comparison could not be accomplished.

3.6.2 Antibiotic resistance of enterococci isolated from shellfish harvesting areas of Cochin estuary

Present study reveals extremely high levels of incidence of MDR enterococci in shellfish harvesting areas of Cochin estuary. Even though most enterococcal species are part of the normal gut microbiota of both humans and animals, some species are opportunistic human pathogens and have been identified as emerging pathogens responsible for many community-acquired and hospital-acquired (nosocomial) super infections such as endocarditis (Munita *et al*., 2013), bacteraemia etc. (Moellering, 1992; Murray, 1990). Because of their inherent as well as acquired resistance to a number of antibiotics, they pose potential therapeutic challenge (Arvanitidou *et al*., 2001). All the enterococcal strains isolated from shellfish growing areas of Cochin estuary were multidrug resistant (100% MDR), which is very high compared to the incidence of drug resistant enterococci (64.7%) from shellfish growing areas in Mediterranean coast of Morocco as reported by Bennani *et al*. (2012).

In our study an exceptionally high level resistance (100%) was observed against ampicillin, among enterococci isolated from shellfish, sediment and harvesting waters from shellfish harvesting areas of Cochin estuary. Resistance greater than 90% was observed against cephalothin ciprofloxacin, penicillin and trimethoprim. Among the 18 various antibiotics tested, resistance against vancomycin teicoplanin, rifampicin and linazolid needs special mention, as these are antibiotics targeted specially against MDR enterococci. Similar studies have reported resistance against antibiotics such as erythromycin,

rifampicin, (Chajecka-Wierzchowska *et al*., 2016; Moore *et al*., 2008); however the frequencies of resistance against most of the antibiotics encountered in our study were found to be exceptionally high. Of particular concern is the intrinsic antibiotic resistance demonstrated against aminoglycosides and cephalosporins (Tendolkar *et al*., 2003; Morrison *et al*., 1997). The emergence of vancomycin resistance enterococci (VRE), particularly *E. faecium* strains is a growing challenge in chemotherapy as it leaves the clinicians treating VRE infections with limited therapeutic options has seriously affected the treatment and infection control of these organisms (Teixeira *et al*., 2011; De Perio, 2006).

Resistance, though in lesser degrees was also exhibited by enterococci against high level streptomycin and high level gentamicin, which is very rare. This is in agreement with the results of similar previous studies (Bennani *et al*., 2012; Moore *et al*., 2008). The degree of general antibiotic resistance was found to be extremely high to the extent that, two strains isolated from sediments were resistant towards all the 18 antibiotics with MAR index as high as 1. Enterococci isolated from sediments showed maximum resistance against 12 out of 18 antibiotics tested which included even teicoplanin, HLS, HLG, linazolid and doxycycline etc. This is in agreement with the study of Bennani *et al*. (2012), where highest antibiotic resistance was reported in enterococci isolated from sediment, compared to those from shellfish and seawater. In this study, enterococcal strains from harvesting waters showed maximum resistance against 7 antibiotics including vancomycin and cotrimoxazole while Bennani *et al*. (2012) reported highest frequencies of resistance against rifampicin among isolates from seawater. In current study, enterococci from shellfish showed maximum resistance against rifampicin and tetracycline while similar studies from Mediterranean coast of Morocco

(Bennani *et al*., 2012) reported highest frequencies against erythromycin among enterococci from shellfish and sediment. Present study revealed exceptionally higher resistances against teicoplanin (52.3%) and vancomycin (77.3%) respectively, which is very high compared to that reported by Bennani *et al*. (2012) (2.3%). Exceptionally high level of resistance observed against ampicillin, among enterococci isolated from shellfish, sediment and harvesting waters from shellfish harvesting areas of Cochin estuary is quite alarming, since possibility exists for the transfer of resistance to other pathogenic and non pathogenic microorganisms in the marine environment (Blom *et al*., 2000).

In addition, enterococci isolated during present study have exhibited co-resistance to beta lactams, chloramphenicol, tetracyclines, rifampin, fluoroquinolones, aminoglycosides (high levels) and glycopeptides such as vancomycin and teicoplanin. This is a matter of great concern, as enterococci has emerged as a potent pathogen especially in nosocomial and iatrogenic infections and their resistance to a plethora of antimicrobial agents is a major hurdle in their therapy (Teixeira *et al*., 2011; Courvalin, 2006).

3.6.3 Antibiotic resistance among *V. parahaemolyticus* from shellfish harvesting areas of Cochin estuary

V. parahaemolyticus isolated from shellfish harvesting areas of Cochin estuary showed maximum resistance against ampicillin and cefpodoxime (93.5% each). This is in agreement with several previous reports where, ampicillin resistance have been widely reported in *V. parahaemolyticus* from a number of sources such as coastal waters (Zanetti *et al*., 2001), wastewater (Okoh and Igbinosa, 2010), fish farms (Labella *et al*., 2013), shrimp farms (Reboucas *et al*., 2011; Tendencia and De la Pena, 2001) and shellfish samples (Ottaviani *et al*., 2013). Eighty seven percentage of

V. parahaemolyticus isolated from shellfish harvesting areas of Cochin estuary was found to be multiple drug resistant which is very high compared to the findings of Manjusha *et al*. (2005) where 54% multiple drug resistance was reported from the study area. *V. parahaemolyticus* isolated from shellfish during present study showed 100% resistance against ampicillin. This is in agreement with that of Silvester *et al*. (2015) and Sudha *et al*. (2014), where similar higher degrees of ampicillin resistance was reported from *V. parahaemolyticus* isolated from the same study area. Sudha *et al*. (2014) reported total sensitivity against antibiotics such as ciprofloxacin, nalidixic acid, streptomycin and tetracycline among *V. parahaemolyticus* isolated from shellfish collected from markets of the study area. However, in our study enhanced resistances against the aforesaid antibiotics were observed. Complete sensitivity was observed against chloramphenicol, which is in agreement with the report of Sudha *et al*. (2014). Majority of the antibiotics classes such as penicillins, cephalosporins, aminoglycosides against which maximum resistance was exhibited are not commonly used in aquaculture practices in India (Manjusha *et al*., 2005). Hence it can be presumed that, anthropogenic factors such as hospital effluents might have been responsible for acquiring drug resistance of *Vibrio*s from shellfish harvesting areas of Cochin estuary. In contrary, all the strains isolated from shellfish harvesting areas of Cochin estuary were maximum sensitive towards tetracyclines and quinolones/ fluoroquinolones which are commonly used in aquaculture farms in India.

3.6.4 Prevalence of antibiotic resistance genes

3.6.4.1 Antibiotic resistance genes in *E. coli*

Bla$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ genes were detected in majority of the *E. coli* strains which showed complete absence of inhibition zone for beta lactam

antibiotics. The presence of extended spectrum beta lactamase (ESBL) genes in sediment, harvesting water as well as shellfish of this estuarine habitat is of great public health concern, as there are possibilities for wide spread of beta lactam and cephalosporin resistance even in food through horizontal transmission (Moremi *et al*., 2016; Abgottspon *et al*., 2014; Tham *et al*., 2010). Similar findings have been made by researchers earlier, where probable horizontal transfer of resistance genes from either human sewage or clinical sources to fish in rivers or lakes in which drainage of wastewater from treatment plants occured has been suggested (Abgottspon *et al*., 2014; Blaak *et al*., 2014; Jiang *et al*., 2012).

Out of the total 36 resistant *E. coli* strains selected for study, 75% exhibited presence of both $bla_{\text{CTX-M}}$ gene and bla_{TEM} genes. $Bla_{\text{CTX-M}}$ gene and bla_{TEM} genes were detected in 91.7% and 77.7% of the strains respectively. Our results are in agreement with that of Brandao *et al*. (2017) who reported high prevalence of bla_{TEM} gene (91.3%) in oysters from urban estuaries. However, Ryu *et al.* (2012) reported lesser prevalence (21.4%) of bla_{TEM} gene compared to our results. It has been reported that, although TEM and SHV variants were the most predominant ESBLs produced by enterobacteriaceae, recently CTX-M has replaced both TEM and SHV as the most common types of ESBLs (Philippon *et al*., 2016). This is in agreement with many previous reports regarding transmission of ESBL producing enterobacteriaceae from farm animals to humans especially through food chain (Lazarus *et al*., 2015; Valentin *et al*., 2014; Kluytmans *et al*., 2013; Leverstein-van Hall *et al*., 2011; Overdevest *et al*., 2011). The prevalence of both genes was highest in strains from shellfish, followed by sediment and the least prevalence was recorded in harvesting waters.

All the identified serogroups which showed the presence of ESBL genes were pathogenic. Distribution of both $bla_{\text{CTX-M}}$ and bla_{TEM} genes among various pathogenic serogroups also followed a similar pattern. Maximum prevalence of both genes was recorded in EHEC, followed by ETEC, UPEC and EPEC serogroups. The present study confirms the increased prevalence of ESBL genes in food animals, which inturn confirms the apprehensions of the Panel on Biologic Hazards of the European Food Safety Authority (EFSA, 2011) which concluded that a risk exists for transmission of extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae from farm animals, especially from poultry, to humans through the food chain (Kluytmans *et al*., 2013; Overdevest *et al*., 2011).

Spatial variation was seen in the presence of ESBL genes in *E. coli* strains isolated. The incidence of both $bla_{\text{CTX-M}}$ and bla_{TEM} genes were highest in *E. coli* isolated from station 4, followed by stations 5, 6, 1 and the least was reported at station 8. Strains from stations 2 and 7 lacked ESBL genes. There are no previous reports regarding prevalence of ESBL genes in Cochin estuary and hence a comparison was not possible.

Another significant finding was the detection of plasmid encoded colistin resistance *mcr-1*gene in both shellfish and sediment samples from station 7 and shellfish sample from station 5. This is indeed a matter of serious concern, as colistin is often opted as a last resort drug to treat carbapenemase resistant bacteria and presence of this plasmid encoded *mcr-1*gene may be responsible for the horizontal transfer of colistin resistance to other pathogenic and non-pathogenic bacteria present in the study area.

Ever since its first report from China in 2015 (Liu *et al*., 2016b), several recent studies from various parts of the world have reported the

isolation of *mcr-1* gene from various food animals such as swine (Falgenhauer *et al*., 2016; Suzuki *et al*., 2016), chicken (Petrillo *et al*., 2016; Perrin-Guyomard *et al*., 2016; Shen *et al*., 2016), turkey (Battisti, 2016; Perrin-Guyomard *et al*., 2016), beef (Mulvey *et al*., 2016), pork meat and sausage (Webb *et al*., 2016; Liu *et al*., 2016b); vegetables (Zurfuh *et al*., 2016); environmental sources such as river water (Zurfuh *et al*., 2016), water (Petrillo *et al*., 2016) etc. The first report of *mcr-1* gene in India was from a urine sample from an inpatient at a university hospital (Kumar *et al*., 2016b). However, since there are no published reports on prevalence of *mcr-1* gene from shellfish growing areas in general or from this study area in particular, a comparison was not possible.

3.6.4.2 Antibiotic resistance genes in enterococci from shellfish harvesting areas of Cochin estuary

Glycopeptide resistance gene *VanA* gene could be detected in one *E. faecalis strain* isolated from sediment from station 1 and *VanB* gene was detected in one *E. faecium* strain isolated from shellfish from station 6. This is in agreement with the result of Goksel *et al*. (2016) where *VanA* gene could be detected in enterococci from a mastitic milk sample. There are similar reports of presence of *VanA* gene in enterococci in clinical specimens from a tertiary hospital in India; however the incidence was comparatively higher than present study (Gangurde *et al*., 2014). The presence of glycopeptide resistance genes confers higher levels of resistance to antibiotics such as vancomycin and teicoplanin used to treat multidrug resistant enterococci.

Several mechanisms for the transfer of resistance genes such as conjugative plasmids, transposons or via bacteriophages in enterococci have been reviewed (Mazaheri *et al*., 2011; Vinodkumar *et al*., 2011). Since

enterococci reside in the gastrointestinal tract they come into contact with both other Gram-negative and Gram-positive organisms and exchange of resistance genes to other bacterial genera may occur (Sung and Lindsay, 2007; Clewell, 1990). Among the glycopeptides resistance genes, *VanA* and *VanB* genes especially are transferable via plasmids or transposons (Woodford *et al*., 1995) and transfer of VanA phenotype from enterococci to virulent pathogen like methicillin-resistant *Staphylococcus aureus* (MRSA) has been demonstrated (Noble *et al*., 1992).

3.6.5 Toxigenic genes in *E. coli* from shellfish harvesting areas of Cochin estuary

High prevalence of shiga toxin genes (stx_1, stx_2), intimin (*eae*) gene and enterohemolysin gene (*hlyA)* from shellfish harvesting areas of Cochin estuary indicates the potential public health hazard involved in consumption of shellfish harvested from Cochin estuary. This is in agreement with the results of Baliere *et al*. (2015), where stx_1, stx_2, and *eae* have been reported from shellfish harvesting areas of French coastal areas. Similarly, prevalence of stx_1 and stx_2 genes in *E. coli* from shellfish from Mediterranean coast of Morocco has been reported by Bennani *et al*. (2011). In present study, the prevalence of both stx_2 and stx_1 genes were higher; however, the prevalence of stx_2 gene (54.3%) was found to be higher than that of stx_1 gene (20%). This is contradictory to the findings of Bennani *et al*. (2011), where lesser prevalence of both genes were reported and the incidence of stx_1 gene was more than that of stx_2 gene. Similarly Brandao *et al*. (2017) also reported lesser prevalence of *eaeA* and *stxA2* virulence genes (16.7%) in *E. coli* strains isolated from oysters harvested from urban estuaries. Majority of the *E. coli* strains (78.1%) isolated from shellfish harvesting areas of Cochin estuary during present study, harboured one or more number of toxigenic

genes whereas significantly lesser incidence of shiga toxin genes (54.1%) were reported from French coastal areas by Baliere *et al*. (2015). Contradictory to our findings several researchers have reported absence of stx_1 *and* stx_2 genes in meat products (Abdel-Rhman *et al*., 2015).

In present study, high prevalence of *eae* gene (36.2%) was reported which probably indicates the increased virulence of these STEC strains in humans (Oswald *et al*., 2000; Barrett *et al*., 1992). Our result was high compared to the report of (Abdel-Rhman *et al*., 2015) where 32% incidence of *eae* gene was reported in meat products. *Eae* gene encodes an outer membrane protein (intimin) required for intimate attachment to intestinal epithelial cells (Jerse *et al*., 1990). It is also a diagnostic marker of a subset of highly virulent STEC for humans termed *LEE* (locus for enterocyte effacement) positive (Paton and Paton, 1998a; Gannon *et al*., 1993). The pathogenicity island termed *LEE,* encodes and renders the capacity to produce attaching and effacing lesions on intestinal mucosa. Thus e*ae* gene is considered as a potential virulence factor for humans, as it is required for the expression of full virulence of STEC (Eklund *et al*., 2002; Schmidt *et al*., 1994). Contradictory to our findings, many researchers have reported either absence or lesser prevalence of *eae* gene, which indicate their lack of virulence in humans (Cortes *et al*., 2005; Orden *et al*., 2003).

Another interesting finding was that, the incidence of shiga toxin genes was more in shellfish samples, followed by sediment and least was detected in harvesting water. Greater number of toxigenic genotypes was identified in *E. coli* strains from shellfish followed by sediment and harvesting waters. Similarly, shellfish samples exhibited the co-occurrence of maximum number of toxigenic genes, followed by sediment samples,

while harvesting waters demonstrated the least, which is summarised in Figure 3.29. Co-existence of all the four toxic genes *stx*$_1$, *stx*$_2$, *eae* and *hlyA* genes was exhibited by 2.3% of the *E. coli* strains from shellfish, which inturn reveals the safety hazard associated with the consumption of shellfish harvested from the study area.

3.6.6 Virulence genes in *V. parahaemolyticus* from shellfish harvesting areas of Cochin estuary

Virulence genes *tdh* and *trh* could be detected in *V. parahaemolyticus* strains isolated from both shellfish and sediment samples collected from shellfish harvesting areas of Cochin estuary. Two strains from sediments showed the co-existence of both *tdh* and *trh* genes. However, the percentage of prevalence was less; 22.5% of *V. parahaemolyticus* strains was positive for *trh* genes, while only 6.4% was positive for *tdh* genes. Many researchers have the opinion that environmental strains have low frequencies of virulence genes and hence are not pathogenic to humans. Our results are in agreement with previous findings where, low frequencies of *tdh* and *trh* genes were reported from most environmental *V. parahaemolyticus* strains (Ceccarelli *et al*., 2013; Vieira *et al*., 2011; Nishibuchi and Kaper, 1995). However, our results were considerably higher than that of Yu *et al.* (2013), where only 2.5% of the 1076 *V. parahaemolyticus* strains isolated from shellfish growing areas of Taiwan, exhibited the presence of virulence genes.

However, there are contradictory previous reports regarding higher frequencies of detection of virulence genes from environmental strains. In present study, primers by Bej *et al*. (1999) was used and the possible low recovery of virulence genes by Bej *et al*. (1999) is also doubted as a possible reason by some researchers (Gutierrez West *et al*., 2013) for the lesser

reporting of virulence genes. Gutierrez West *et al*. (2013), reported that usage of their new PCR primers has drastically improved amplification of *tdh* and *trh* genes in environmental *V. parahaemolyticus* strains. Klein and Lovell (2017) detected virulence genes in 50% environmental *V. parahaemolyticus* strains isolated from oysters. Velazquez-Roman *et al*. (2012) detected *tdh* and or *trh* in 52% of *V. parahaemolyticus* strains isolated from a heavily impacted area of shrimp mariculture.

The relative prevalence of *trh* genes was found to be more than that of *tdh* gene in this study. This is in agreement with the findings of Nakaguchi (2013), where higher comparative prevalence of *trh* gene than *tdh* gene was reported in molluscan shellfish from Malaysia and Indonesia. Baker-Austin *et al*. (2008) has also made similar observations in environmental strains from highly populated areas of South Carolina and Georgia coasts. Highest prevalence of *trh* gene was detected in *V. parahaemolyticus* strains from sediment samples, than those from shellfish samples in present study.

Even though it is widely accepted that, virulence genes are associated with pathogenic *V. parahaemolyticus*, nontoxigenic *V. parahaemolyticus* strains causing acute gastroenteritis have been reported (Ottaviani *et al*., 2012). Absence of the aforesaid virulence factors from some clinical strains (Ronholm *et al*., 2015; Ottaviani *et al*., 2012), as well as their occurence in numerous environmental isolates that are apparently not acquired from infected humans (Gutierrez West *et al*., 2013; West *et al*., 2013), suggests the possibility that *tdh* and *trh* may serve some environmental function and has nothing to do with human disease (Raghunath, 2015; Gutierrez West *et al*., 2013), though these functions remain unknown. Another possibility is that, some other virulence factors which are yet to be identified may be

associated with disease outbreaks. Virulent genes study by Vongxay *et al*. (2008), suggested that hemolysins *tdh* and/or *trh* may not be necessarily the only virulence factors of pathogenic *V. parahaemolyticus* isolates.

The detection of virulence factors in *V. parahaemolyticus* strains from sediment and shellfish samples of Cochin estuary highlights the possible safety hazard associated with consumption of shellfish harvested from the study area. The reason is shellfish tend to accumulate *V. parahaemolyticus* in their tissues and it has been reported by many researchers that their numbers sometimes even exceeds that required for an infectious dose (Klein and Lovell, 2017), which can probably result in a disease outbreak if the strains are potentially virulent.

In summary, our results confirm that the shellfish harvesting areas along Cochin estuary act as a significant environmental reservoir of antibiotic resistant bacteria, which harboured a pool of antibiotic resistance genes, ready to be disseminated to other human pathogens or environmental organisms. In addition, they also have been found to harbour several toxigenic/virulence genes; most of which are located on mobile genetic elements which could also be horizontally transmitted. It may thus serve as a permanent reservoir of antibiotic resistant genes, resulting in antimicrobial resistance, cycling through environment, food and human sources. Another disturbing fact is that the prevalence of drug resistance as well as resistance genes was highest among bactrerial strains from shellfish samples when compared to those from sediment and harvesting water samples. Same is the case of toxigenic and other virulence genes also, where highest incidence was observed in strains from shellfish samples compared to those from sediments and water samples. Present study reveals the poor sanitary quality

of the shellfish harvesting areas along Cochin estuary and the probable health hazards associated with consumption of shellfish harvested from this estuary. Hence, the necessity of regulatory interventions in improving the sanitary quality of the shellfish harvesting areas such as monitoring and certification is strongly recommended, for which the present study may form a baseline reference.

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Chapter 4

SURVIVAL KINETICS OF INDICATOR AND PATHOGENIC BACTERIA IN ESTUARINE WATER AND SEDIMENT MICROCOSMS

Contents

4.1 Introduction

Sanitary water quality attracts worldwide attention due its substantial role in fish and shellfish consumption associated disease outbreaks, recreation disruptions and contamination of crops eaten raw (Painter *et al*., 2013; Rippey, 1994). Faecal contamination is the main route of entry for enteric pathogens into water bodies. Often the magnitude of these faecal polluted urban discharges exceeds the self-purifying limits of the receiving water bodies. This results in an alarming increase in the number of faecal indicator organisms as well as pathogens in the receiving waters, which becomes unfit for all the aforesaid beneficial purposes.

Contamination of shellfish growing areas in particular is a matter of great concern since shellfish is a delicacy preferred to be eaten either raw or partially cooked in many parts of the world. Being filter feeders, they filter

large quantities of water and during this process tend to accumulate all the pollutants present in the environment, including microbial pathogens in their tissues (Prato *et al*., 2013; Lalitha and Surendran, 2005). In developed countries, continuous monitoring of the harvesting areas is mandatory; noncompliance of which invites stringent measures like closure of the harvesting areas (NSSP, 2015). Cochin estuary, due to its proximity to the town ship, is highly polluted which may adversely affect the sanitary quality of the fish and shellfish resources harvested from it. In this context, the survival of faecal coliforms and pathogenic bacteria in aquatic environments is a potential topic of extensive research. Prolonged survival of faecal indicator as well as pathogenic bacteria has been demonstrated in aquatic sediments (Chandran *et al*., 2011; An *et al*., 2002; Alkan *et al*., 1995), resuspension of which due to natural turbulence or anthropogenic activities like boating and dredging will make it a permanent source of contamination.

Natural environments are dynamic systems influenced by an array of variables, and hence the survival of indicator as well as pathogenic bacteria is largely affected by the conditions that prevail in such environments. Survival of these organisms is largely influenced by a number of physical, chemical, and biological factors such as solar radiation (Sinton *et al*., 2002; Davies and Evison, 1991), temperature (Zhang *et al*., 2014; Abhirosh *et al*., 2009; Anderson *et al*., 1983), salinity), nutrients (Chandran *et al*., 2011; An *et al*., 2002), bacteriophages (Letchumanan *et al*., 2016; Ricca and Cooney, 1999), and protozoan predation (Korajkic *et al*., 2013; Hahn and Hofle, 2001; Rhodes and Kator, 1990) etc. In addition to these aforementioned factors, several researchers have also acknowledged the role of turbidity, heavy metals, adsorption and sedimentation processes (Auer and Niehaus,

1993), competition with autochthonous microbiota (McCambridge and McKeekin, 1981; Enzinger and Cooper, 1976) and antibiosis (Colwell, 1978) in survival of allochthonous microorganisms in the natural waters.

4.2 Review of literature

The factors controlling the survival of enteric pathogens as well as indicators in aquatic environments have been explored by a number of researchers. From the extensive literature available on the survival studies, it is apparent that bacterial survival in the natural environment is contingent on a variety of physical, chemical, and biological parameters. While extensive literature is available on survival studies of *E. coli*, the same is not true for *V. parahaemolyticus* and enterococci. A wide range of survival times have been reported for bacteria outside the human host, in the natural environments, which can be attributed to the impact of the afore-said factors and also the probable variation in the analytical methodology and bacterial strains used during experiments.

4.2.1 Effect of temperature on survival

The optimum temperature required for growth of enteric bacteria is found to be 37 °C. However bacterial survival has been found to occur at wide temperature ranges as it exists in natural environments (Rozen and Belkin, 2001; Delille and Delille, 2000). Some researchers have reported that lower temperatures favour increased growth of bacteria in fresh and marine waters (An *et al*., 2002; Rozen and Belkin, 2001; Auer and Niehaus, 1993) whereas, some others have reported contradictory findings that higher temperatures favour increase in bacterial survival (Crabill *et al*., 1999; Buckhouse and Gifford, 1976). The impact of temperature on faecal coliform

(FC) survival has been analysed by a number of researchers (Nasser and Oman, 1999; Dan *et al*., 1997; Alkan *et al*., 1995). Alkan *et al*. (1995) noted no significant impact on FC survival, when the temperature increased from 10 to 30 °C and it was also found that when both temperature and light were present, the impact of light outweighed that of temperature.

A proportional increase in FC mortality has been reported with increasing temperature (An *et al*., 2002; Auer and Niehaus, 1993). A decreasing exponential relationship was found to exist between seawater temperature and FC levels. The time required for 90% reduction in bacteria (T90) had decreased by approximately 55% for each 10 °C increase in water temperature (Solic *et al*., 1992). Lower *E. coli* densities were observed during summer season, probably due to lower loading and warmer temperatures. Higher temperatures are in turn found to promote protozoan predation which also synergistically impacted bacterial mortality (Mezrioui *et al*., 1995; Barcina *et al*., 1997; Sherr *et al*., 1988; Anderson *et al*., 1983). Higher densities of *E. coli* are reported in bottom waters, may be due to their adsorption onto colloidal size particles, and also cooler water temperatures which favours growth (Delille and Delille, 2000). *E. coli* exhibit differential survival rates in different water sources; faster decay rates in marine environments than that in lakes (Pachepsky *et al*., 2014; Blaustein *et al*., 2013)

Similar to *E. coli*, several studies have been conducted to determine the survival of *Vibrio* species at various temperatures (Gooch *et al*., 2002; Muntada-Garriga *et al*., 1995). The incidence of *V. parahaemolyticus* and other *vibrios* correlated well with the temperature of water. *V. parahaemolyticus* has optimum growth temperatures ranging from 35 °C to 39 °C (Igbinosa *et al*., 2008; Miles *et al*., 1997; Jackson, 1974). *V. parahaemolyticus* did not survive

well during frozen treatment or low temperature storage (<5 °C) and heating treatment at 60 °C is found to inactivate *V. parahaemolyticus* (Zhang *et al*., 2014). Several studies have been carried out to determine the effect of temperature on survival of *V. parahaemolyticus* in various media such as raw oyster meat, raw oyster meat slurry and broth (Kim *et al*., 2012; Fernandez-Piquer *et al*., 2011); however, relatively only a few studies have been conducted in laboratory microcosms (Abhirosh *et al*., 2009; Hood and Ness, 1982). During winter months, the isolation of *V. parahaemolyticus* was less, but improved during the summer. In winter, when the water temperature is very low, the bacterium survives in sediments and in summer it increases to detectable levels in the water. When the temperatures increased during summer to favourable levels, the organisms in the sediments are re-suspended and could be detected in the water (Kaneko and Colwell, 1973).

The incidence of high *V. parahaemolyticus* densities in oysters also has been found to be influenced by environmental factors such as temperature, a positive correlation has been found between the organism's survival and temperature (Julie *et al*., 2010; Parveen *et al*., 2008; Duan and Su, 2005). Temperature has been found to affect the survival of *V. parahaemolyticus* more than starvation. Starvation often rendered the cells viable but non-culturable. Variant strains demonstrated differential starvation response (Liu *et al*., 2016a); Kanagawa negative strains lost culturability faster than Kanagawa positive strains (Jiang and Chai, 1996). Restoration of favourable temperatures helped the cultures to regain their culturability. Microcosm studies to evaluate the impact of various factors on *V. parahaemolyticus* survival are comparatively few. Abhirosh *et al*. (2009) reported greater reduction of *V. parahaemolyticus* cell numbers at 30 °C than at 20 °C in both

cycloheximide treated and untreated water. At higher temperatures such as 30 °C a possible reason for greater reduction in count is the increased protozoan predation. Many researchers have thus concluded that temperature had comparatively more influence than salinity on survival rates and the response varied with strains, sources and genotypes (Liu *et al*., 2016a; Jiang and Chai, 1996).

4.2.2 Effect of salinity on survival

Salinity has been found to be an important factor influencing the survival of bacteria in marine and estuarine environments (Davies *et al*., 1995; Auer and Niehaus, 1993). Saline conditions impact the survival of bacteria by posing unwanted stress on cells. Enzinger and Cooper (1976) reported bactericidal effect of marine water on non-marine bacteria.

The inverse relationship between osmotic stress and survival of FIB has been well documented by many researchers (Sinton *et al*., 2002; Munro *et al*., 1994; Gauthier *et al*., 1992). Rapid death rate of coliforms has been reported in marine environments whereas enterococci are believed to survive well in marine waters (Anderson *et al*., 2005). Anderson *et al*. (1979) reported that due to the stress caused by salinity, cells become nonviable at higher salinities within the first two days itself, whereas slower death rates were reported at lesser salinities. Many researchers observed FIB (e.g. Enterococci and *E. coli*) survived better in fresh water environments than marine environments (Byappanahalli *et al*., 2012; Anderson *et al*., 2005; Davies *et al*., 1995). Anderson *et al*. (2005) found that the decay of enterococci was two times higher in marine mesocosms than in fresh water mesocosms in both water and sediment. Pre-incubation of FIB with sea water prior to incubation in seawater was shown to reduce the negative

effects of osmotic stress (Troussellier *et al*., 1998; Munro *et al*., 1994; Gauthier *et al*., 1992). Gradual introduction of *E. coli* into brackish water was found to enhance its survival, since it helped better acclimatisation to such environments. This was probably because of the expression of stress response genes initiated by the sigma factor rpoS, which facilitated a higher tolerance for stressful conditions (Troussellier *et al*., 1998).

The decay rate of the enterococci was higher in fresh water than in marine water whereas *E. coli* exhibited lower decay rate in fresh water compared to marine water (Ahmad *et al*., 2014). The ability for enterococccci to grow in higher salinities (6.5%) probably explains their better performance as indicators in marine environments compared to faecal coliforms and *E. coli* (Wade *et al*., 2003). Korajkic *et al*. (2013a) reported differential survival rates of FIB from different sources. Using microcosms they studied extended survival of enterococci and *E. coli* from cattle manure, in freshwater and in marine waters respectively, when compared to those from sewage sources.

On the contrary, some other researchers have reported the reduced impact of salinity on bacterial survival when compared with other factors (Liu *et al*., 2016; Delille and Delille, 2000; Fujioka *et al*., 1981). Fujioka *et al*. (1981) observed that sunlight and not salinity or other factors impacted FC survival in marine environments. Abhirosh and Hatha (2005) have also reported remarkable inactivation of *E. coli* and *S. typhimurium* in estuarine water in presence of sunlight. Liu *et al*. (2016) observed that compared to temperature effects, the impact of salinity was found to be less. Varying growth rates were observed for strains from different sources and with different genotypes. The strains isolated from fresh water showed more conspicuous

growth variations than those from sea water. There are contradictory reports stating inverse relationship between *Vibrio* survival and salinity, where reduced survival was observed on increasing the salinity (McCarthy, 1996).

4.2.3 Role of biotic factors on survival

Abiotic and biotic factors are found to influence bacterial survival; yet there are many recent reports which state that natural microbiota (predation and competition) plays significant role in survival of FIB in natural environments (Korajkic *et al*., 2013a; Staley *et al*., 2011). Diverse groups of the food web which includes protozoa, lytic phages and predatory bacteria have been found to regulate bacterial concentrations in marine and fresh environments (Barcina *et al*., 1997).

4.2.3.1 Effect of protozoan bacteriovory on survival

Protozoan grazing accounts for the majority of the bacterial mortality in aquatic habitats (Jousset, 2012; Pernthaler, 2005; Anderson *et al*., 2005; An *et al*., 2002; Davies *et al*., 1995). Bacterivorous protozoa accounts for up to 90 % of bacterial mortality (Menon *et al*., 2003). Among the protozoan grazers, major contribution is by nanoflagellates and ciliates (Pernthaler, 2005), and by amoebas in some other environments such as soils (Rodríguez-Zaragoza, 1994). Enzinger and Cooper (1976) reported dramatic decrease in *E. coli* concentrations, with a simultaneous increase in the protozoan population. However, it was noted that the combined impact of solar radiation and predators on *E. coli* mortality was greater than the impact of each factor alone (McCambridge and McMeekin, 1981). The grazing rates of protozoa are found to depend on several factors including prey density, prey characteristics and temperature (Iriberri *et al*., 1994; Gonzalez *et al*., 1990).

A direct positive correlation between temperature and predation rates has been proposed by many researchers (Barcina *et al*., 1991; Sherr *et al*., 1988; Anderson *et al*., 1983; McCambridge and McMeekin, 1980). Mezrioui *et al*. (1995) proved that the low T_{90} values for *E. coli* in summer compared to winter might be due to increased zooplankton activity in summer. Sherr *et al*. (1988) reported a linear increase in grazing rates between 12 ºC and 22 ºC, after which they levelled off when a balance between uptake by protozoa and growth by the bacteria was attained. Anderson *et al*. (1983) reported an increase in *E. coli* decline with increase in eukaryotic grazing and temperature. Some authors reported enhanced ingestion rates for Gram-negative bacteria than Gram-positive prey (Iriberri *et al*., 1994; Gonzalez *et al*., 1990). Higher elimination rates reported for *E. coli* compared to *E. faecalis* and *Staphylococcus epidermis*, attributed to differences in cell morphology of the bacterial prey (Iriberri *et al*., 1994). Thus, the extended survival periods of bacteria in autoclaved sea water compared to raw water, can be attributed to inactivation of the biotic factors that inhibit their survival (McCarthy, 1996).

4.2.3.2 Effect of competing autochthonous bacteria on survival

Many researchers have reported the role of competing autochthonous bacteria on bacterial survival (Staley *et al*., 2011; Korajkic, 2013a; McCambridge and McMeekin, 1981). The poor capacity of Enterobacteriaceae to outgrow the competitors in seawater has been reported by Jannasch (1968). Long *et al*. (2001) reported more than one-half of the isolates from marine pelagic zone expressed antagonistic activity, and this characteristic was more common with particle-associated bacteria than with free-living bacteria. Lytic bacteria have been proposed to prey on Gram negative bacteria such as

Vibrio spp. (Chen *et al*., 2012; Sockett, 2009). In contrary, many researchers have demonstrated that interactions like bacterial competition, antagonism and even bacterial predation were relatively less important in removing coliforms from estuarine water than indigenous protozoa (Marino and Gannon, 1991; Enzinger and Cooper, 1976). Korakjic *et al*. (2013b) reported that *E. coli* decay was more impacted by natural microbiota (predation and competition) than sunlight, when the former was removed by disinfection.

4.2.3.3 Role of bacteriophages on survival

Bacteriophages are another category of biotic factors capable of infecting and controlling bacterial population in natural environments. They form an important component of the self-purifying ability of aquatic habitats and their regulation of bacterial population is by cell lysis. They are invariably present in all places where their hosts are present (Ackermann, 1996). Some researchers proposed that bacteriophage mediated mortality predominates in oxygen poor conditions or in highly productive systems (Pernthaler, 2005). Penon *et al*. (1991) made contradictory finding that seawater fraction passed through 0.2 µm filters containing bacteriophages had no deleterious effect on bacterial mortality. Carlucci and Pramer (1960) have shown that bacteriophages effectively reduced *E. coli* populations only under nutrient rich conditions and hence its role in natural environments is negligible.

Baross *et al*. (1978) have shown that bacteriophages are ubiquitous in seawater. *Vibr*io bacteriophages were isolated from marine samples of molluscan shellfish, seawater and sediments. The predominant bacteriophage type that was isolated was specific for *V. parahaemolyticus* whereas those against other species could not be detected. Increased incidence of

V. parahaemolyticus bacteriophages was reported during warmer temperatures, probably due to the increase in number of mesophilic *vibrios*. Some researchers reported that bacteriophages of *V. parahaemolyticus* isolated from seawater exhibited a broad host range towards other *Vibrio* species also (Letchumanan *et al*., 2016; Demeng *et al*., 2014; Matsuzaki *et al*., 2000). Because of their ability to lyse bacterial cells there are many reports about application of bacteriophages as an alternative to antibiotics; the principle behind phage therapy as applied in medical, agriculture, aquaculture and food industries (Srinivasan *et al*., 2007; Withey *et al*., 2005; Nakai and Park, 2002). These applications can be even extended to decontamination of mussels using specific phages targeted against specific human pathogens.

4.2.4 Increased incidence and survival of indicators and pathogens in sediments compared to overlaying water

Increased bacterial survival rates have been observed in sediments compared to overlaying water, due to the increased availability of nutrients (An *et al*., 2002; Alkan *et al*., 1995). The availability of nutrients is an essential factor for the survival and probable growth of enteric bacteria in natural environments (Delille and Delille, 2000; Alkan *et al*., 1995).

It has been observed that sediments not only improve survival of *E. coli* but also promotes its growth. The removal of FC from water mainly occurs by adsorption onto particles, which later settle and accumulate in the sediments (Ghoul *et al*., 1990). It has been reported that 90.5 % of FC bacteria were found to be associated to particles ranging from 0.45 to 10 µm whereas the remaining 9.5% are associated to particles larger than 10 µm (Auer and Niehaus, 1993). Another probable reason for increased bacterial incidence in sediments is the anaerobic condition that prevails, which does

not provide a suitable environment for protozoa that prey on FC (Crabill *et al*., 1999). Absence of competing organisms as well as the abundance of nutrients may be the additional favourable factors (Hood and Ness, 1982). Sediments and submerged aquatic vegetation also harboured higher densities of enterococci than water, not only due to increased nutrient availability but also by providing protection for the organism from UV radiation (Suter *et al*., 2011; Badgley *et al.,* 2010). Hence, the load was higher in the bottom sediments and near shore environments compared to the mid channel region, where they would be available for later resuspension. Various researchers have reported varying FC sediment to water ratios: 10:1 to greater than 100:1 (Rozen and Belkin, 2001; Gerba and McLeod, 1976) 100:1 to 1000:1 (Davies *et al*., 1995) and 2000:1 (Crabill *et al*., 1999).

E. coli showed improved survival in fresh water sediments containing more clay and small particle size due to its high organic content, than in fresh water sandy systems probably due to its low organic content (Jung *et al*., 2014; Omrane, 2013; Garzio-Hadzick *et al*., 2010; Crabill *et al*., 1999; Burton *et al*., 1987). Contradictory observation was made by LaLiberte and Grimes (1982) who found that in unsterile sediments greater number of FC was found in sandy texture than in silty clay sediment and hence the organic fraction does not contribute much to FC survival in sediments.

Sediment may also act as source of contamination to the above water body releasing pathogens when re-suspended and hence FC presence in water does not essentially indicate recent faecal contamination. The bacterial concentration in sediments can be used as a relatively stable indicator of long term mean bacterial concentration in the water column above. (Chandran *et al*., 2011; Haller *et al*., 2009; Craig *et al*., 2004). Chandran *et al*. (2011) performed

microcosm studies to evaluate survival of *V. parahaemolyticus* in water and sediment collected from Vembanad Lake along south west coast of India. Enhanced survival was observed in sediments compared to overlying water that can be attributed to the increased organic content present in it.

Among the different strategies adopted by microorganisms to survive in nutrient limited environments size reduction is an important one. Starvation induced morphological changes in *V. parahaemolyticus* cells which change their shapes from rod to irregular shapes and finally spherical or coccoid forms. Upon starvation, the cells entered viable but non culturable form (VBNC) but retained all their characteristics and on return of favourable conditions, such as optimum temperature or nutrient availability, resuscitation of cells took place (Jiang and Chai, 1996). Sediments and submerged aquatic vegetation harboured higher densities of enterococci than water, mainly due to increased nutrient availability and protection from UV radiation (Suter *et al*., 2011; Badgley *et al*., 2010). Hence, the load was high in the bottom sediments and near shore environments compared to the mid channel region, where they would be available for later re-suspension.

4.2.5 Relative survival of faecal indicator and pathogenic bacteria in aquatic environments

For practical convenience, microbial water quality monitoring systems rely on the use of FIB such as *E. coli* (APHA, 1995) and enterococci (US EPA, 2004), to predict the incidence and survival of pathogens in aquatic habitats. Though this approach is convenient and less laborious many studies have proved lack of precision, since FIB levels did not always correlate well with pathogen types (bacterial, viral and protozoan) and numbers. Some researchers have found that enterococci cannot be always considered as reliable indicators

in marine waters and waste stabilisation pond effluent (Sinton *et al*., 2002, 1994) and they even grow in some aquatic environments (Hartz *et al*., 2008). In connection with this issue, a number of reports have been published on the relative survival of indicator as well as selected pathogens in fresh and saline waters (Garrido-Pérez *et al*., 2008; Burton *et al*., 1987).

Burton *et al.* (1987) compared survival of *E. coli*, with pathogens such as *Klebseilla pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella newport* in freshwater sediment microcosms and found that except *S. newport* all of the bacteria tested had prolonged survival in sediments compared to the overlying water. Hence it was concluded that among the above mentioned bacteria only the survival of *S. newport* can be predicted precisely using *E. coli* as the indicator.

Hartz *et al*. (2008) studied the survival of FIB (*E. coli* and enterococci) in beach sand and experimental microcosms. Prolonged survival of faecal indicators in sediments could be demonstrated, compared to water and even growth took place in the absence of predators. The authors also postulated that the high numbers of indicators re-suspended in overlying water may be misinterpreted as recent contamination. Whether the pathogens also followed the same pattern or possessed the ability to grow in sediments was also not known. Touron *et al*. (2007) based on their studies on intertidal sediments in U.K, reported that sediment could act as a possible reservoir for faecal indicators but not necessarily for pathogens such as *Salmonella*. Another fact is that faecal coliforms and faecal streptococci were isolated throughout the year without any obvious seasonal trends in their numbers whereas pathogens like *V. parahaemolyticus* showed seasonal variation with their maximum incidence in summer months

(DePaola and Nordstrom, 2003). Pathogens like *V. parahaemolyticus* are halophilic and form a member of autochthonous microflora of brackish saline waters (Khouadja *et al*., 2014; Makino *et al*., 2003) whereas *E. coli* prefers fresh water environments.

Hence, it is not surprising that indicator and pathogen counts are frequently poorly correlated in the water column. There is high probability for consistent presence of indicators in water since they enter via faecal contamination whereas pathogens tend to be present intermittently, depending on disease incidence in the upstream communities, or carrier live stocks in the catchment. Furthermore, it is a fact that no single indicator alone can be expected to simulate the behaviour of the wide range of pathogen types (bacterial, viral, protozoan).

4.3 Objectives

1) Microcosm studies to determine the effect of temperature on the survival of *E. coli*, *E. faecalis* and *V. parahaemolyticus* in estuarine water.

2) Microcosm studies to determine the effect of varying salinities on the survival of *E. coli*, *E. faecalis* and *V. parahaemolyticus* in estuarine water.

3) To study the impact of biological factors (include protozoans, autochthonous bacteria and bacteriophages) on the survival of *E. coli*, *E. faecalis* and *V. parahaemolyticus* in estuarine water and sediment microcosms.

4.4 Materials and Methods

4.4.1 Bacterial strains used in the study

All the strains used in the present study were isolated from sediment and shellfish (*Villorita cyprinoides*) harvested from the shellfish growing areas of Cochin estuary. The strains used for the studies included *Escherichia coli* strain ES2P4 (Genbank accession no. KT804408) isolated from sediment, *Vibrio parahaemolyticus strain* V10M1 (GenBank accession no. KT163390) isolated from sediment and *Enterococcus faecalis* strain SF2K2 (GenBank accession no. KT804408) isolated from *Villorita cyprinoides.* Preliminary cultural, biochemical and molecular confirmation of the isolates were done as described in detail, elsewhere in this thesis (Refer section 2.4.3).

4.4.2 Preparation of bacterial inoculum and estimation of initial inoculum density

The selected strains of *Escherichia coli, Enterococcus faecalis* and *Vibrio parahaemolyticus* were inoculated into Tryptone Soy Broth and incubated for 24 hours at 37 °C. After incubation, the cells were harvested by centrifugation at 13,000 rpm for 5 min. The pellet obtained was washed thrice using physiological saline and resuspended in 10 mL physiological saline. One mL (approximately10^8 CFU/mL) of the culture was inoculated into 250 mL Erlenmeyer flasks containing100 mL test solutions to give an initial inoculum density of 10^6 CFU/mL.

4.4.3 Setting up of microcosms

Microcosms were prepared using water and sediment samples freshly collected from the Cochin estuary using Niskin sampler and Van Veen grabs respectively. Water microcosms were prepared by adding 100 mL estuarine water to 250 mL sterile conical flasks designated survival chambers and

sediment microcosms by overlaying 50 mL estuarine water over 50 gm sediment in 250 mL sterile survival chambers (Hood and Ness, 1982). Due to the proximity of the laboratory to the estuary, various microcosms could be set up within one hour of sample collection. In order to study the effect of different parameters on bacterial survival, microcosms with different conditions were set up. Survival of the test organisms were monitored up to 28 days.

4.4.3.1 Microcosm to study the effect of temperature on survival

Autoclaved estuarine water from the study area was used to study the effect of temperature. One hundred mL of the sterile estuarine water was taken in sterile 250 mL Erlenmeyer flask. Based on the temperature range which prevailed in the estuary during the three seasons pre-monsoon, monsoon and post-monsoon, three different temperatures such as 25 °C, 30 °C and 35 °C respectively were selected to study its effect on the survival of test microorganisms.

4.4.3.2 Microcosm to study the impact of salinity.

To study the impact of salinity water microcosms containing autoclaved estuarine water from the study area with three different salinities were set up- 0 ppt, 15 ppt and 30 ppt respectively, selected based on the salinity range that prevailed in the estuary during the three seasons pre-monsoon, monsoon and post-monsoon.

4.4.3.3 Microcosm to study the impact of biological factors

Raw estuarine water (RW) and raw sediment (RS) microcosms were used to study the effect of all self-contained biological factors such as protozoan predators, competing autochthonous bacteria and bacteriophages on the test organisms.

In order to study the effect of protozoan bacteriovory, eukaryotic inhibitor cycloheximide (500 mg/l) was added to the raw estuarine water and sediment (Davies et al., 1995). Effect of competing autochthonous bacteria was determined by estimating the load of total viable bacteria.

The bacteriophages in estuarine water were enumerated by plaque assay using double-layer agar method (Kennedy *et al*., 1986). Forty-five mL of the sample and 5 mL of *E. coli* / *V. parahaemolyticus*/ *E. faecalis* was inoculated into 45 mL of Deca Strength Phage Broth (DSPB) and incubated at 37 °C for 24 hours. After incubation, the cells were centrifuged at 2500 rpm for 10 minutes and the supernatant filtered through 0.45 µm filter. Then 0.1 mL of the filtrate was mixed with 1 mL of *E. coli* / *V.parahaemolyticus* / *E. faecalis* culture and 5 mL of 0.6 % nutrient agar (used as top agar) and poured over nutrient agar plates containing 1.2% agar (basal agar). The plates were incubated at 37 °C for 24 h and the plaques were counted and expressed as plaque forming units (PFU)/mL.

4.4.3.4 Microcosm to study the relative survival rates

Relative survival rates of the test organisms under the above selected parameters were studied by inoculating all the three test organisms *Escherichia coli* (ES2P4), *Enterococcus faecalis* (SF2K2) and *Vibrio parahaemolyticus* (V10M1) simultaneously into the microcosms set up with varying parameters. Microcosms with both estuarine water and estuarine sediment were used for the study.

4.4.4 Enumeration of bacteria

Survival of the test organisms in various microcosms were monitored up to 28 days using drop plate method (Hoben and Somasegaran, 1982).

Initial inoculum load at the time of inoculation (time zero) was estimated. Subsequent sampling and analysis from each test solution was done after 1st, 3rd, 5th, 7th, 14th, 21st and 28th day intervals by the drop plate method.

Selective media such as Eosin methylene blue (EMB) agar and Hicrome *E. coli* agar were used for the enumeration of *E. coli*, Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar and Hicrome *Vibrio* agar for the enumeration of *V. parahaemolyticus* and Kenner Faecal (KF) Streptococcal agar was used for the enumeration of *E. faecalis*. The plates were incubated at 37 °C and number of colonies counted after 24 h. All the enumerations were done in triplicates and the mean values were used to plot the survival curves.

4.4.5 Statistical analysis

Statistical analysis was performed using SPSS software 20 (Statistical Package for Social Science). The differences in the survival of the test organisms under varying conditions were analysed for significance using one-way analysis of variance (ANOVA) or Student's t- test wherever applicable. Significance level was set at $\alpha = 0.05$.

4.5 Results

In this study, survival of test microorganisms such as *Escherichia coli*, *Enterococcus faecalis* and *Vibiro parahaemolytics* in Cochin estuary as a function of temperature, salinity, chemical composition of the estuarine water and the various biological factors such as competing autochthonous bacteria, predation by protozoans and bacteriophages was assessed. The microcosms were prepared in order to study the effect of each one of the parameters at a time. Temperature and salinity were chosen according to the seasonal variations encountered in the estuary. The results are presented in various figures.

4.5.1 Survival of test organisms at various temperatures

Survival of *E. coli* in sterile estuarine water incubated at 3 different temperatures, is represented in Figure 4.1. *E. coli* exhibited a slow and gradual increase in cell numbers throughout the experiment at both 25 °C and 30 °C. However, at 35 °C *E. coli* showed a steep decline after 7 days. One log reduction was observed after 21 days and further 2 log reductions by 28th day. The results indicated that for extended periods *E. coli* showed better survival at 25 °C and 30 °C. However, the results were found to be statistically insignificant.

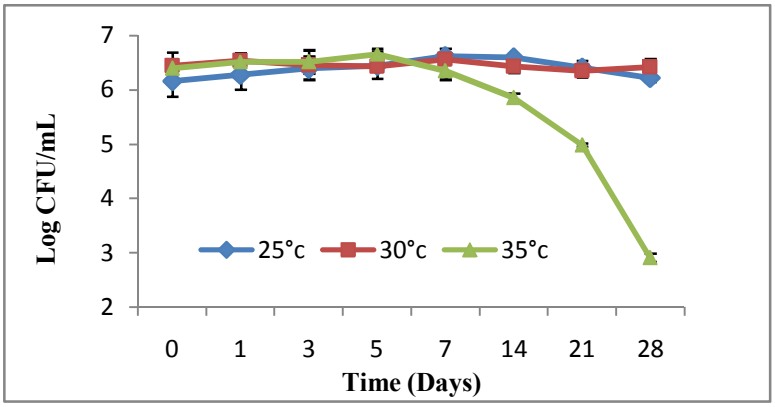

Figure 4.1 Survival curves of *Escherichia coli* at different temperatures

At 25 °C *V. parahaemolyticus* exhibited a gradual increase up to one log was observed by the 7th day which then gradually declined 2.5 logs from the initial load by 28th day (Figure 4.2). Similar growth pattern was observed at 30 °C also but the decline was steeper after the 5th day resulting in a final reduction of 3.5 logs from the initial load within 28th day. At 35 °C there was a steep increase of 1.5 logs after the first day then decreasing by 3.5 logs from the initial load within 28th day. When survival at the three temperatures was compared, least reduction of *V. parahaemolyticus* was

seen at 25 °C. For rapid growth at shorter intervals, higher temperatures such as 30 °C and 35 °C were preferred; however the results were found to be statistically insignificant.

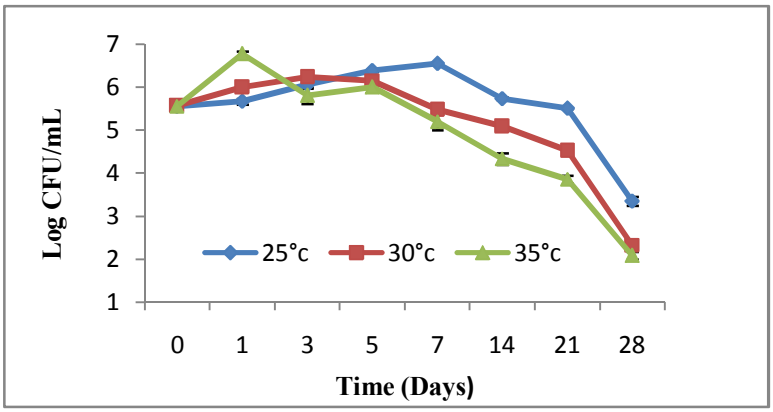

Figure 4.2 Survival curves of *V. parahaemolyticus* at different temperatures

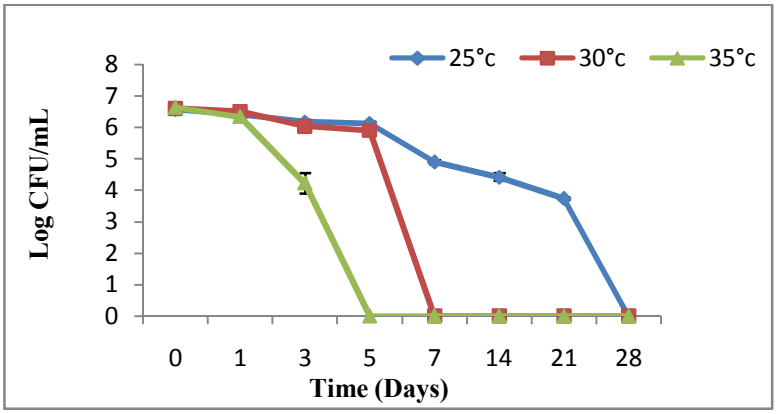

Figure 4.3 Survival curves of *Enterococcus faecalis* at different temperatures

The effect of temperature on the growth of *E. faecalis* is represented in Fig.4.3. At 25 °C and 30 °C, the growth of *E. faecalis* showed a slight decline until the 5th day. At 30 °C there was no viable count after 7 days, whereas survival could be observed up to 28th day at 25 °C. At 35 °C steep

decline was observed after the first day and survival was seen only up to 5th day; however, the results were found to be statistically insignificant.

4.5.1.1 Relative survival of test organisms at various temperatures

The three test organisms exhibited different survival rates at 25 °C; however the differences were statistically insignigicant (Fig. 4.4). *E. coli* and *V. parahaemolyticus* survived up to the 28th day while *E. faecalis* survival was limited only upto the 28th day. *E. coli* maintained a steady growth throughout the entire experiment. *V. parahaemolyticus* exhibited steady growth until the 7th day of incubation and then started declining; with a total reduction of 2.2 logs throughout the entire period of experiment.

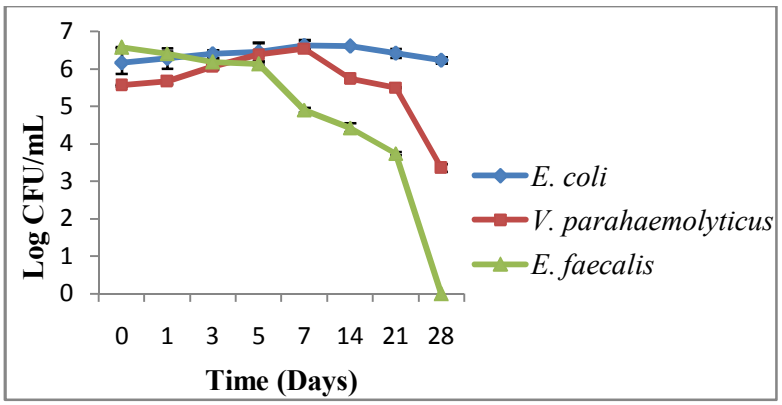

Figure 4.4 Relative survivals of test organisms at 25 °C

Figure 4.5 represents the significant differential survival of the three test organisms at 30 °C (p=0.014). Both *E. coli* as well as *V. parahaemolyticus* survived up to the 28th day, while *E. faecalis* survived only up to the 7th day. *E. coli* demonstrated a steady growth throughout the entire period of the experiment. *V. parahaemolyticus* showed a gradual growth up to the 3rd day and then gradually started declining, with a total reduction of 3.2 logs until the 28th day.

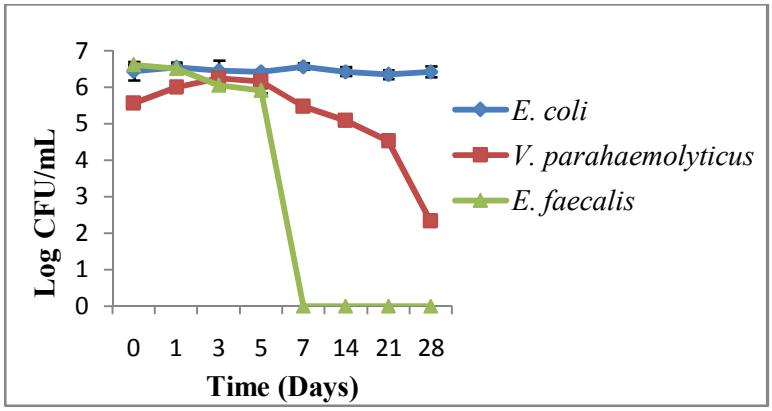

Figure 4.5 Relative survivals of test organisms at 30 °C

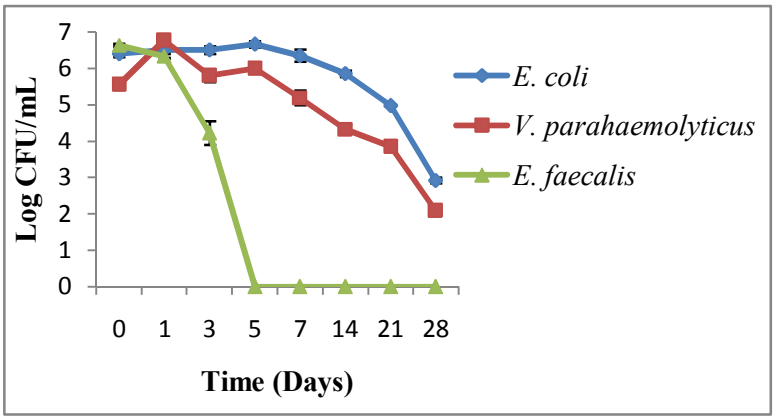

Figure 4.6 Relative survivals of test organisms at 35 °C

At 35 °C, *E. coli* and *V. parahaemolyticus* exhibited survival up to 28th day, whereas *E. faecalis* survived only up to 5 days. Though their survival rates were similar, *E. coli* and *V. parahaemolyticus* varied in their respective growth patterns. Throughout the experiment *E. coli* showed a gradual decline, whereas *V. parahaemolyticus* showed an initial spurt of growth during the first day, which later declined until the 28th day. *E. faecalis* showed a steep decline throughout the experiment with least survival of only up to 5 days. The differences in survival rates were found to be statistically significant (p=0.005).

4.5.2 Survival of test organisms at various salinities

The effect of varying salinities on the growth of *E. coli* is demonstrated in Fig.4.7. A gradual decline in the growth was observed at all the three salinities 0 ppt, 15 ppt and 30 ppt respectively. At 0 ppt even though a slight decline was noted until the 14th day, the growth picked a steady pace and at 21st day half log increase and later at 28th day a half log reduction was observed. At 0 ppt salinity, *E. coli* exhibited an overall reduction of only one log reduction. At 15 ppt and 30 ppt salinities similar growth curves were observed and throughout the entire experiment the bacterial load progressively decreased by 2.2-2.5 logs at 15 ppt and 30 ppt salinities respectively. However, the results were found to be statistically insignificant ($p>0.05$).

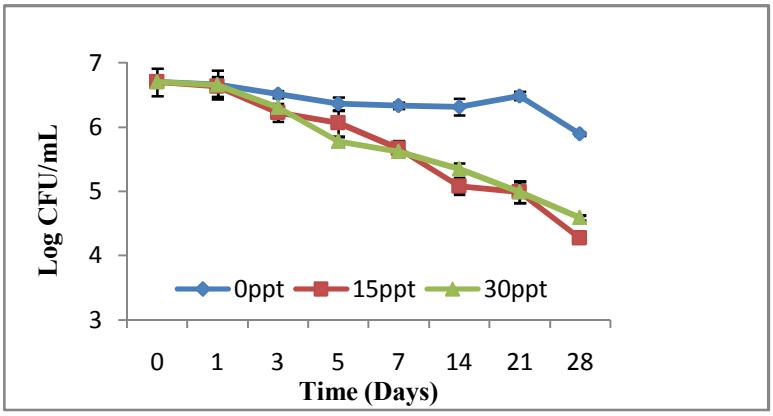

Figure 4.7 Survival rates of *Escherichia coli* at varied salinities

V. parahaemolyticus showed different survival rates at various salinities as shown in Figure 4.8. At 0 ppt salinity there was a small increase of 0.4 logs after the first day and later declined exponentially by 3.8 logs. Growth patterns were similar at 15 ppt and 30 ppt salinities. An increase of 0.5-1 log growth was observed until the 3rd day and then a steady growth was maintained until the 14th day. On the 21st day a slight decline was

observed, but picked up until the 28th day. At 15 ppt and 30 ppt a total reduction of 0.5 and 0.3 logs from the initial load were observed whereas at 0 ppt a reduction of 3.4 logs could be observed within 28 days. The results were found to be statistically insignificant (p=0.152).

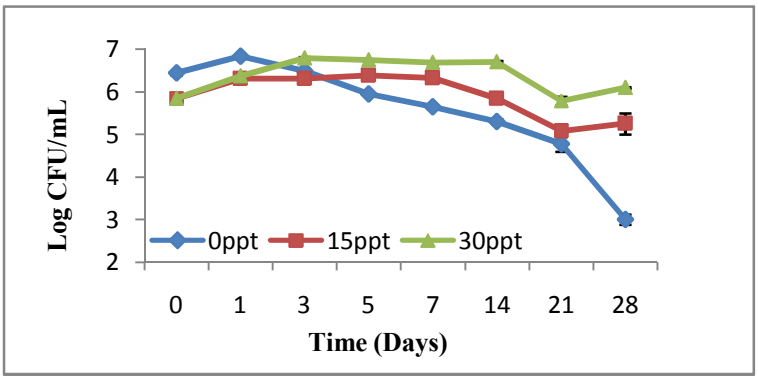

Figure 4.8 Survival rates of *V. parahaemolyticus at* varied salinities

The effect of varying salinities on *E. faecalis* is represented in Figure 4.9. *E. faecalis* showed more or less identical survival patterns at all salinities; a gradual decline in the number of cells was observed from the first day itself and a total reduction of 4 logs from the initial load took place within 28 days at all three salinities *viz.* 0ppt, 15 ppt and 30 ppt respectively. However, the results were found to be statistically insignificant.

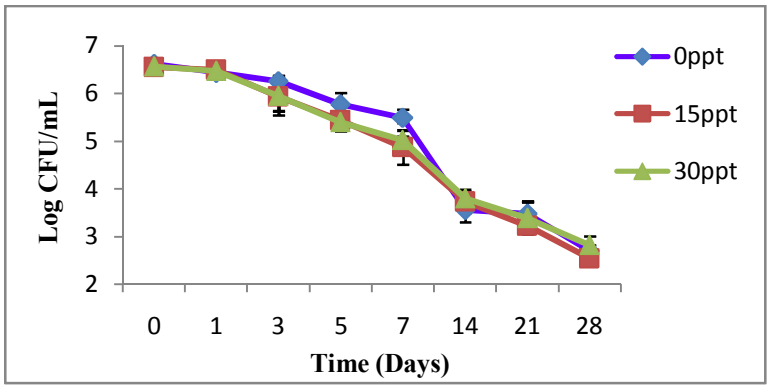

Figure 4.9 Survival curves of *E. faecalis* at different salinities

4.5.2.1 Relative survival of test organisms at various salinities.

Cochin estuary has often demonstrated high fluctuations in salinity ranging from 0 ppt salinity during monsoon season to 30-35 ppt during pre-monsoon season. The relative survival study is useful in predicting the survival thresholds of the pathogens as well as indicators in this uryhaline estuary which in turn helps to assess the health hazard associated during various seasons.

The growth of the three test organisms at 0 ppt salinity is represented in Fig. 4.10. All the three organisms under study showed survival up to the 28th day. However, out of the three test organisms, *E. coli* showed the least reduction in cell population of only 0.9 logs, followed by *V. parahaemolyticus* which exhibited a reduction of 3.4 logs. *E. faecalis* exhibited the maximum reduction of 3.9 logs till the end of the experiment. The differences in survival rates were found to be statistically insignificant (p=0.079).

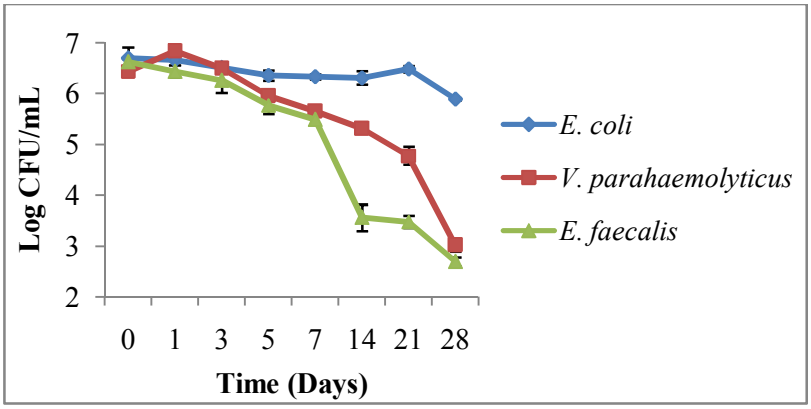

Figure 4.10 Relative survival of test organisms at 0 ppt salinity

The three test organisms survived up to the 28th day of the experiment at 15 ppt salinity as represented in Fig. 4.11. However no significant differences existed with respect to their survival rates (p=0.123). *V. parahaemolyticus* exhibited slight growth up to the 5th day and then started declining with a

total reduction of only 0.6 log throughout the entire period of the experiment. *E. coli* exhibited a decline of 2.4 logs while *E. faecalis* exhibited the maximum decline of 4 logs throughout the entire experiment.

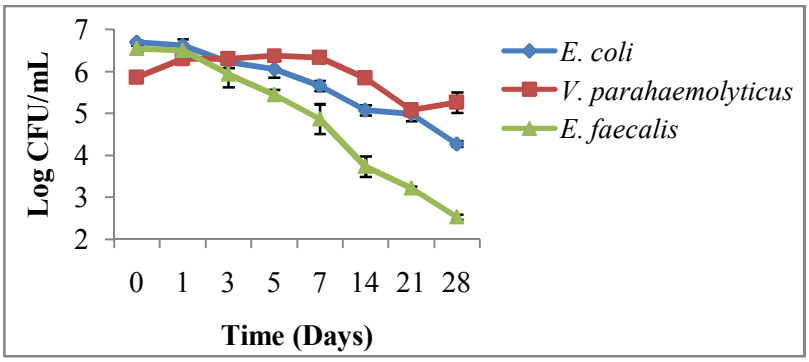

Figure 4.11 Relative survival of test organisms at 15 ppt salinity

At 30 ppt salinity highly significant variation was found with respect to the survival rates of the 3 test organisms (p=0.02) (Figure 4.12).

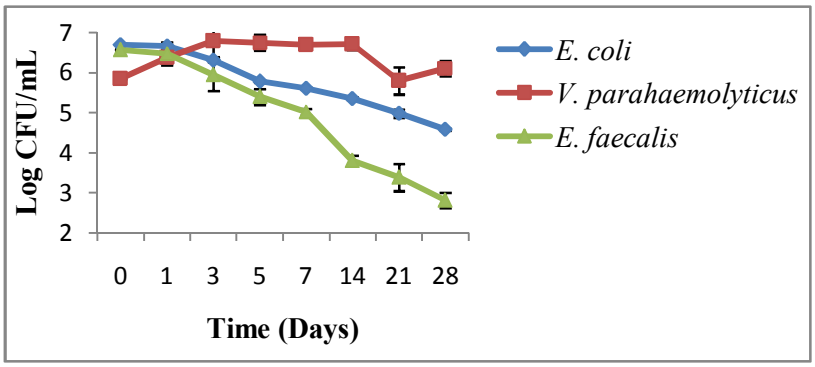

Figure 4.12 Relative survival of test organisms at 30 ppt salinity

V. parahaemolyticus showed relatively better survival rates compared to the other two organisms under study. Exponential growth of *V. parahaemolyticus* until the 3rd day was observed and then maintained a steady rate till the 14th day and then started declining and later started increasing with a net increase of approximately 0.26 log towards the end of the experiment.

4.5.3 Impact of biotic factors on survival of test organisms

4.5.3.1 Impact of biotic factors on survival of test organisms in estuarine sediments

The effect of biological factors on the survival of *E. coli* in estuarine sediments is evident from Figure 4.13. *E. coli* demonstrated comparatively greater decline in cell population in raw sediment when compared to autoclaved sediment. In autoclaved sediment, 2 logs increase in growth was seen soon after the first day, then started declining, finally reaching approximately the same load as the initial *E. coli* concentration, after 28 days. In raw sediment, there was continuous reduction in growth throughout the experiment with a total reduction of 3.7 logs from the initial load. The difference in survival of *E. coli* in autoclaved and raw sediment was highly statistically significant ($p<0.001$).

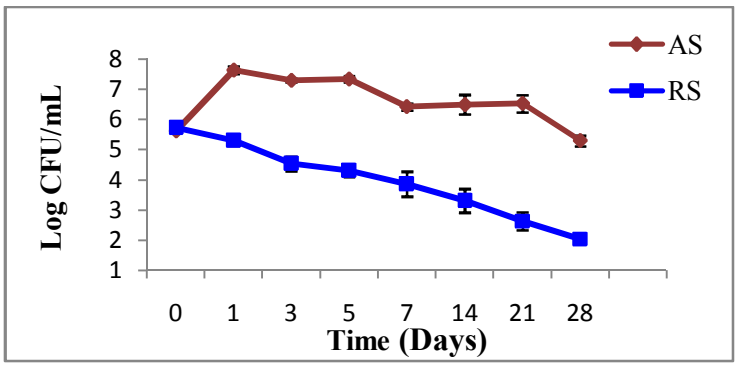

Figure 4.13 Impact of biotic factors on survival of *E. coli* in estuarine sediments

V. parahaemolyticus exhibited significant reduction in cell populations in raw sediment compared to autoclaved sediment ($p<0.001$) as shown in Figure 4.14. In raw sediment growth started declining from the first day itself and a total reduction of 3.6 logs from the initial level was observed until the 28th day, whereas in autoclaved sediment 2 increase of growth was

observed within the first day itself and then showed a sight declining trend until the last day of the experiment.

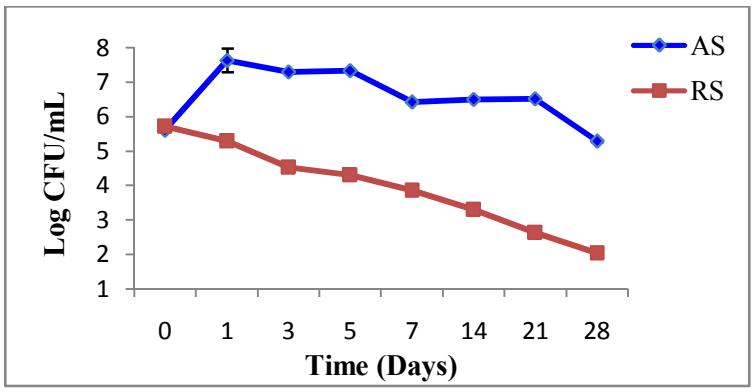

Figure 4.14 Impact of biotic factors on survival of *V. parahaemolyticus* in estuarine sediments

Biotic factors had significant impact on the survival of *E. faecalis* in raw estuarine sediment (Figure 4.15). Significant reduction ($p<0.05$) in survival rates was exhibited in raw sediment compared to autoclaved sediment. In raw sediment, the survival was limited to only 21 days, whereas in autoclaved sediment, survival was shown until the 28th day of the experiment; with an overall reduction of only one log throughout the experiment.

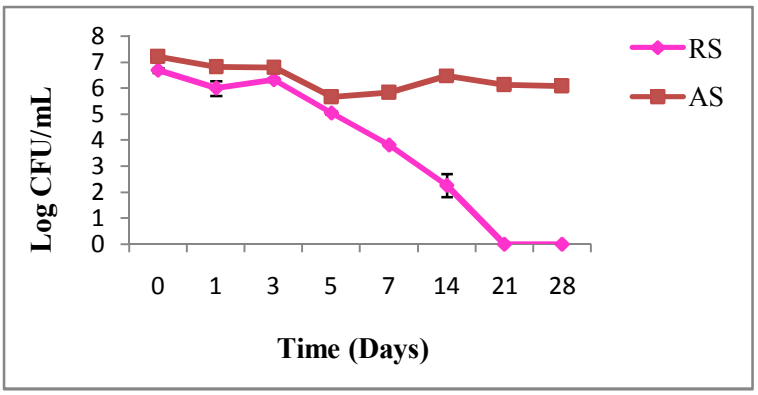

Figure 4.15 Impact of biotic factors on survival of *E. faecalis* in estuarine sediments

4.5.3.2 Impact of biotic factors on survival of test organisms in estuarine water

E. coli demonstrated significant reduction (p=0.003) in survival in raw water, when compared to autoclaved water (Figure 4.16). In autoclaved water growth was observed until the 28th day of the experiment whereas in raw water *E. coli* survived only up to 7 days. The drastic reduction in survival of *E. coli* in raw water could be attributed to the significant impact of biological factors.

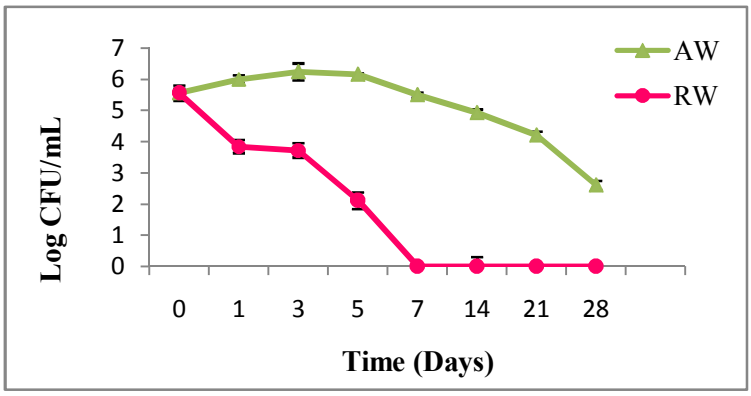

Figure 4.16 Impact of biotic factors on the survival of *E. faecalis* in estuarine water

Figure 4.17 shows the significant reduction in survival of *V. parahaemolyticus* exhibited in raw water compared to autoclaved water (p=0.003). In raw water, the survival was limited to 7 days, whereas in autoclaved water growth was demonstrated throughout the experiment. In raw water steep decline was observed from the first day itself whereas in autoclaved water initially growth was observed until the the 3rd day and then started declining to 2.6 logs until the 28th day.

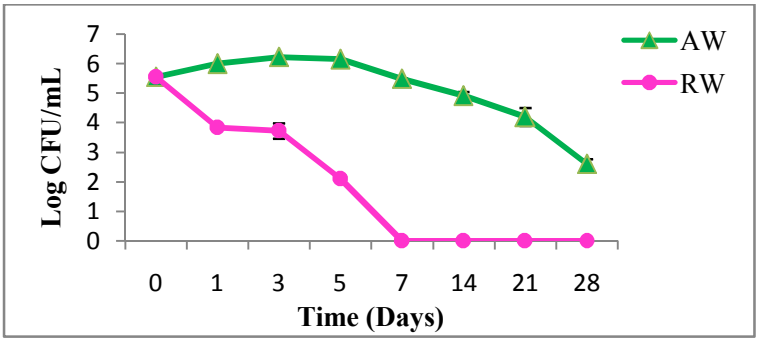

Figure 4.17 Impact of biotic factors on the survival of *V. parahaemolyticus* in estuarine water

The survival of *E. faecalis* in raw estuarine water was influenced by biotic factors as demonstrated in Figure 4.18. In raw water the growth of *E. faecalis* declined continuously and was limited to only 5 days, whereas in autoclaved water the decline in growth was very gradual and extended survival could be observed up to the 21st day. However, the results were found to be statistically insignificant (p=0.25).

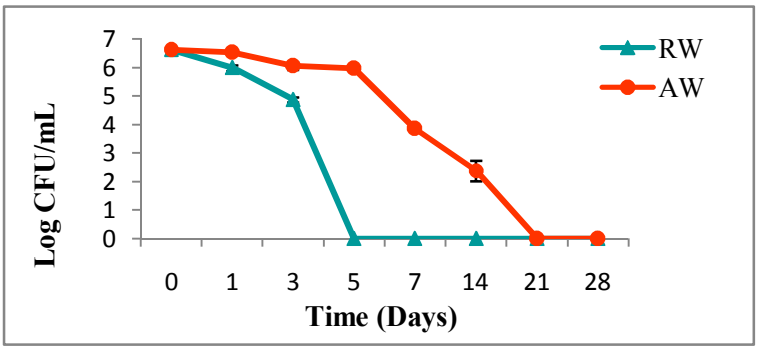

Figure 4.18 Impact of biotic factors on the survival of *Enterococcus faecalis* in estuarine water

4.5.4 Effect of protozoan bacteriovory on the survival of test organisms

4.5.4.1 Effect of protozoan bacteriovory on the survival of test organisms in estuarine sediment

E. coli exhibited slightly extended survival in estuarine sediment microcosm untreated with cycloheximide, rather than in treated sediment

microcosm as given in Figure 4.19. In cycloheximide untreated sediment microcosm, extended survival of up to 21 days was exhibited whereas in case of raw sediment treated with cycloheximide 14 days of survival was observed; however the results were found to be statistically insignificant.

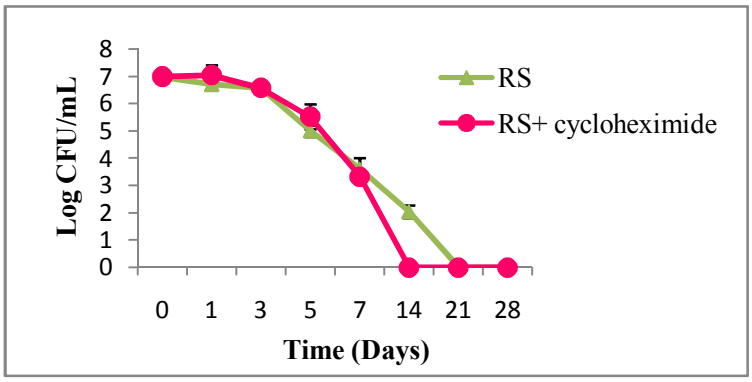

Figure 4.19 Effect of protozoan bacteriovory on the survival of *E. coli* in estuarine sediment

V. parahaemolyticus did not show any significant difference ($p>0.05$) in survival, in cycloheximide-treated and untreated sediment microcosms as shown in (Figure 4.20).

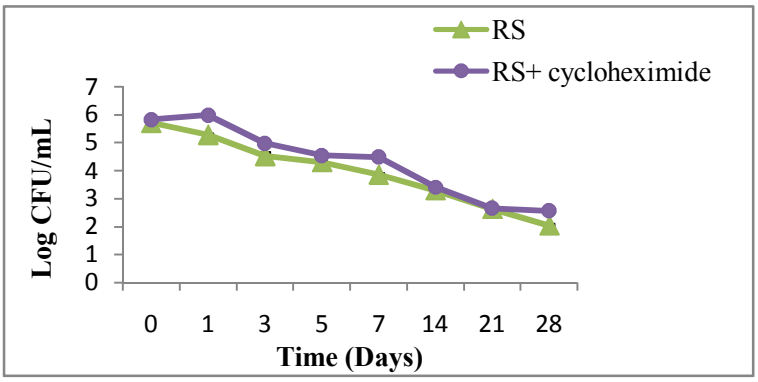

Figure 4.20 Effect of protozoan bacteriovory on the survival of *V. parahaemolyticus* in estuarine sediment

Figure 4.21 shows no difference in survival rates of *E. faecalis* in cycloheximide treated and untreated sediment microcosms. In both the microcosms survival was observed up to 21 days.

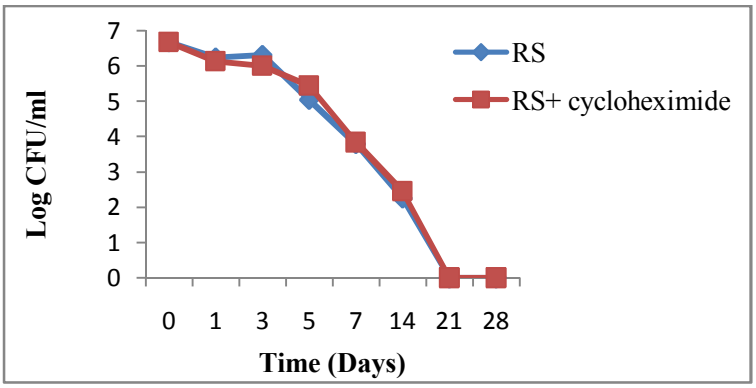

Figure 4.21 Effect of protozoan bacteriovory on the survival of *E. faecalis* in estuarine sediment

4.5.4.2 Effect of protozoan bacteriovory on the survival of test organisms in estuarine water

E. coli exhibited no difference in survival rates in cycloheximide treated and untreated water (Figure 4.22). In both microcosms the survival could be observed only up to the 14th day of the experiment.

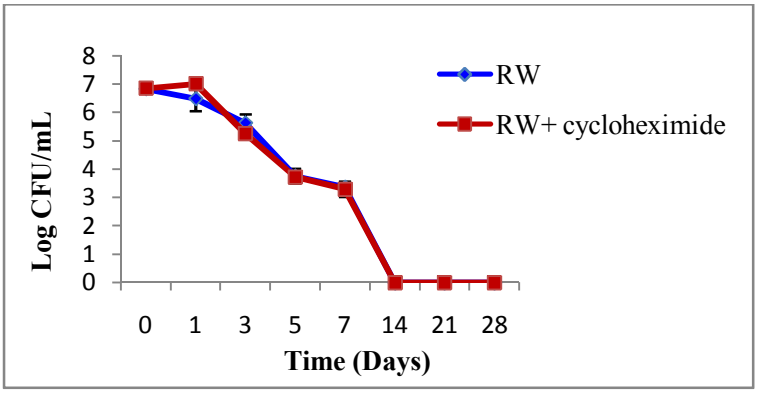

Figure 4.22 Effect of protozoan bacteriovory on the survival of *E. coli* in estuarine water

V. parahaemolyticus exhibited extended survival in cycloheximide-treated estuarine water, compared to untreated water as shown in figure 4.23. In cycloheximide-treated water microcosm *V. parahaemolyticus* survived for 7 days while 5 days of survival could be observed in untreated water microcosm. However, the differences in survival rates were found to be statistically insignificant ($p>0.05$).

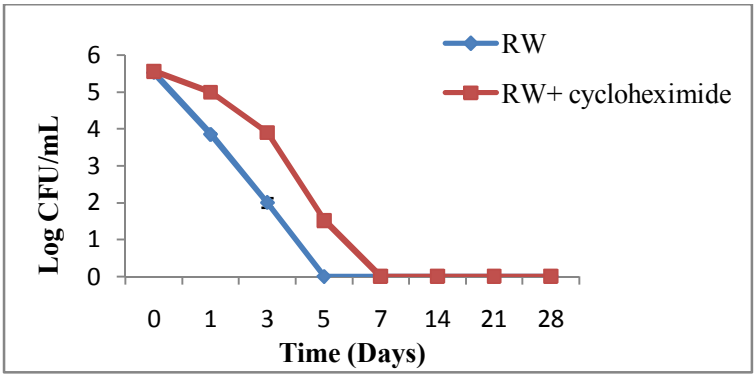

Figure 4.23 Effect of protozoan bacteriovory on the survival of *V.parahaemolyticus* in estuarine water

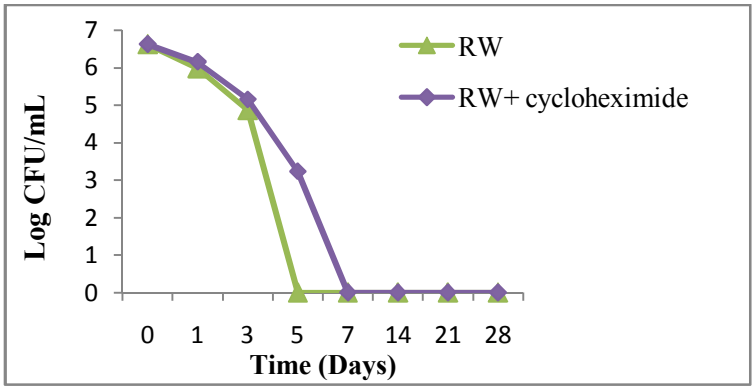

Figure 4.24 Effect of protozoan bacteriovory on the survival of *E. faecalis* in estuarine water

Figure 4.24 shows differences observed in survival periods of *E. faecalis* in cycloheximide treated and untreated water. In cycloheximide treated water, *E. faecalis* survived up to 7 days while in untreated water,

survival was reduced to 5 days. However, the differences in survival rates in the two water microcosms were found to be statistically insignificant ($p>0.05$).

4.5.5 Impact of bacteriophages on the survival of test organisms

Bacteriophages are important biotic factors that influence bacterial survival in estuarine environments. Coliphages and vibriophages were detected from Cochin estuary in present study. The mean coliphage count observed in the present study was 7.5×10^3 PFU/mL. Vibriophages were also detected and the mean count was 2×10^3 PFU/mL. However, phages specific for *E. faecalis* could not be detected during this study.

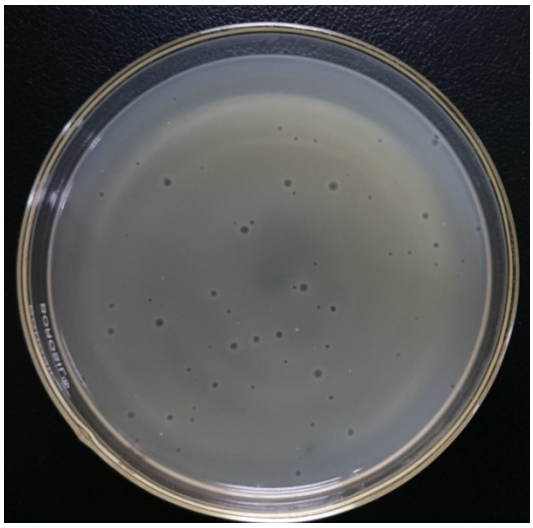

Figure 4.25 Plate showing coliphage plaques

4.6 Discussion

4.6.1 Survival at varying temperatures

Microcosm studies on effect of temperature on survival of the test pathogens revealed that *E. coli*, *V. parahaemolyticus* and *E. faecalis* preferred lower temperatures such as 25 °C and 30 °C for better survival for prolonged

periods. Higher temperatures such as 35 °C favoured the growth of *E. coli* and *V. parahaemolyticus* only for shorter durations. This can be one of the probable reasons for their increased isolation rates from shellfish harvesting waters during warmer months, compared to winter months (Julie *et al*., 2010; Kaneko and Colwell, 1973). However, after the initial spurts, growth started declining to the very low level at higher temperatures, compared to lower temperatures. This is in agreement with the findings of many researchers that lower temperatures favour increased survival of bacteria in fresh and marine waters (An *et al*., 2002; Rozen and Belkin, 2001; Delille and Delille, 2000; Auer and Niehaus, 1993). Present study agrees well with the findings of Abhirosh *et al*. (2009) who also reported better survuval of *E. coli* and *V. parahaemolyticus* at lower temperatures say 20 °C, compared to higher temperatures such as 30 °C. However, present findings are not in agreement with the previous reports that higher temperatures favour increased bacterial growth, or that, temperature does not have any correlation with bacterial growth (Crabill *et al*., 1999; Alkan *et al*., 1995).

Even though many researchers have studied the impact of temperature on survival of *V. parahaemolyticus*, *E. coli* and *E. faecalis* (Kim *et al*., 2012; Fernandez-Piquer *et al*., 2011), very few reports are available on studies in laboratory microcosms (Abhirosh *et al*., 2009; Hood and Ness, 1982).

Unlike the other two organisms under study, *E. faecalis* demonstrated the least survival at all the 3 temperatures selected. This is in agreement with the reports of Anderson *et al*. (2005), who found higher decay rates for enterococci than faecal coliforms. Growth was not at all exhibited at any of the 3 temperatures at any point of time, but declined throughout the study

period. It was also observed that a negative correlation existed between temperature and enterococccal population reduction; least survival was observed at 35 °C, followed by 30 °C and at 25 °C, survival could be seen up to the 28th day. Increased protozoan predation during warmer months has been attributed to increased bacterial reduction in warmer periods (Liu *et al*., 2016; Solic *et al*., 1992).

4.6.2 Survival at varying salinities

Salinity is considered to be an important factor influencing the survival of bacteria in marine and estuarine environments (An *et al*., 2002; Davies *et al*., 1995; Auer and Niehaus, 1993). In present study, salinity was found to have a negative correlation with survival of *E. coli*. *E. coli* exhibited better survival at lower salinities and decay rates increased with increase in salinity levels. This is in agreement with the findings of Ahmad *et al*. (2014) that *E. coli* showed better survival in fresh water compared to marine waters. Saline conditions had adverse impact on survival of bacteria by posing unwanted stress on cells (Enzinger and Cooper, 1976).

V. parahaemolyticus showed a positive correlation with salinity. Decay rates decreased as salinity increased, which agrees well with the fact that *V. parahaemolyticus* is a halophilic microorganism and forms a member of indigenous microbiota of estuarine and marine habitats (Zhang and Orth, 2013).

Out of the three microorganisms under study, *E. faecalis* exhibited reduced survival at all the three different salinities. Salinity variation was not found to have any apparent relation with the survival of *E. faecalis.* This is not in agreement with the findings of Ahmad *et al*. (2014), where enterococci demonstrated better survival in marine waters than in fresh waters.

At lower salinities, *E. coli* showed the least decline in growth rate followed by *V. parahaemolyticus* and *E. faecalis.* At higher salinities *V. parahaemolyticus* exhibited least decay rates followed by *E. coli* whereas *E. faecalis* showed the maximum decline. Thus at lower salinity *E. coli,* and at higher salinities *V. parahaemolyticus* exhibited the maximum survival, while *E. faecalis* demonstrated least survival at all the three different salinities.

4.6.3 Impact of biotic factors on bacterial survival

Among the factors simulated to study bacterial survival in the natural aquatic environments, biotic factors (predation and competition) were found to be the most significant one. This was evident from the fact that extended survival of all the 3 organisms could be seen in autoclaved water and sediment, compared to raw water and sediment. Among autoclaved sediment and water, greater survival of all the organisms was supported in autoclaved sediment, due to its nutrient rich nature, additionally enriched by the release of extra nutrients during autoclaving (Hood and Ness, 1982; Gerba and McLeod, 1976). Among raw water and raw sediment, latter demonstrated better growth not only due to its nutrient rich nature but also due to reduced level of predation. This may be attributed to the anaerobic condition that prevailed in the sediments which decreased the protozoan predation, as well as certain degree of protection rendered by the particles from the predators (Abhirosh and Hatha, 2005; Auer and Niehaus, 1993; Crabill *et al*., 1999).

E. coli and *V. parahaemolyticus* exhibited similar growth patterns in all the four microcosms (autoclaved sediment, raw sediment, autoclaved water and raw water). Both *E. coli* and *V. parahaemolyticus* exhibited least survival in raw water, better survival rates were observed in raw sediment,

autoclaved water and autoclaved sediment. In raw sediment, the growth rate consistently decreased until the last day. Considerable increase in growth was observed in autoclaved sediment during the first day and thereafter declined slightly and maintained at the initial level throughout the experiment. In autoclaved water, slight growth was observed during the initial day, which later declined significantly throughout the experiment which can be attributed to the progressive depletion of nutrients (Delille and Delille, 2000; Alkan *et al*., 1995).

Unlike the other two organisms under study, *E. faecalis* exhibited least survival in raw water, autoclaved water as well as raw sediment. Extended survival of *E. faecalis* was observed until the last day of the experiment only in autoclaved sediment; however, the rate of decline was relatively higher than the other two organisms.

Relatively higher number of all the 3 test organisms could be recovered from sediment microcosms compared to water, irrespective of whether it was autoclaved or raw. This is probably due to the nutrient rich nature of the sediment compared to water. This is in agreement with other reports where, extended survival in sediments compared to water has been reported (Delille and Delille, 2000; Alkan *et al*., 1995). In addition to nutrients, other factors like reduced protozoan predation in the anaerobic sediments (Crabill *et al*., 1999), protection conferred by sediment particles from predators and protection from UV radiation (Suter *et al*., 2011; Badgley *et al*., 2010) are other factors which have been reported to support bacterial survival. Hence, it can be concluded that sediment may also act as a permanent repository to the overlying water body releasing pathogens when re-suspended.

4.6.3.1 Survival in cycloheximide-treated and untreated raw sediment and water

In present study, cycloheximide treatment which was meant to inhibit the predatory protozoa, was found to have no impact on *E. coli* and *E. faecalis* survival in both water and sediment microcosms. No significant improvement in bacterial survival was observed even after cycloheximide treatment. This can be attributed to bacteriophage mediated cell lysis as significant number of coliphages (7.5×10^3 PFU/mL) and vibriophages 2×10^3 PFU/mL could be isolated during present study. This is contradictory to the findings of several workers who reported significant increase in bacterial survival in different cycloheximide treated microcosms such as water (McCambridge and McMeekin, 1981), sewage (Mallory *et al*., 1983) and sediment (Davies *et al*., 1995; Marino *et al*., 1991). Abhirosh *et al.* (2009) also reported decreased *E. coli* mortality in raw estuarine water microcosms treated with cycloheximide compared with those untreated with cycloheximide, even though the difference was found to be statistically insignificant. The above studies state that protozoan grazing is an important factor regulating bacterial populations in natural aquatic environments (Jousset, 2012; Anderson *et al*., 2005; Pernthaler, 2005). However, the observations of present study do not agree with the above findings. Cycloheximide treatment helped to prolong the survival of *V. parahaemolyticus* slightly by two days in raw water microcosm; however, cycloheximide treatment did not impact much the survival rate of *V. parahaemolyticus* in sediment microcosm.

4.6.3.2 Impact of bacteriophages on bacterial survival

Increased bacterial mortality, even after the inhibition of protozoan predators using eukaryotic inhibitor cycloheximide, indicates the increased activity of predatory bacteria or bacteriophages in Cochin estuary. Several

researchers have identified the role of bacteriophages in the removal of coliforms from natural environments (Jacquet *et al*., 2005; Alonso *et al*., 2000). Suttle and Chen (1992) estimated that considerable population of bacterial community in marine ecosystems was infected daily and 8-26% of the bacterial mortality in marine ecosystems could be explained by viral lysis. In freshwater ecosystems, also viruses have been found to be one of the important agents responsible for bacterial mortality, with daily bacterial removal reaching up to 97% (Weinbauer and Hofle, 1998). In the present study, high concentrations of coliphages and vibriophages could be isolated which confirms the probable role of bacteriophages in removal of test pathogens in the microcosms. Protozoan predators and bacteriophages have been found to exert maximum influence on *E. coli* and *S. typhimurium* mortality in estuarine water (Abhirosh *et al*., 2009; Abhirosh and Hatha, 2005). Because of their ability to lyse bacterial cells, there are many reports regarding use of bacteriophages as an alternative to antibiotics; the principle behind phage therapy as applied in medical, agriculture, aquaculture and food industries (Srinivasan *et al*., 2007; Withey *et al*., 2005; Nakai and Park, 2002). These applications can be extended to decontamination of mussels using specific phages targeted against specific human pathogens (Pereira *et al*., 2017).

There are certain previous reports contradictory to the observations of present study regarding the predominant role of bacteriophages in bacterial mortality. Penon *et al*. (1991) found that seawater fraction which contained bacteriophages had no deleterious effect on bacterial motility whereas Carlucci and Pramer (1960) showed that bacteriophages effectively reduced bacterial cells only in nutrient rich conditions and hence it is not possible in natural environments. However, it is established fact the bacteriophage

mediated lysis is one of the key factor that limits the bacterial numbers in natural waters. They also play critical role in microbial loop.

Microbial water quality monitoring systems depend on the use of FIB such as *E. coli* and enterococci to predict the incidence and survival of pathogens in aquatic habitats. Usually drinking water (APHA, 1995) and shellfish growing water quality (NSSP, 2015) is assessed based on the presence of faecal coliforms or *E. coli* in particular, whereas recreational water quality is assessed based on the presence of enterococci (US EPA, 2004). Nevertheless, there are contradictory reports regarding the efficacy of enterococci and faecal coliforms/*E. coli* as faecal indicators. Many researchers consider enterococci as better single indicators especially in marine environments (Jin *et al*., 2004; Cabelli *et al*., 1982). In contrary, some researchers have reported that enterococci cannot be always considered as reliable indicators in marine waters and waste stabilisation pond effluent (Sinton *et al*., 2002, 1994) and they are even found to grow in some aquatic environments (Hartz *et al*., 2008). However, in present study *E. coli* exhibited better survival than other organisms in both raw water and sediment microcosms.

From present study, it can be concluded that survival of indicators and pathogens is greatly influenced by the conditions that are prevailing in the natural environments. Factors such as temperature, salinity, nutrient availability and biotic factors have found to have profound influence on bacterial mortality in natural environments. Among all the aforesaid factors which form part of the inherent self purifying capacity of natural water bodies, biotic factors which encompass protozoa, competing autochthonous bacteria and bacteriophages play the dominant role. In the present study,

among all the biotic factors bacteriophages have been largely found responsible for bacterial mortality. Bacteriophages have been exploited to control pathogenic bacteria, the principle behind therapy as applied in medical, agriculture, aquaculture and food industries. Since the microcosms used in the present study were constructed using water and sediments collected from shellfish harvesting areas of Cochin estuary, the applications can be extended to decontamination of clams using specific phages targeted against specific human pathogens. Both indicators and pathogens had comparatively lesser decay rates and extended survival in sediments than in the overlying water. Extended survival of bacteria in sediments can be attributed to several favourable factors like lower temperatures, protection from ultraviolet light, lesser predatory activity due to the prevailing anaerobic condition and slight degree of protection conferred by sediment particles and nutrient abundance. As a result the underlying sediments can act as a permanent source of contamination to the overlying water column if disturbed by anthropogenic activities like drudging or natural phenomena such as storm, which may adversely affect the intended beneficial uses of the estuary like shellfish growing and other aquaculture practices, recreational and tourism activities etc.

Chapter 5

DEPURATION STUDIES OF *VILLORITA CYPRINOIDES* VAR. *COCHINENSIS HARVESTED* FROM COCHIN ESTUARY USING A CLOSED WATER DEPURATION SYSTEM ATTACHED WITH ACTIVATED CARBON FILTER

Contents

5.1 Introduction

Depuration can be defined as the process by which harvested shellfish is placed in land-based plants containing clean estuarine water, to permit the purging of their gastrointestinal contents under controlled conditions (Oliveira *et al*., 2011; Jackson and Ogburn, 2009; Richards, 1998). It is sometimes referred to by some authors as purification (Ayres, 1991). Food safety is the major concern associated with bivalve consumption. It is one of the delicacies preferred to be eaten raw by people in many parts of the world. Most of the shellfish growing areas are situated in shallow, nutrient rich, near-shore waters which often receive river and land run-off as well as other sewage discharges. Being filter feeders they accumulate all sorts of contaminants (Pavoni *et al*., 2013; Pinto and Bosch, 2013) including pathogens and bio-toxins in the surrounding waters and thus have been responsible for many food borne disease outbreaks worldwide. Realising the

potential risk factor associated with consumption of bivalves, remedial measures are being implemented mainly at two stages- pre-harvest and post-harvest levels. At pre-harvest level, harvesting of shellfish is permitted only if the sanitary quality of the growing area meet the standards prescribed by the regulatory agencies, whereas at post-harvest level various strategies such as depuration in land based plants or relaying in clean estuarine waters for longer periods is adopted.

5.2 Review of literature

5.2.1 Shellfish and infectious disease

Shellfish associated disease outbreaks have been recognised and documented for over a century. Historically bacterial diseases such as cholera, typhoid, viral diseases such as hepatitis and even algal bio-toxins have been linked with bivalve consumption (Odeyemi, 2016; Letchumanan *et al*., 2014; Costa, 2013).

In United Kingdom, nearly 80 disease outbreaks between 1981 and 1988 were attributed to shellfish, which exceeded the total number of such outbreaks recorded in the previous 40 years (West, 1991). According to FAO reports (FAO, 2014), 10% of all food borne disease outbreaks are caused by seafood. As per CDC reports, in 2013 (CDC, 2013b), the food category mostly implicated in food borne disease was related to molluscs (11%) and the most implicated bacterial pathogen coupled with this food source was *Vibrio parahaemolyticus*. However, food related disease outbreaks, are likely to be under reported as they often occur as sporadic cases (Scallan *et al*., 2011; Cliver, 1994). Consequently, the true number of clinical cases attributable to shellfish consumption may be somewhat higher.

Contamination of shellfish growing waters with human faecal material, poses the significant health risk to consumers. The pathogens implicated mostly in such illnesses include both viral as well as bacterial agents. Among viral disease outbreaks, Norwalk virus gastroenteritis and hepatitis A are the most reported cases. *Vibrio* spp., natural constituent of marine waters may be associated with shellfish associated disease outbreaks. *V. cholerae* and *V. vulnificus* related cases were reported rarely (Cantet *et al*., 2013; Carnahan *et al*., 1994; Hansen *et al*., 1993) associated with shellfish linked disease cases whereas, *V. parahaemolyticus* has been identified as an important agent (Odeyemi, 2016; Letchumanan *et al*., 2014; Li *et al*., 2014). Pathogenic serogroups of *E. coli* such as enteropathogenic and enterohaemorrhagic strains including O157:H7 have been rarely isolated from bivalves (Brandao *et al*., 2017; Bennani *et al*., 2011). Contamination of shellfish by other pathogenic bacteria which are not indigenously found in estuarine environments may be due to either faecal contamination of shellfish growing areas, contamination of shellfish products by infected food handlers, or storage in inappropriate unhygienic conditions. Outbreaks due to other pathogens such as *Clostridium perfringens, Salmonella,* and *Shigella* etc. have not been identified as significant threat to shellfish associated food safety (Lunestad *et al*., 2016; Rahimi *et al*., 2013; Kvenberg 1991; Grau, 1989).

In addition, the contamination of shellfish with bio-toxins, most of them potentially lethal derived from toxic algal blooms, also represents a significant threat to public health (Hall, 1991; Shumway, 1990).

5.2.2 Shellfish sanitation programmes

Depuration was originally introduced in North America in an attempt to reduce outbreaks of typhoid fever associated with the consumption of raw

shellfish (Canzonier, 1991). During the 1920's, several outbreaks of typhoid fever had occurred in USA due to consumption of contaminated Eastern oysters (*Crassostrea virginica*). Subsequently, microbiological standards were established in USA for shellfish growing waters, ultimately implementing growing area classification (Dressel and Snyder, 1991).

Statutory bodies of various countries such as United States (National Shellfish Sanitation Programme (NSSP), 2015), European Union (EC No 854/2004), New Zealand (New Zealand Food Safety Authority (NZFSA), 2006) Korea (Korea Ministry of Food and Drug Safety (KMFDS, 2015; Ministry of Oceans and Fisheries (MOF), 2015), Australian Shellfish Sanitation Program (ASQAP) etc. have formulated guidelines to classify growing areas, based on which post-harvest treatments of shellfish have to be planned. The classification is based on faecal coliform or *E. coli* levels either in shellfish harvesting waters (NSSP, 2015) or in bivalve tissue and intravalvular fluids (EC No 854/2004). (Refer section 2.2.3.1).

Nowadays depuration procedures, quality assurance and sanitation procedures have been adopted in shellfish producing areas in various other geographical locations also, such as Canada, Spain, France, Denmark, Italy, Turkey, South East Asia, etc. as reviewed by Otwell *et al*. (1991).

Eventhough depuration is not mandatory in India there are a few published reports from India, on the depuration of oysters and clams (Chinnadurai *et al*., 2014; Nambudiri *et al*., 1995; Balachandran and Surendran, 1984). Nambudiri *et al*. (1995) reported complete depuration of *V. cyprinoides* within 15 h in a UV based closed depuration system. In contrast, Balachandran and Surendran (1984) reported only one log reduction of FC and FS in *V. cyprinoides* during the first 18 h of depuration.

However, they did not employ any depuration water disinfection processes, except for initial chlorination. Pillai and Selvan (1987) attempted various depuration methods for *Crassostrea madrasensis* and found chlorination was the most effective method. Chinnadurai *et al*. (2014) also attempted the depuration of the aforementioned oyster using catridge filtered and UV treated seawater and found that differential rates of elimination of faecal coliforms from surface held and bottom held oysters; the former were purified within 24 h whereas the latter took 48 h for purification.

5.2.3 Indicators of shellfish sanitary quality

Total coliforms, faecal coliforms and *E. coli* are the most commonly used indicators of faecal contamination in shellfish as reviewed by Hackney and Pierson (1994). However, of the three indicators listed, total coliforms may be present in other environmental sources also, hence provide least information regarding faecal contamination, whereas faecal coliforms and *E. coli* assays give reliable information on faecal pollution. However, care should be taken while making these assays as sometimes, the cells subjected to sub lethal stress may be in the viable but non-culturable state (VBNC), as a result of which under-estimation of cells may occur, which is a limitation of existing methodologies (Kator and Rhodes, 1994).

Though most of the regulatory agencies rely on FC or *E. coli* levels to determine the suitability of a growing area for shellfish growing or sanitary quality of the harvested shellfish, it has been found to not always correlate well with the presence of other pathogens such as *Vibrio* spp. or *Salmonella* spp. (Kfir *et al*., 1993), enteric viruses such as Hepatitis A (Le Guyader *et al*., 1993; Cole *et al*., 1986) etc. Two studies by DePaola *et al*. (2010) and Brands *et al*. (2005), reported the presence of *Salmonella* in live oysters collected

from waters approved for shellfish harvesting in the US. The presence of pathogenic *Vibrio* species such as *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* also cannot be predicted using faecal coliforms as they are believed to be part of the indigenous microflora of estuarine waters and their numbers have been found to increase during warmer months. Hence in Washington State, in 1997 several shellfish growing areas were remained closed during summer months (Anon, 1997). Several other closures of shellfish harvesting areas have been reported (MDE, 2016; US EPA, 2013; VDH, 2012). These findings questions the reliability of the system of approving areas for shellfish growing based on faecal coliform levels alone and intensive research to identify more reliable indicators of sanitary quality of shellfish has to be carried out.

Even though many attempts for other suitable indicators such as male-specific bacteriophage (Dore and Lees, 1995) have been made, Kator and Rhodes (1994) concluded that there was little prospect of identifying a universally applicable indicator of human faecal contamination.

Most of the European Union members followed stipulated minimum period of 48 hours depuration before the regulations came into existence. None of the present regulations insist any particular period of depuration to be done; however, it is stated that the period must be sufficient to meet the microbiological as well as general shellfish quality standards. As stated by WHO (Rees *et al*., 2010), live bivalves sold should meet following requirements:

1) they should be fresh and viable;

2) they must not contain marine biotoxins above prescribed limits and

3) *E. coli* levels in depurated bivalve tissue should be ≤230/100 g and *Salmonella* spp. not detected in 25 g (EC Regulation No. 2074/2005).

5.2.4 Factors affecting depuration

The most important factor that affects the success of a depuration process is the well being of the animals themselves as the process exploits the natural physiological functions of the gastrointestinal tract of shellfish. Only healthy and actively feeding animals can be depurated effectively. Other factors that influence depuration are the following;

5.2.4.1 Particle size and abundance

Particle size and abundance are found to influence feeding activity in oysters. Attached bacteria are more efficiently consumed because of larger particle size compared to free bacteria (Kramer *et al*., 2016). Particles greater than ca. 5 μm are captured with about 100% efficiency compared to smaller ones and the efficiency decreased asymptotically with decreasing particle size (Riisgard, 1988).

5.2.4.2 Temperature and salinity

The temperature and salinity of depuration water have been found to significantly influence the efficacy of depuration by modulating shellfish physiological processes such as filtration rate, gastric emptying, spawning etc. (Phuvasate and Su 2013, 2012; Shumway, 1996; Fleet, 1978). Uncontrolled temperature increases have been demonstrated as a stimulus to induce spawning of Sydney rock oysters (Frankish, 1989) and Pacific oysters (Arakawa, 1990) and may promote a build up of microorganisms in the shellfish or depuration water. Phuvasate and Su (2012) found that depuration at 7-15 °C was found to be more effective than very low

temperatures and can be used as a post harvest treatment. Increasing the salinity of process water by 10-20% has been found to inhibit oyster spawning, especially when coupled with effective temperature control. Phuvasate and Su (2013) observed that faster depuration of Pacific oysters (*Crassostrea gigas*) was facilitated by higher salinities such as 20-30 ppt compared to lower salinities such as 10 ppt. Buisson *et al*. (1981) demonstrated the influence of temperature on the removal of faecal coliforms from Pacific oysters; the oysters were successfully depurated within three days to acceptable levels of <230 faecal coliforms per 100 g of tissue at 17 to 21 °C whereas at 10.2 °C only a marginal reduction in bacteria was achieved and little or no reduction was observed at 5 °C.

5.2.4.3 Turbidity

Turbidity of the depuration water is also associated with reduced depuration efficacy. Excessive levels of suspended particles may adversely affect depuration by concentrating the pathogens thus increasing their uptake by shellfish (Millard *et al*., 1987; Landry *et al*., 1983; Metcalf *et al*., 1979), reducing the filtration rate, and interfering with the penetration of UV light or other disinfecting agents. Spawning has been found to increase water turbidity which in turn adversely affects depuration efficiency (Frankish, 1989). However many researchers have reported that despite turbidity the depuration rates were not affected (Sobsey and Jaykus, 1991; Bird *et al*., 1990).

5.2.4.4 Effect of initial load

Initial shellfish microbe loading has been found to significantly affect the ability of the shellfish to subsequently remove microbes during depuration (De Mesquita *et al*., 1991); Cook and Ellender, 1986). De Mesquita *et al*. (1991) reported that successful depuration of naturally

contaminated mussels with an initial load > 5,000 *E. coli* per 100 g of mussel could not be accomplished to meet the bacteriological standard of 230 *E. coli* per 100 g tissue. Similar observation was made by Cook and Ellender (1986), who found that it was not possible to remove polio virus from oysters with high initial viral loads during relaying.

5.2.4.5 Tissue distribution of microbes in shellfish

The differential distribution of microbes within shellfish tissues has been reported to influence their subsequent elimination through depuration. Viral agents associated with food or faecal pellets depurated (Hay and Scotti, 1986; Di Giralamo *et al*., 1975), or closely associated with epithelial cells (Romalde *et al*., 1994) may be effectively purified while those disseminated into tissues other than gastrointestinal tract may not be effectively depurated (Di Giralamo *et al*., 1975).

5.2.4.6 Differential depuration of various microbes

Several authors have reported that different types of microbes are purged from shellfish at different rates. Nishio *et al*. (1981) reported that low numbers of *Salmonella typhi* persisted in *C. gigas* after more than 9 days of depuration which may be due to the capability of the organism to attach to the intestinal epithelium and subsequently disseminate to other tissues. Several authors have reported that the potentially pathogenic *Vibrio* spp. are not effectively eliminated by depuration (Jones *et al*., 1991b) or even the number of *Vibrio* spp. were reported to increase during depuration (Murphree and Tamplin, 1995; Tamplin and Capers, 1992). Persistence of Hepatitis A virus after depuration in re-circulating systems incorporating UV disinfection has been reported by Sobsey *et al*. (1987).

5.2.4.7 Comparative removal rates of viral and bacterial agents during depuration

The rate of elimination of viruses during depuration is slower compared to the rate of removal of bacteria (De Mesquita *et al*., 1991). Dore and Lees (1995) compared the depuration rates of bacteriophages to three bacterial indicators (*E. coli*, faecal streptococci and *Clostridium* spores) and found that bacteriophages had slower removal rates.

5.2.5 Depuration systems

Several types of depuration systems have been designed by various researchers. Fish box-type systems which consist of a number of high density polyethylene containers stacked on top of one another, Tray-type systems which consist of a number of trays positioned above one another and Pool-type systems which consist of a large tank constructed with a gradient towards one end are some of the popular types as reviewed by reviewed by Ayres (1991). Water is usually re-circulated after passing through an appropriate disinfection unit. Different approaches such as use of single or cocktail of bacteriophages (Pereira *et al*., 2017) and probiotic bacteria isolated from shellfish have been attempted in depuration by several researchers (Fajardo *et al*., 2014).

5.2.5.1 Various methods of depuration process water disinfection

Appropriate water disinfection system capable of inactivating microbes, is extremely important for effective shellfish depuration. As reviewed by Otwell *et al*. (1991) the currently used disinfection systems employ ultraviolet radiation (Australia, Denmark, Malaysia, New Zealand, Philippines, Singapore, United Kingdom and United States), ozone (France, Spain and Australia), chlorine (France, Italy and Spain) and iodophors (Italy and Spain).

5.2.5.1.1 Ultraviolet radiation

Usage of UV radiation for disinfection of depuration water has the advantage of producing few residuals in the water that may contaminate the shellfish. For this reason UV disinfection is preferred by US FDA to chlorine or ozone. However several variables such as dosage of radiation, flow rate of water past the lamp, the penetration of UV light etc. may affect the efficacy of disinfection process (Souness and Fleet, 1991). Suspended particles and shellfish derived particulate matter especially that observed during spawning etc. may interfere with UV mediated disinfection (Rowse and Fleet, 1984). Ramos *et al*. (2012) attempted depuration of oyster with a closed depuration system using a combination of UV light and chlorinated water and found it was useful in elimination of *V. parahaemolyticus* and *V. vulnificus*.

5.2.5.1.2 Ozone

Ozone is a potent chemical oxidant that reacts with organic materials, resulting in deactivation of microbes. It is known to be effective against Enteroviruses such as rotaviruses (Bitton, 1994; Roy *et al*., 1981). However due to factors such as increased cost and maintenance difficulties (Herrington, 1991), possible generation of mutagens (Bitton, 1994), and causing rancidity of shellfish tissue due to its oxidising action, it is not widely used in depuration water disinfection.

5.2.5.1.3 Chlorine

Chlorine acts as a disinfecting agent by causing oxidative damage to the external microbe surface and nucleic acids. Enteric bacteria are considered sensitive to chlorine, enteric viruses less so whereas protozoan cysts are resistant to chlorine (Bitton, 1994). However, due to certain disadvantages such as secondary production of carcinogenic trihalomethane,

interference with shellfish pumping (Blogoslawski, 1991) and imparted taste to shellfish which in turn reduces its marketability (Rodrick and Schneider, 1991) it is not a widely used disinfecting agent. However, Pillai and Selvan (1987) employed different depuration methods for *Crassostrea madrasensis* and observed that chlorination was the best method.

5.2.5.1.4 Iodine/Iodophors

Scientific literature describing the use of iodine or iodophors for disinfection of depuration water is limited. Even though iodine can be used to disinfect water supplies, the possible health implications due to higher doses of iodine in the body (Troyan and Hansen, 1989) have discouraged its use as disinfectant. However, iodophors have been used in Italy for some molluscan depuration operations.

5.2.5.1.5 Activated charcoal/carbon filters

The practice of using activated carbon to remove harmful impurities from water has existed since Roman times. Activated carbon refers to a family of carbonaceous adsorbents with a highly amorphous form and extensively developed internal pore structure. The sources of activated carbon are mostly, coal, coconut shells, peat and petroleum based residues. Activated carbon from coconut shell has predominantly pores in micro pore range (diameter in the range of less than 4 nm), which forms almost 85-90% surface area of coconut shell. Coconut shell-based activated carbon has the advantages of being least dusty and derived from renewable resources. Activated carbon filters are used in a number of home or municipal (pre-treated or chlorinated) water treatment systems to reduce or eliminate bad tastes, odours, chlorine, and many organic contaminants. The removal of contaminants from water is believed to be due to two principal mechanisms;

adsorption, and catalytic reduction, a process involving the attraction of negatively-charged contaminant ions to the positively-charged activated carbon. It is found to remove particles down to 0.5 micron, including *Giardia* and *Cryptosporidium* but does not eliminate bacteria or viruses.

No literature regarding use of activated carbon to disinfect process water in depuration systems is available, except that it was used to remove chlorine residues after disinfection of process water in a depuration plant in Italy (Rees *et al*., 2010; Casali *et al*., 1981).

In this present study, we evaluated efficiency of a simple home based closed water depuration system, using coconut shell based activated carbon filter to purify naturally contaminated *V. cyprinoides*.

5.3 Objectives

- To study depuration of naturally contaminated *Villorita cyprinoides* using a closed water depuration system fitted with activated carbon filter with special reference to reduction of total coliforms (TC), faecal coliforms (FC) and faecal streptococci (FS).
- To compare the relative depuration rates of total coliforms, faecal coliforms and faecal streptococci to evaluate their suitability as indicators of effective bivalve depuration

5.4 Materials and Methods

5.4.1 Collection of bivalve shellfish (*Villorita cyprinoides*)

Bivalve shellfish (*Villorita cyprinoides*) collected from Cochin estuary was used for depuration experiments. Sample collection was done as described in detail in section 2.4.2.1.

5.4.2 Bacteriological analysis of the sample

5. 4.2.1 Sample processing and enumeration of total coliforms

Total coliform levels in shellfish, sediment and harvesting waters were enumerated by 3 tube three decimal dilution (1 g, 0.1 g and 0.01 g) most probable number (MPN) method using EC broth (Hi-media, India), as described in detail elsewhere (BAM, 2011; Hitchin *et al*., 1995; APHA, 1970). Approximately 15-20 medium sized shellfishes were surface cleaned, aseptically shucked and about 25 g of meat and liquor was transferred to a sterile stomacher bag and blended with 225 mL of sterile peptone water in a stomacher (IUL Instruments, Spain). Ten mL samples were inoculated into 10 mL double strength EC broth; 1mL and 0.1mL samples were inoculated into single strength EC broth of 9 mL and 9.9 mL respectively, all containing inverted Durham's tubes. Similarly, appropriately diluted sediment and water samples (1: 9 ratio) in 10 mL, 1 mL and 0.1 mL quantities were also inoculated into respective dilution tubes with inverted Durham's tubes, as mentioned above. The inoculated tubes were incubated at 37 °C for 24 h and observed for growth and gas production. Tubes showing growth and gas production were recorded as TC positive and compared with the MPN table to calculate the MPN index. The results were expressed as MPN index/100 g of shellfish/sediment or MPN index/100 mL of harvesting waters. One loopful from positive EC broth tubes showing growth and gas production was streaked simultaneously onto Eosin methylene blue (EMB) agar (Hi-media, India) and Hicrome *E. coli* agar (Hi-media, India) plates and incubated at 37 °C for 24 h. After incubation, the plates were examined, typical colonies from EMB (green metallic sheen) and Hicrome *E. coli* agar (blue-green) were re-streaked and purified and transferred to nutrient agar (Hi-media, India) slants, for further characterization.

5.4.2.2 Enumeration of faecal coliforms

Faecal coliforms in shellfish samples were enumerated as described in section 2.4.3.1.1

5.4.2.3 Enumeration of faecal streptococci

Faecal streptococci in shellfish samples were enumerated as described in section 2.4.3.2.1

5.4.3 Depuration experiments

Prior to the experiments, the clams (*Villorita cyprinoides*) were observed to confirm that the specimens were alive and actively feeding.

5.4.3.1 Design of depuration tank and depuration process.

The depuration system consisted of closed water holding glass tank with nominal capacity of 53 litres and dimensions 51x36x34 cm. A wall hung immersion water pump (Dophin P-708, China), placed 15 cm above the bottom of the tank (to avoid recirculation of settled faecal material) re-circulated (18 litres/min) the sea water in the depuration tank which was then passed through an activated carbon filter (coconut shell based charcoal) held within a holder placed above the tank. Before starting the experiment, activated charcoal was backwashed to remove the fine carbon particles that may be present. For every batch of depuration, 45 litres of autoclaved natural seawater with salinity adjusted to 10 ppt and pH 7.3 was used. The experiment was carried out at ambient temperature (29-30 °C).

Approximately, 80 medium sized clams were arranged in monolayer on a plastic mesh tray which was suspended 20 cm above, from the tank bottom to prevent re-contamination from the faecal material settled at the bottom. About 4-5 shellfishes were taken out at various intervals of 0, 6, 12, 24, 72 and 96 h using a sterile spatula and total coliforms, faecal coliforms and faecal streptococci

were enumerated as described above. Clams survived well throughout the experiment, however, any dead ones if found were removed from the system immediately. Clams were not fed during the entire period of depuration process.

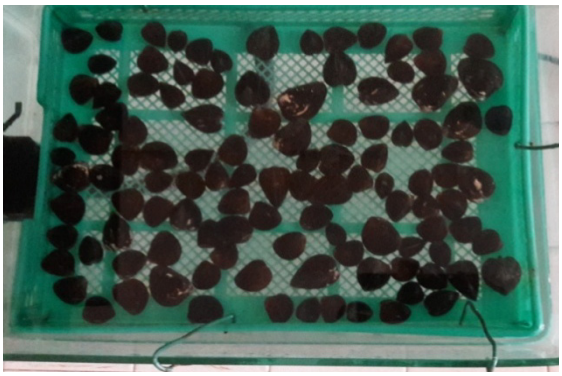

Figure 5.1 Bivalves arranged in monolayer on a plastic mesh tray for depuration

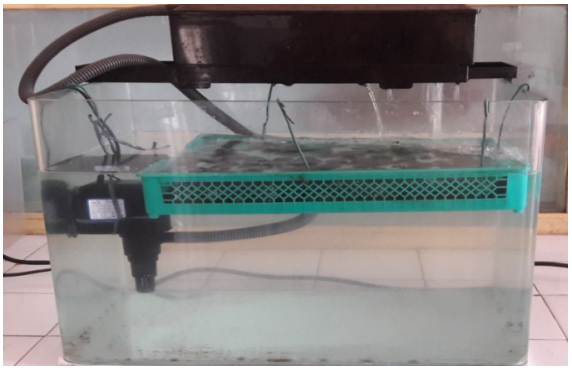

Figure 5.2 Depuration tank with oysters undergoing depuration

5.4.4 Statistical analysis

Statistical analysis of the results in this study was performed using SPSS software 20 (Statistical Package for Social Science). The differences in the reduction of individual test organisms under varying time intervals and the relative reduction of various test organsisms were analysed for significance using one-way analysis of variance (ANOVA) with Duncan's multiple range test. Significance level was set at $\alpha = 0.05$.

5.5 Results

Closed water depuration system using activated carbon filter was found to be effective in reducing the TC, FC and FS counts of naturally contaminated *V. cyprinoides* to acceptable limits.

5.5.1 Depuration of total coliforms (TC) using closed water depuration system fitted with activated carbon filter

The initial TC count was found to be 5.04 logs (1.1×10^5 MPN/100 g). One log reduction was obtained within the first 6 hours of depuration, and further 2.5 logs reduction was observed after 96 hours of depuration to a final TC count of 1.4 logs (Figure 5.3). Thus during the entire 96 h depuration process using activated carbon, a total reduction of 3.6 logs could be accomplished. However, complete depuration of TC could not be attained and even after 96 h depuration TC load of 1.4 logs remained in shellfish. The rates of reduction was found to be significant up to 12 hours (p=0.01) whereas during the remaining intervals, reduction was not statistically significant.

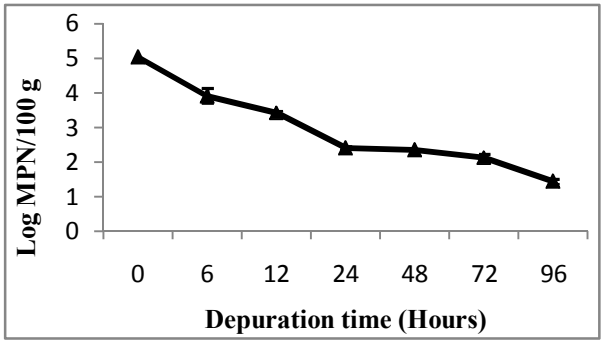

Figure 5.3 Reduction of total coliforms in naturally contaminated *V. cyprinoides* from Cochin estuary during closed water depuration using activated carbon filter

5.5.2 Depuration of faecal coliforms (FC) using closed water depuration system fitted with activated carbon filter

The initial FC count in naturally contaminated *V. cyprinoides* from Cochin estuary was found to be 4.7 logs (4.6 x 10^4 MPN/100 g) which conformed only to the microbiological standards of class C shellfish growing area of EU regulation. One log reduction was achieved within the first 6 hours of depuration; later the rate of removal slowed down until the 12th hour (Figure 5.4). Within 24 hours, the FC load in shellfish could be reduced to < 230MPN/100 g, while total depuration of FC was observed within 48 h. Thus using closed water depuration using activated carbon, a total reduction of 4.7 logs in FC count could be obtained within 48 hours. The rates of reduction of FC was found to be statistically significant (p=0.01).

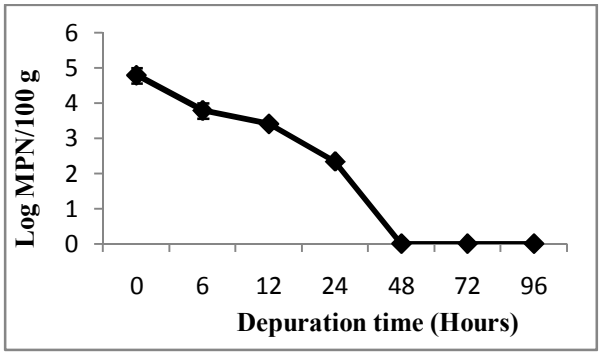

Figure 5.4 Reduction of faecal coliforms in naturally contaminated *V. cyprinoides* from Cochin estuary during closed water depuration using activated carbon filter

5.5.3 Depuration of faecal streptococci (FS) using closed water depuration system fitted with activated carbon filter

Figure 5.5 shows the complete depuration of FS levels in *V. cyprinoides* samples from an initial count of 5.04 logs (1.1 × 10^5 MPN/100 g). Initial

reduction of 1.4 logs was obtained within the first 6 hours of depuration, and further reduction of 3.5 logs to complete depuration took place within total 48 hours of depuration. The rates of reduction of FS at various time intervals was found to be statistically significant (p=0.01).

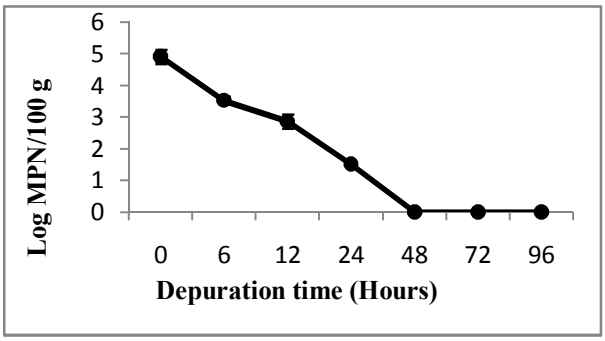

Figure 5.5 Reduction of faecal streptococci in naturally contaminated *V. cyprinoides* from Cochin estuary during closed water depuration using activated carbon filter

5.5.4 Relative depuration of TC, FC and FS using closed water depuration system fitted with activated carbon filter

The relative depuration of TC, FC and FS from naturally contaminated *V. cyprinoides* is given in Figure 5.6. Complete depuration of FC and FS were achieved within 48 hours, whereas TC could not be depurated completely even after 96 h. However the TC count was reduced by 3.64 logs to a final count of 1.4 logs (30 MPN/100 g). Among FC and FS, the former was reduced by 2.4 logs within 24 hours whereas the latter underwent a reduction by 3.4 logs. In case of TC, FC as well as FS the maximum rate of depuration was observed within the initial 6 h alike. The rates of reduction between various test organisms was found to be statistically significant (p=0.01).

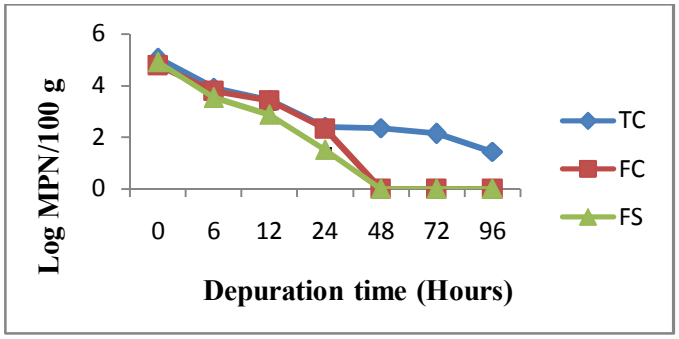

Figure 5.6 Relative depuration of TC, FC and FS from naturally contaminated *V. cyprinoides* using closed water depuration using activated carbon filter

5.6 Discussion

The closed water depuration system attached with activated carbon filter made from locally available material (coconut shell) attempted in present study was found to be effective for purification of naturally contaminated *V. cyprinoides* harvested from Cochin estuary. Depuration and relaying have been shown to reduce faecal coliforms and other enteric bacteria to acceptable regulatory levels (Obadai *et al*., 2010; Jones *et al*., 1991a; Power and Collins, 1989) which will help to minimise the public health risk from faecal-borne bacterial pathogens in purified shellfish (Oliveira *et al*., 2011; Richards, 1988).

Maximum rate of reduction of all the three groups of organisms was observed in the first 6 hours of depuration during which TC and FC exhibited 1 log reduction while FS exhibited slightly higher reduction of 1.4 logs. Within 24 h of depuration, shellfish FC levels could be reduced to < 230MPN/100 g (reduced by 2.5 logs i.e. 53% reduction) which falls in the acceptable FC regulatory limits of depurated shellfish. This indicates very good efficiency of the depuration system. This is in agreement with the

results of several previous researchers where shellfish were depurated to regulatory limits within 24 h (Andritsos *et al*., 2016; Power and Collins, 1989). In agreement to our findings Andritsos *et al*. (2016) also observed steep decline during the initial 10 h of depuration. Similar finding was made by (Nambudiri *et al*., 1995) where complete depuration of *V. cyprinoides* was accomplished in a UV based depuration system within 15 h of depuration. In contrast, in another study from India, Balachandran and Surendran (1984) reported one log reduction of FC and FS in *V. cyprinoides* within the first 18 h which was lower compared to our results. However they did not employ any water disinfection processes, except for initial chlorination. Greater reduction accomplished during our study may probably point to the efficiency of coconut shell based charcoal for process water treatment.

Among the three categories of sanitary quality indicators used (TC, FC and FS), removal of FC and FS demonstrated almost similar trends, whereas TC took comparatively longer depuration time. However this is not in agreement with the findings of Love *et al*. (2010) who demonstrated that *E. coli* depurated faster than *E. faecalis* from oysters (*Crassostrea virginica*) and hard shell clams (*Mercinaria mercinaria*) in a flow-through depuration system under variable environmental conditions. In present study, we could remove FS as well as FC completely within 48 h depuration. This is in agreement with the findings of Casali *et al*. (1981) where 99% elimination of *Enterococcus* from hen clams could be achieved within 48 h. Our results are in agreement with the findings of Chinnadurai *et al*. (2014), where differential rates of purification were observed for total coliforms, faecal coliforms and faecal streptococci. Maffei *et al*. (2009) also observed differential elimination rates for *E. coli* and faecal coliforms; faecal coliforms reduced faster than *E. coli*.

However, several previous studies have proved faster rates of depurations of both these faecal indicators compared to viruses such as poliovirus and Hepatitis A virus (Le Guyader *et al*., 1993; Cole *et al*., 1986). These findings question the reliability of faecal indicators such as *E. coli* as indicators of depurated shellfish sanitary quality, as they poorly correlate with viral. Similarly, poor correlation of faecal coliforms with presence of *Vibrio* spp. or *Salmonella* spp. have been reported by many researchers which again questions their reliability as sanitary indicators (DePaola *et al*., 2010; Brands *et al*., 2005; Kfir *et al*., 1993).

Careful maintenance of optimum conditions such as temperature, pH, salinity, rate of water circulation etc. needs special mention as optimum conditions, minimise the stress to the oysters which would have otherwise impaired the depuration process (Power and Collins, 1989). Favourable tropical temperature is an important factor that influences depuration efficiency (Phuvasate *et al*., 2012), as lower temperatures reduced the efficiency compared to elevated temperatures (Buisson *et al*., 1981). However elevated temperatures may induce spawning which increases the water turbidity and reduce filtration rates which in turn impede depuration (Arakawa, 1990; Frankish, 1989).

In conclusion, our closed water recirculation depuration system with activated charcoal made from locally available coconut shell waste was found to be effective for the depuration of naturally contaminated *V. cyprinoides*. Though it is effective in purifying moderate levels of microbial contamination, its efficiency in purifying higher levels of microbial contamination has to be thoroughly assessed. This is suitable for household purposes as it uses simple, cost effective, easily available, natural and renewable water treating agent

such as coconut shell based activated carbon. However, for commercial purposes activated charcoal based depuration could be coupled with a suitable antimicrobial agent that kills bacteria or viruses. Commercial establishments require much more accurate and efficient water treatment systems since the sanitary quality of the product to be marketed should meet global standards. However in India, this bivalve species does not have much export value, rather it is largely consumed in the domestic market itself and provide livelihood for thousands of fishermen community. Moreover, in India bivalve is not a commodity preferred to be eaten raw, hence less stringent, cost effective, simple depuration measures as mentioned above may be sufficient enough to meet the required sanitary quality as it is consumed only after proper cooking.

Chapter 6

SUMMARY AND CONCLUSION

Contents

6.1 Summary

Present study deals with the assessment of the bacteriological quality of water, sediment as well as shellfish (*Villorita cyprinoides* var. *cochinensis*) from selected shellfish harvesting areas of Cochin estuary. Bacteriological quality is assessed in terms of faecal indicator bacteria and specific pathogens such as diarrheagenic *E. coli, V. parahaemolyticus* and *Salmonella*. Samples were collected on a seasonal basis from eight stations in order to study the temporal and spatial variations in quality. Risk assessment of the isolated bacterial species in terms of prevalence of antibiotic resistance among these strains, presence of specific antibiotic resistance genes and other pathogenic/virulence genes was also undertaken. Microcosm studies were undertaken to determine the effect of various environmental factors such as temperature, salinity, biotic factors etc. on the survival of the selected strains of pathogens in estuarine water and sediment. Finally, depuration of naturally contaminated *Villorita cyprinoides* from Cochin estuary using a simple closed water depuration system, fitted with activated carbon filter (made from natural, locally available, eco-friendly material such as coconut shell waste) was also attempted, in order to check its efficacy to be used as a simple household depuration system.

Salient findings of the study are summarized as follows:

- High levels of faecal coliform and faecal streptococci were found in shellfish, sediment and harvesting waters in monsoon and post monsoon seasons compared to pre-monsoon season ($p<0.001$).
- Shellfish and sediment samples demonstrated higher faecal coliform and streptococci levels compared to harvesting waters during pre-monsoon season while the difference was not so prominent in the other two seasons.
- According to EU criteria stations 1, 3, 4, 5, 6 and 7 were classified under 'C'class shellfish harvesting area during all the seasons. Stations 2 and 8 were classified at 'B' level during pre-monsoon season and 'C' during monsoon and post-monsoon seasons. According to NSSP criteria all the stations were classified under 'prohibited areas' during all the 3 seasons.
- The prevalence of pathogenic *E. coli* serogroups in *V. cyprinoides*, sediment and water samples were 80.77%, 100% and 90.91% respectively.
- Enterotoxigenic strains of *E. coli* exhibited the maximum prevalence in *V. cyprinoides* and water samples, whereas in sediment samples enterohaemorrhagic strains were encountered more frequently.
- Among the identified serogroups of *E. coli*, the most prevalent one in *V. cyprinoides* was O8 (18.18%), where as O157:H7 and O121 (11.10% each) was more prevalent in sediment samples. In water samples O141 and O8 (21.40% each) were more frequently encountered.

- Incidence of *V. parahaemolyticus* was very high in shellfish samples (83.33%), followed by sediment samples (50%) and water samples (33.33%).
- Prevalence of *Salmonella* was very low. Only one water sample from station 1 yielded positive isolation of *Salmonella*.
- Prevalence of MDR *E. coli* strains in *V. cyprinoides*, sediment and water samples were 98%, 97.4% and 97.3% respectively.
- *E. coli* from shellfish showed maximum overall antibiotic resistance followed by those from sediments and harvesting waters ($p<0.001$).
- *E. coli* from station 3 exhibited the maximum overall antibiotic resistance, while station 2 exhibited the least ($p<0.001$).
- *E. coli* strains collectively exhibited resistance towards all the antibiotics tested and strains showed high MAR indices.
- High level resistance was shown towards the last line drug of choice colistin (78.4%) and resistance against meropenem (24%) also was quite alarming.
- Percentage of *E. coli* from *V. cyprinoides*, sediment and water samples which showed resistance to ≥ 8 antibiotics were 84%, 73.7% and 51.4 % respectively.
- All the enterococci strains isolated from *V. cyprinoides*, sediment and water samples were multidrug resistant, with average MAR indices of 0.63, 0.68 and 0.55 respectively.
- All enterococci from all the samples were resistant towards ampicillin; two strains from sediment were resistant towards all the 19 antibiotics tested (MAR index=1).

- Remarkable resistance shown towards drugs meant to treat drug resistant enterococci such as vancomycin, teicoplanin, high level gentamicin, high level streptomycin, linazolid and rifampicin is quite alarming.
- The prevalence of MDR *V. parahaemolyticus* in *V. cyprinoides*, sediment and water was found to be 90%, 84.6% and 85.7% respectively.
- Enterococci strains from sediment showed maximum overall antibiotic resistance followed by those from shellfish and harvesting waters ($p<0.001$).
- Enterococci strains station 1 collectively exhibited maximum antibiotic resistance, while those from stations 5 and 7 exhibited the least ($p<0.001$).
- *V. parahaemolyticus* strains from *V. cyprinoides* and sediment showed an average MAR index of 0.37 while those from water showed an index of 0.47.
- *V. parahaemolyticus* strains from harvesting waters showed maximum overall antibiotic resistance followed by those from sediment and shellfish ($p<0.001$).
- *V. parahaemolyticus* strains from station 3 exhibited the maximum overall antibiotic resistance, while those from station 6 exhibited the lowest ($p<0.001$).
- *V. parahaemolyticus* strains from all the samples showed relatively low resistance towards tetracyclines. All the strains were sensitive to doxycycline.

- ESBL genes, $bla_{\text{CTX-M}}$ and bla_{TEM} were detected in 91.7% and 77.7% *E. coli* strains (screened among strains which showed complete phenotypic resistance to beta lactam antibiotics) respectively.
- The prevalence of both $bla_{\text{CTX-M}}$ and bla_{TEM} was in the order *E. coli* strains from shellfish> sediment >harvesting waters.
- All the serogroups which showed the presence of ESBL genes were pathogenic; maximum prevalence was recorded in EHEC, followed by ETEC, UPEC and EPEC serogroups.
- Among the various serogroups, the prevalence of both ESBL genes was in the order O157>O121> O8 followed by other minor serogroups.
- Plasmid encoded colistin resistance *mcr-1* gene was detected in one *E. coli* strain each isolated from shellfish and sediment from station 7, and one strain from shellfish harvested from station 5.
- Vancomycin resistance *VanA* gene was detected in one *E. coli* from sediment from station 1, and *vanB* was detected in shellfish from station 6.
- High prevalence of toxigenic genes was detected in *E. coli* and the prevalence among *E. coli* from shellfish, sediment and water samples were 84%, 78.1% and 69% respectively.
- Among the four toxigenic genes screened shiga toxin gene, stx_2 showed the maximum overall prevalence (54.3%), followed by intimin gene (*eae*) (36.2%), shiga toxin gene, stx_1 (20%), and enterohemolysin gene (*hlyA*) showed the least prevalence (6.7%).

- Maximum diverse toxigenic genotypes as well as co-occurrence of maximum number of toxigenic genes were exhibited by *E. coli* strains in the order shellfish> sediment >harvesting waters.
- Around 2% of *E. coli* strains showed co-occurence of all the four toxigenic genes screened- stx_1, stx_2, *eae* and *hlyA* and the sample implicated was shellfish while the serogroup identified was O121.
- Virulence genes were detected in 25.8% of total *V. parahaemolyticus* strains.
- *V. parahaemolyticus* strains from sediment showed maximum prevalence of virulence genes, followed by strains from shellfish whereas strains from water lacked virulence genes.
- Around 19% of total *V. parahaemolyticus* strains exhibited the presence of *trh* gene, 3.2% strains showed *tdh* gene, while 3.2% (isolated from sediment) showed co-occurence of both *tdh* and *trh* genes
- Better survival of all the three test organisms was observed at lower temperatures such as 25 °C compared to higher temperatures.
- *E. coli* showed maximum survival at 0 ppt salinity whereas *V. parahaemolyticus* showed better survival at 30 ppt salinity. Salinity variation had no effect on survival of *E. faecalis*.
- Biotic factors had significant impact on survival of all the three test organisms.
- Protozoan predation was found to have not much effect on survival of test organisms in sediment and water

- Bacteriophages were found to be an important biological factor affecting survival of both *E. coli* and *V. parahaemolyticus* in Cochin estuary.
- Coliphage count in harvesting water from Cochin estuary was found to be 7.5×10^3 PFU/mL and vibriophage count was 2×10^3 PFU/mL.
- The closed water depuration system attached with activated carbon filter made from locally available material (coconut shell), attempted in present study was found to be effective for purification of naturally contaminated *V. cyprinoides* harvested from Cochin estuary.

6.2 Conclusion

Present study reveals the poor sanitary quality of the shellfish growing areas located along Cochin estuary, which can be attributed to the high level of faecal contamination the estuary has undergone. Consequently, the sanitary quality of the shellfish harvested from Cochin estuary also did not conform to legal standards during most of the seasons. Moreover, high prevalence of pathogenic *E. coli* serogroups, which included even the most virulent enterohaemorrhagic *E. coli* O157:H7 from sediments as well as shellfish from this estuary is quite alarming. Presence of pathogenic strains in sediments has to be seriously dealt with because even if the overall water quality is improved, the underlying sediment may act as permanent repository, releasing pathogens to the overlying water column, in case of events such as storms or dredging. Risk assessment revealed that Cochin estuary is a significant environmental reservoir of antibiotic resistant bacteria, which harboured a pool of antibiotic resistance genes, ready to be disseminated to other human pathogens or environmental organisms. It may

thus serve as a permanent reservoir of antibiotic resistant genes resulting in antimicrobial resistance, cycling through environment, food and human sources. In addition, they also have been found to harbour several toxigenic/ virulence genes; most of which are located on mobile genetic elements which can also be horizontally transmitted.

Another disturbing fact is that the prevalence of drug resistance, resistance genes, toxigenic as well as virulence genes was high in bacterial pathogens from shellfish samples compared to sediments and water samples. This can be attributed to their peculiar filter-feeding habit, due to which they concentrate the pathogens and contaminants in their tissues above threshold limits, while it is present in the surrounding waters in comparatively lower levels. Though the only way to improve the microbial quality of shellfish is to improve the sanitary quality of the growing areas, at present no such monitoring of shellfish growing areas exist in India. Present study reveals the probable health hazards associated with consumption of shellfish harvested from this estuary and emphasises on the necessity of regulatory interventions in this regard, for which present findings may form a baseline reference. The simple closed water depuration system, attached with activated carbon filter made from natural, locally available, eco-friendly material (coconut shell waste) attempted in present study was found to be an effective household depuration system for purification of naturally contaminated *V. cyprinoides* harvested from Cochin estuary.

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GenBank Submissions

a) *Vibrio alginolyticus* strain VKF44 16S ribosomal RNA gene, partial sequence

GenBank: KT005561.1

LOCUS KT005561 1379 bp DNA linear BCT 05-OCT-2015
DEFINITION Vibrio alginolyticus strain VKF44 16S ribosomal RNA gene, partial sequence.
ACCESSION KT005561
VERSION KT005561.1
KEYWORDS .
SOURCE Vibrio alginolyticus
ORGANISM Vibrio alginolyticus
Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1379)
AUTHORS Antony,A.C., Silvester,R., Alexander,D. and Hatha,M.
TITLE Genotyping of Vibrio sp. using GRO EL RFLP
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1379)
AUTHORS Antony,A.C., Silvester,R., Alexander,D. and Hatha,M.
TITLE Direct Submission
JOURNAL Submitted (03-JUN-2015) Department of Marine Biology, Microbiology and Biochemistry., Cochin University of Science and Technology, School of Marine Sciences, Lake Side Campus, Fine Arts Avenue, Ernakulam, Kerala 682016, India
COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
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rRNA <1..>1379
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b) *Vibrio fluvialis* strain 8M1 16S ribosomal RNA gene, partial sequence

GenBank: KT163389.1

LOCUS	KT163389 1425 bp DNA linear BCT 05-OCT-2015
DEFINITION	Vibrio fluvialis strain 8M1 16S ribosomal RNA gene, partial sequence.
ACCESSION	KT163389
VERSION	KT163389.1
KEYWORDS.	
SOURCE	Vibrio fluvialis
ORGANISM	Vibrio fluvialis Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE	1 (bases 1 to 1425)
AUTHORS	Antony,A.C. and Hatha,M.
TITLE	Incidence of Vibrio parahaemolyticus in shellfish

JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1425)
AUTHORS Antony,A.C. and Hatha,M.
TITLE Direct Submission
JOURNAL Submitted (11-JUN-2015) Department of Marine Biology, Microbiology and Biochemistry, Cochin Univsersity of Science and Technology, Fine Arts Avenue, Ernakulam, Kerala 682016, India
COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

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c) *Vibrio parahaemolyticus* strain V10M1 16S ribosomal RNA gene, partial sequence

GenBank: KT163390.1

LOCUS KT163390 884 bp DNA linear BCT 05-OCT-2015
DEFINITION Vibrio parahaemolyticus strain V10M1 16S ribosomal RNA gene, partial sequence.
ACCESSION KT163390
VERSION KT163390.1
KEYWORDS.
SOURCE Vibrio parahaemolyticus
ORGANISM Vibrio parahaemolyticus
Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 884)
AUTHORS Antony,A.C. and Hatha,M.A.
TITLE vibrio parahaemolyticus O3:K6 in shellfish
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 884)
AUTHORS Antony,A.C. and Hatha,M.A.
TITLE Direct Submission
JOURNAL Submitted (11-JUN-2015) Department of Marine Biology, Microbiology and Biochemistry, Cochin Univsersity of Science and Technology, Fine Arts Avenue, Ernakulam, Kerala 682016, India
COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

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d) *Myroides odoratimimus* strain MJ1 16S ribosomal RNA gene, partial sequence

GenBank: KT163391.1

LOCUS	KT163391 1384 bp DNA linear BCT 05-OCT-2015
DEFINITION	Myroides odoratimimus strain MJ1 16S ribosomal RNA gene, partialsequence.
ACCESSION	KT163391
VERSION	KT163391.1
KEYWORDS.	
SOURCE	Myroides odoratimimus
ORGANISM	Myroides odoratimimus Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Myroides.
REFERENCE	1 (bases 1 to 1384)
AUTHORS	Antony,A.C. and Hatha,M.
TITLE	Better E.coli recovery
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 1384)
AUTHORS	Antony,A.C. and Hatha,M.
TITLE	Direct Submission
JOURNAL	Submitted (11-JUN-2015) Department of Marine Biology, Microbiology

and Biochemistry, Cochin Univsersity of Science and Technology,
Fine Arts Avenue, Ernakulam, Kerala 682016, India

COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

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e) *Vibrio alginolyticus* strain 39V7A2 16S ribosomal RNA gene, partial sequence

GenBank: KT163392.1

LOCUS	KT163392 1423 bp DNA linear BCT 05-OCT-2015
DEFINITION	Vibrio alginolyticus strain 39V7A2 16S ribosomal RNA gene, partialsequence.
ACCESSION	KT163392
VERSION	KT163392.1
KEYWORDS.	
SOURCE	Vibrio alginolyticus
ORGANISM	Vibrio alginolyticus Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE	1 (bases 1 to 1423)
AUTHORS	Antony,A.C., Hatha,M. and Paul,M.K.
TITLE	Vibrio species in shellfish
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 1423)
AUTHORS	Antony,A.C., Hatha,M. and Paul,M.K.
TITLE	Direct Submission
JOURNAL	Submitted (12-JUN-2015) Department of Marine Biology, Microbiology and Biochemistry, Cochin Univsersity of Science and Technology, Fine Arts Avenue, Ernakulam, Kerala 682016, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
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f) *Escherichia coli* strain EF2P4 16S ribosomal RNA gene, partial sequence

GenBank: KT804408.1

LOCUS	KT804408 1352 bp DNA linear BCT 10-FEB-2016
DEFINITION	Escherichia coli strain EF2P4 16S ribosomal RNA gene, partial sequence.
ACCESSION	KT804408
VERSION	KT804408.1
KEYWORDS.	
SOURCE	Escherichia coli
ORGANISM	Escherichia coli Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Enterobacteriaceae; Escherichia.
REFERENCE	1 (bases 1 to 1352)
AUTHORS	Antony,A.C. and Hatha,M.
TITLE	Prevalence of E.coli O157:H7 in shellfish

JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1352)
AUTHORS Antony,A.C. and Hatha,M.
TITLE Direct Submission
JOURNAL Submitted (18-SEP-2015) Department of Marine Biology, Microbiology and Biochemistry, Cochin Univsersity of Science and Technology, Fine Arts Avenue, Ernakulam, Kerala 682016, India
COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

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481 ctgggcgtaa agcgcacgca ggcggtttgt taagtcagat gtgaaatccc cgggctcaac
541 ctgggaactg catctgatac tggcaagctt gagtctcgta gaggggggta gaattccagg
601 tgtagcggtg aaatgcgtag agatctggag gaataccggt ggcgaaggcg gccccctgga
661 cgaagactga cgctcaggtg cgaaagcgtg gggagcaaac aggattagat accctggtag
721 tccacgccgt aaacgatgtc gacttggagg ttgtgccctg aggcgtggct tccggagcta
781 acgcgttaag tcgaccgcct ggggagtacg gccgcaaggt taaaactcaa atgaattgac
841 gggggcccgc acaagcggtg gagcatgtgg tttaattcga tgcaacgcga agaaccttac
901 ctggtcttga catccacaga actttccaga gatggattgg tgccttcggg aactgtgaga
961 caggtgctgc atggctgtcg tcagctcgtg ttgtgaaatg ttgggttaag tcccgcaacg
1021 agcgcaaccc ttatcctttg ttgccagcgg tccggccggg aactcaaagg agactgccag
1081 tgataaactg gaggaaggtg gggatgacgt caagtcatca tggcccttac gaccagggct
1141 acacacgtgc tacaatggcg catacaaaga gaagcgacct cgcgagagca agcggacctc
1201 ataaagtgcg tcgtagtccg gattggagtc tgcaactcga ctccatgaag tcggaatcgc
1261 tagtaatcgt ggatcagaat gccacggtga atacgttccc gggccttgta cacaccgccc
1321 gtcacaccat gggagtgggt tgcaaaagaa gt
//

g) *Enterococcus faecalis* strain SF2K2 16S ribosomal RNA gene, partial sequence

GenBank: KT804409.1

LOCUS KT804409 1403 bp DNA linear BCT 10-FEB-2016
DEFINITION Enterococcus faecalis strain SF2K2 16S ribosomal RNA gene, partialsequence.
ACCESSION KT804409
VERSION KT804409.1
KEYWORDS.
SOURCE Enterococcus faecalis
ORGANISM Enterococcus faecalis
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus.
REFERENCE 1 (bases 1 to 1403)
AUTHORS Antony,A.A. and Hatha,M.
TITLE Prevalence of vancomycin resistant enterococci in shellfish, harvesting waters and underlying sediments
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1403)
AUTHORS Antony,A.A. and Hatha,M.
TITLE Direct Submission
JOURNAL Submitted (19-SEP-2015) Department of Marine Biology, Microbiology and Biochemistry, Cochin Univsersity of Science and Technology, Fine Arts Avenue, Ernakulam, Kerala 682016, India
COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

FEATURES Location/Qualifiers
source 1..1403
/organism="Enterococcus faecalis"
/mol_type="genomic DNA"
/strain="SF2K2"
/host="shellfish"
/db_xref="taxon:1351"
/country="India"
/collection_date="12-Feb-2015"
rRNA <1..>1403
/product="16S ribosomal RNA"

ORIGIN

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     61 acacgtgggt aacctaccca tcagaggggg ataacacttg gaaacaggtg ctaataccgc
    121 ataacagttt atgccgcatg gcataagagt gaaaggcgct ttcgggtgtc gctgatggat
    181 ggacccgcgg tgcattagct agttggtgag gtaacggctc accaaggcca cgatgcatag
    241 ccgacctgag agggtgatcg gccacactgg gactgagaca cggcccagac tcctacggga
    301 ggcagcagta gggaatcttc ggcaatggac gaaagtctga ccgagcaacg ccgcgtgagt
    361 gaagaaggtt ttcggatcgt aaaactctgt tgttagagaa gaacaaggac gttagtaact
    421 gaacgtcccc tgacggtatc taaccagaaa gccacggcta actacgtgcc agcagccgcg
    481 gtaatacgta ggtggcaagc gttgtccgga tttattgggc gtaaagcgag cgcaggcggt
    541 ttcttaagtc tgatgtgaaa gcccccggct caaccgggga gggtcattgg aaactgggag
    601 acttgagtgc agaagaggag agtggaattc catgtgtagc ggtgaaatgc gtagatatat
    661 ggaggaacac cagtggcgaa ggcggctctc tggtctgtaa ctgacgctga ggctcgaaag
    721 cgtggggagc aaacaggatt agataccctg gtagtccacg ccgtaaacga tgagtgctaa
    781 gtgttggagg gtttccgccc ttcagtgctg cagcaaacgc attaagcact ccgcctgggg
    841 gagtacgacc gcaaggttga aactcaaagg aattgacggg ggcccgcaca agcggtggag
    901 catgtggttt aattcgaagc aacgcgaaga accttaccag gtcttgacat cctttgacca
    961 ctctagagat agagctttcc cttcggggac aaagtgacag gtggtgcatg gttgtcgtca
   1021 gctcgtgtcg tgagatgttg ggttaagtcc cgcaacgagc gcaaccctta ttgttagttg
   1081 ccatcattca gttgggcact ctagcgagac tgccggtgac aaaccggagg aaggtgggga
   1141 tgacgtcaaa tcatcatgcc ccttatgacc tgggctacac acgtgctaca atgggaagta
   1201 caacgagtcg ctagaccgcg aggtcatgca aatctcttaa agcttctctc agttcggatt
   1261 gcaggctgca actcgcctgc atgaagccgg aatcgctagt aatcgcggat cagcacgccg
   1321 cggtgaatac gttcccgggc cttgtacaca ccgcccgtca caccacgaga gtttgtatac
   1381 tacccgaagt cggtgaggta aca
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h) *Salmonella enterica* subsp. *enterica* serovar *Typhi* strain R1L3 16S ribosomal RNA gene, partial sequence

GenBank: KT804410.1

LOCUS	KT804410 1339 bp DNA linear BCT 10-FEB-2016
DEFINITION	Salmonella enterica subsp. enterica serovar Typhi strain R1L3 16Sribosomal RNA gene, partial sequence.
ACCESSION	KT804410
VERSION	KT804410.1
KEYWORDS.	
SOURCE	Salmonella enterica subsp. enterica serovar Typhi (Salmonella typhi)
ORGANISM	Salmonella enterica subsp. enterica serovar Typhi Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Enterobacteriaceae; Salmonella.
REFERENCE	1 (bases 1 to 1339)
AUTHORS	Ally,A. and Hatha,M.
TITLE	Occurence of salmonella in shellfish
JOURNAL	Unpublished

REFERENCE 2 (bases 1 to 1339)
AUTHORS Ally,A. and Hatha,M.
TITLE Direct Submission
JOURNAL Submitted (19-SEP-2015) Department of Marine Biology, Microbiology and Biochemistry, Cochin Univsersity of Science and Technology, Fine Arts Avenue, Ernakulam, Kerala 682016, India
COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

FEATURES Location/Qualifiers
source 1..1339
/organism="Salmonella enterica subsp. enterica serovar Typhi"
/mol_type="genomic DNA"
/strain="R1L3"
/serovar="Typhi"
/host="shellfish"
/sub_species="enterica"
/db_xref="taxon:90370"
/country="India"
/collection_date="14-Jan-2015"
rRNA <1..>1339
/product="16S ribosomal RNA"

ORIGIN
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61 ggaaacggtg gctaataccg cataacgtcg caagaccaaa gaggggacc ttcgggcctc
121 ttgccatcag atgtgcccag atgggattag ctagttggtg aggtaacggc tcaccaaggc
181 gacgatccct agctggtctg agaggatgac cagccacact ggaactgaga cacggtccag
241 actcctacgg gaggcagcag tggggaatat tgcacaatgg gcgcaagcct gatgcagcca
301 tgccgcgtgt atgaagaagg ccttcgggtt gtaaagtact ttcagcgggg aggaaggtgt
361 tgtggttaat aaccgcagca attgacgtta cccgcagaag aagcaccggc taactccgtg
421 ccagcagccg cggtaatacg gagggtgcaa gcgttaatcg gaattactgg gcgtaaagcg
481 cacgcaggcg gtctgtcaag tcggatgtga aatccccggg ctcaacctgg gaactgcatt
541 cgaaactggc aggcttgagt cttgtagagg ggggtagaat tccaggtgta gcggtgaaat
601 gcgtagagat ctggaggaat accggtggcg aaggcggccc cctggacaaa gactgacgct
661 cagtgcgaaa gcgtggggag caaacaggat tagataccct ggtagtccac gccgtaaacg
721 atgtctactt ggaggttgtg cccttgaggc gtggcttccg gagctaacgc gttaagtaga
781 ccgcctgggg agtacggccg caaggttaaa actcaaatga attgacgggg gcccgcacaa
841 gcggtggagc atgtggttta attcgatgca acgcgaagaa ccttacctgg tcttgacatc
901 cacagaactt tccagagatg gattggtgcc ttcgggaact gtgagacagg tgctgcatgg
961 ctgtcgtcag ctcgtgttgt gaaatgttgg gttaagtccc gcaacgagcg caacccttat
1021 cctttgttgc cagcggtccg gccgggaact caaaggagac tgccagtgat aaactggagg
1081 aaggtgggga tgacgtcaag tcatcatggc ccttacgacc agggctacac acgtgctaca
1141 atggcgcata caaagagaag cgacctcgcg agagcaagcg gacctcataa agtgcgtcgt
1201 agtccggatt ggagtctgca actcgactcc atgaagtcgg aatcgctagt aatcgtggat
1261 cagaatgcca cggtgaatac gttcccgggc cttgtacaca ccgcccgtca caccatggga
1321 gtgggttgca aaagaagta
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i) *Enterobacter asburiae* strain E19 16S ribosomal RNA gene, partial sequence

GenBank: KT804411.1

LOCUS KT804411 1357 bp DNA linear BCT 10-FEB-2016
DEFINITION Enterobacter asburiae strain E19 16S ribosomal RNA gene, partialsequence.
ACCESSION KT804411
VERSION KT804411.1
KEYWORDS.
SOURCE Enterobacter asburiae
ORGANISM Enterobacter asburiae
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Enterobacteriaceae; Enterobacter; Enterobacter cloacae complex.
REFERENCE 1 (bases 1 to 1357)
AUTHORS Ally,A. and Hatha,M.
TITLE Comparative evaluation of EMB agar and Chromogenic agar in isolation of E. coli
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1357)
AUTHORS Ally,A. and Hatha,M.
TITLE Direct Submission
JOURNAL Submitted (19-SEP-2015) Department of Marine Biology, Microbiology and Biochemistry, Cochin Univsersity of Science and Technology, Fine Arts Avenue, Ernakulam, Kerala 682016, India
COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

FEATURES	Location/Qualifiers
source	1..1357
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	/mol_type="genomic DNA"
	/strain="E19"
	/host="shellfish"
	/db_xref="taxon:61645"
	/country="India"
	/collection_date="12-Feb-2015"
rRNA	<1..>1357
	/product="16S ribosomal RNA"

ORIGIN

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      1 tttgccggcg agcggcggac gggtgagtaa tgtctgggaa actgcctgat ggaggggggat
     61 aactactgga aacggtagct aataccgcat aacgtcgcaa gaccaaagag ggggaccttc
    121 gggcctcttg ccctcagatg tgcccagatg ggattatcta gtaggtgggg taacggctca
    181 cctaggcgac gatccctatc tggtctgaga ggatgaccac ccacactgga actgagacac
    241 ggtccagact cctacgggag gcagcagtgg ggaatattgc acaatgggcg caagcctgat
    301 gcacccatgc cgcgtgtatg aaaaaggcct tcgggttgta aagtactttc agcggggagg
    361 aaggtgttga ggttaataac ctcagcgatt gacgttaccc gcaaaaaaag caccggctaa
    421 ctccgtgcca gcagccgcgg taatacggag ggtgcaagcg ttaatcggaa ttactgggcg
    481 taaagcgcac gcaggcggtc tgtcaagtcg gatgtgaaat ccccgggctc aacctgggaa
    541 ctgcattcga aactggcagg ctagagtctt gtagaggggg gtagaattcc aggtgtagcg
    601 gtgaaatgcg tagagatctg gaggaatacc ggtggcgaag gcggcccccc ggacaaagac
    661 tgacgctcag gtgcgaaagc gtggggagca aacaggatta gataccctgg tagtccacgc
    721 cgtaaacgat gtcgacttgg aggttgtgcc cttgaggcgt ggcttccgga gctaacgcgt
    781 taagtcgacc gcctggggag tacggccgca aggttaaaac tcaaatgaat tgacgggggc
    841 ccgcacaagc ggtggagcat gtggtttaat tcgatgcaac gcgaagaacc ttacctactc
    901 ttgacatcca gagaactttc cagagatgga ttggtgcctt cgggaactct gagacaggtg
    961 ctgcatggct gtcgtcagct cgtgttgtga aatgttgggt taagtcccgc aacgagcgca
   1021 acccttatcc tttgttgcca gcggttcggc cgggaactca aaggagactg ccagtgataa
   1081 actggaggaa ggtggggatg acgtcaagtc atcatggccc ttacgagtag ggctacacac
   1141 gtgctacaat ggcgcataca aagagaagcg acctcgcgag agcaagcgga cctcataaag
   1201 tgcgtcgtag tccggattgg agtctgcaac tcgactccat gaagtcggaa tcgctagtaa
   1261 tcgtagatca gaatgctacg gtgaatacgt tcccgggcct tgtacacacc gcccgtcaca
   1321 ccatgggagt gggttgcaaa agaagtaggt agcttaa
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j) *Kluyvera georgiana* strain E3 16S ribosomal RNA gene, partial sequence

GenBank: KT804412.1

LOCUS	KT804412 1395 bp DNA linear BCT 10-FEB-2016
DEFINITION	Kluyvera georgiana strain E3 16S ribosomal RNA gene, partial sequence.
ACCESSION	KT804412
VERSION	KT804412.1
KEYWORDS.	
SOURCE	Kluyvera georgiana
ORGANISM	Kluyvera georgiana Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Enterobacteriaceae; Kluyvera.
REFERENCE	1 (bases 1 to 1395)
AUTHORS	Ally,A. and Hatha,M.
TITLE	comparative evaluation of EMB agar and chromogenic medium in isolation of E.Coli from shellfish
JOURNAL	Unpublished

REFERENCE 2 (bases 1 to 1395)
AUTHORS Ally,A. and Hatha,M.
TITLE Direct Submission
JOURNAL Submitted (19-SEP-2015) Department of Marine Biology, Microbiology and Biochemistry, Cochin Univsersity of Science and Technology, Fine Arts Avenue, Ernakulam, Kerala 682016, India
COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

FEATURES Location/Qualifiers
source 1..1395
/organism="Kluyvera georgiana"
/mol_type="genomic DNA"
/strain="E3"
/host="shellfish"
/db_xref="taxon:73098"
/country="India"
/collection_date="23-Mar-2014"
rRNA <1..>1395
/product="16S ribosomal RNA"

ORIGIN
1 acggtagcac agagagcttg ctctcgggtg acgagtggcg gacgggtgag taatgtctgg
61 gaaactgccc gatggagggg gataactact ggaaacggta gctaataccg cataacgtcg
121 caagaccaaa gtgggggacc ttcgggcctc acaccatcgg atgtgcccag atgggattag
181 ctagtaggtg gggtaacggc tcacctaggc gacgatccct agctggtctg agaggatgac
241 cagccacact ggaactgaga cacggtccag actcctacgg gaggcagcag tggggaatat
301 tgcacaatgg gcgcaagcct gatgcagcca tgccgcgtgt atgaagaagg ccttcgggtt
361 gtaaagtact ttcagcgagg aggaaggcgt tgtggttaat aaccacagcg attgacgtta
421 ctcgcagaag aagcaccggc taactccgtg ccagcagccg cggtaatacg gagggtgcaa
481 gcgttaatcg gaattactgg gcgtaaagcg cacgcaggcg gtttgttaag tcagatgtga
541 aatccccggg ctcaacctgg gaactgcatt tgaaactggc aagcttgagt ctcgtagagg
601 ggggtagaat tccaggtgta gcggtgaaat gcgtagagat ctggaggaat accggtggcg
661 aaggcggccc cctggacgaa gactgacgct caggtgcgaa agcgtgggga gcaaacagga
721 ttagataccc tggtagtcca cgccgtaaac gatgtctatt tggaggttgt gcccttgagg
781 cgtggcttcc ggagctaacg cgttaaatag accgcctggg gagtacggcc gcaaggttaa
841 aactcaaatg aattgacggg ggcccgcaca agcggtggag catgtggttt aattcgatgc
901 aacgcgaaga accttaccta ctcttgacat ccagagaact tagcagagat gctttggtgc
961 cttcgggaac tctgagacag gtgctgcatg gctgtcgtca gctcgtgttg tgaaatgttg
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1261 catgaagtcg gaatcgctag taatcgtaga tcagaatgct acggtgaata cgttcccggg
1321 ccttgtacac accgcccgtc acaccatggg agtgggttgc aaaagaagta ggtagcttaa
1381 ccttcgggag ggcgc
//

k) *Pantoea agglomerans* strain E30 16S ribosomal RNA gene, partial sequence

GenBank: KT804413.1

LOCUS	KT804413 1374 bp DNA linear BCT 10-FEB-2016
DEFINITION	Pantoea agglomerans strain E30 16S ribosomal RNA gene, partial sequence.
ACCESSION	KT804413
VERSION	KT804413.1
KEYWORDS.	
SOURCE	Pantoea agglomerans
ORGANISM	Pantoea agglomerans Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Erwiniaceae; Pantoea; Pantoea agglomerans group.
REFERENCE	1 (bases 1 to 1374)
AUTHORS	Ally,A. and Hatha,M.
TITLE	comparative evaluation of EMB agar and chromogenic agar in isolation of E.coli from shellfish
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 1374)
AUTHORS	Ally,A. and Hatha,M.
TITLE	Direct Submission
JOURNAL	Submitted (19-SEP-2015) Department of Marine Biology, Microbiology and Biochemistry, Cochin Univsersity of Science and Technology, Fine Arts Avenue, Ernakulam, Kerala 682016, India
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##

FEATURES	Location/Qualifiers
source	1..1374 /organism="Pantoea agglomerans" /mol_type="genomic DNA" /strain="E30" /host="shellfish" /db_xref="taxon:549" /country="India" /collection_date="08-Sep-2013"
rRNA	<1..>1374 /product="16S ribosomal RNA"

ORIGIN

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    121 cttcgggcct cttgccatca tatgtgccca gatgggatta tctagtaggt ggggtaacgg
    181 ctcacctagg cgacgatccc tatctggtct gagaggatga ccacccacac tggaactgac
    241 acacggtcca cactcctacg ggaggcagca gtggggaata ttgcacaatg ggcgcaagcc
    301 tgatgcaccc atgccgcgtg tatgaaaaag gccttcgggt tgtaaagtac tttcagcggg
    361 gaggaaggtg ttgaggttaa taacctcagc gattgacgtt acccgcaaaa aaagcaccgg
    421 ctaactccgt gccagcagcc gcggtaatac ggagggtgca agcgttaatc ggaattactg
    481 ggcgtaaagc gcacgcaggc ggtctgtcaa gtcggatgtg aaatccccgg gctcaacctg
    541 ggaactgcat tcgaaactgg caggctagag tcttgtagag gggggtagaa ttccaggtgt
    601 agcggtgaaa tgcgtagaga tctggaggaa taccggtggc gaaggcggcc ccctggacaa
    661 agactgacgc tcaggtgcga aagcgtgggg agcaaacagg attagatacc ctggtagtcc
    721 acgccgtaaa cgatgtcgac ttggaggttg tgcccttgag gcgtggcttc cggagctaac
    781 gcgttaagtc gaccgcctgg ggagtacggc cgcaaggtta aaactcaaat gaattgacgg
    841 gggcccgcac aagcggtgga gcatgtggtt taattcgatg caacgcgaag aaccttacct
    901 actcttgaca tccagagaac tttccagaga tggattggtg ccttcgggaa ctctgagaca
    961 ggtgctgcat ggctgtcgtc agctcgtgtt gtgaaatgtt gggttaagtc ccgcaacgag
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   1201 aaagtgcgtc gtagtccgga ttggagtctg caactcgact ccatgaagtc ggaatcgcta
   1261 gtaatcgtag atcagaatgc tacggtgaat acgttcccgg gccttgtaca caccgcccgt
   1321 cacaccatgg gagtgggttg caaaagaagt aggtagctta acctcgggag ggcg
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l) *Vibrio vulnificus* strain JWV13 16S ribosomal RNA gene, partial sequence

GenBank: KT005560.1

LOCUS	KT005560 921 bp DNA linear BCT 05-OCT-2015
DEFINITION	Vibrio vulnificus strain JWV13 16S ribosomal RNA gene, partial sequence.
ACCESSION	KT005560
VERSION	KT005560.1
KEYWORDS.	
SOURCE	Vibrio vulnificus
ORGANISM	Vibrio vulnificus Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE	1 (bases 1 to 921)
AUTHORS	Silvester,R., Alexander,D., Antony,A.C. and Mohamed,H.

TITLE Genotyping of Vibrio sp. using GRO EL RFLP

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 921)

AUTHORS Silvester,R., Alexander,D., Antony,A.C. and Mohamed,H.

TITLE Direct Submission

JOURNAL Submitted (03-JUN-2015) Department of Marine Biology, Microbiology and Biochemistry., Cochin University of Science and Technology, School of Marine Sciences, Lake Side Campus, Fine Arts Avenue, Ernakulam, Kerala 682016, India

COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..921
/organism="Vibrio vulnificus"
/mol_type="genomic DNA"
/strain="JWV13"
/isolation_source="shrimp farm water"
/db_xref="taxon:672"
/country="India"
/collection_date="05-Jun-2012"

rRNA <1..>921
/product="16S ribosomal RNA"

ORIGIN

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    121 tgatgcctac gggccaaaga gggggacctt cgggcctctc gcgtcaggat atgcccaggt
    181 gggattagct agttggtgag gtaagggctc accaaggcga cgatccctag ctggtctgag
    241 aggatgatca gccacactgg aactgagaca cggtccagac tcctacggga ggcagcagtg
    301 gggaatattg cacaatgggc gcaagcctga tgcagccatg ccgcgtgtgt gaagaaggcc
    361 ttcgggttgt aaagcacttt cagttgtgag gaaggtggtg tcgttaatag cggcatcatt
    421 tgacgttagc aacagaagaa gcaccggcta actccgtgcc agcagccgcg gtaatacgga
    481 gggtgcgagc gttaatcgga attactgggc gtaaagcgca tgcaggtggt ttgttaagtc
    541 agatgtgaaa gccccggggct caacctcgga actgcatttg aaactggcag actagagtac
    601 tgtagagggg ggtagaattt caggtgtagc ggtgaaatgc gtagagatct gaaggaatac
    661 cggtggcgaa ggcggccccc tggacagata ctgacactca gatgcgaaag cgtggggagc
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GenBank: KU240006.1

LOCUS KU240006 1327 bp DNA linear BCT 25-MAY-2016

DEFINITION Phytobacter diazotrophicus strain 2SS1B3 16S ribosomal RNA gene,partial sequence.

ACCESSION KU240006

VERSION KU240006.1

KEYWORDS.

SOURCE Phytobacter diazotrophicus

ORGANISM Phytobacter diazotrophicus
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; unclassified Enterobacterales; Phytobacter.

REFERENCE 1 (bases 1 to 1327)

AUTHORS Suresh,K., Hatha,M., Ally,A., Christo,D., Arun,A. and Bini,F.

TITLE Prevalence and survival of pathogenic bacteria on food contact surfaces

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1327)

AUTHORS Suresh,K., Hatha,M., Ally,A., Christo,D., Arun,A. and Bini,F.

TITLE Direct Submission

JOURNAL Submitted (05-DEC-2015) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Ernakulam, Kerala 682016, India

COMMENT ##Assembly-Data-START##

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Appendices

APPENDIX 1

Table 1.1a: ANOVA for prevalence of faecal coliforms in shellfish during pre-monsoon, monsoon and post-monsoon seasons

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.805	2	.403	16.650	.000
Within Groups	.508	21	.024		
Total	1.313	23			

Table 1.1b: ANOVA for prevalence of faecal coliforms in sediment during pre-monsoon, monsoon and post-monsoon seasons

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.274	2	.637	41.200	.000
Within Groups	.325	21	.015		
Total	1.599	23			

Table 1.1c: ANOVA for prevalence of faecal coliforms in harvesting water during pre-monsoon, monsoon and post-monsoon seasons

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.216	2	.608	27.529	.000
Within Groups	.464	21	.022		
Total	1.680	23			

Table 1.2a: ANOVA for prevalence of faecal coliforms in shellfish, sediment and harvesting water during pre-monsoon season

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.045	2	.022	.565	.577
Within Groups	.827	21	.039		
Total	.871	23			

Table 1.2b: ANOVA for prevalence of faecal coliforms in shellfish, sediment and harvesting water during monsoon season

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.005	2	.002	.226	.800
Within Groups	.224	21	.011		
Total	.229	23			

Table 1.2c: ANOVA for prevalence of faecal coliforms in shellfish, sediment and harvesting water during post-monsoon season

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.014	2	.007	.618	.549
Within Groups	.245	21	.012		
Total	.260	23			

Table 1.3a: ANOVA for prevalence of faecal streptococci in shellfish during pre-monsoon, monsoon and post-monsoon seasons

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.062	2	.031	1.000	.385
Within Groups	.655	21	.031		
Total	.718	23			

Table 1.3b: ANOVA for prevalence of faecal streptococci in sediment during pre-monsoon, monsoon and post-monsoon seasons

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.020	2	.010	.887	.427
Within Groups	.239	21	.011		
Total	.259	23			

Table 1.3c: ANOVA for prevalence of faecal streptococci in harvesting water during pre-monsoon, monsoon and post-monsoon seasons

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.958	2	.979	16.868	.000
Within Groups	1.219	21	.058		
Total	3.177	23			

Table 1.4a: ANOVA for prevalence of faecal streptococci in shellfish, sediment and harvesting water during pre-monsoon season

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.547	2	4.274	78.341	.000
Within Groups	1.146	21	.055		
Total	9.693	23			

Table 1.4b: ANOVA for prevalence of faecal streptococci in shellfish, sediment and harvesting water during monsoon season

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.730	2	1.365	64.112	.000
Within Groups	.447	21	.021		
Total	3.177	23			

Table 1.4c: ANOVA for prevalence of faecal streptococci in shellfish, sediment and harvesting water during post-monsoon season

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.060	2	1.530	61.764	.000
Within Groups	.520	21	.025		
Total	3.580	23			

APPENDIX 2

Table 2.1 Chi-square test to study sample-wise association of antibiotic resistance among *E-coli*

Antibiotics	Shellfish	Sediment	Water	p - value
Ak	38	29	16	0.002
Amc	84	76	89	0.047
A	34	47	38	0.157
Ctx	70	68	57	0.116
Cx	80	74	54	0.000
Cpd	98	97	97	0.879
C	20	13	11	0.168
Cip	20	21	14	0.384
Cl	90	68	73	0.001
Cot	14	8	22	0.019
Do	62	55	38	0.002
Gen	26	32	22	0.274
Ipm	92	79	84	0.034
Mrp	22	24	27	0.709
Na	60	53	43	0.054
Nit	66	66	49	0.018
S	48	53	46	0.594
Te	66	66	35	0.000
Tr	18	13	22	0.247
Total	53	50	44	0.000

Table 2.2 Chi-square test to study sample-wise association of antibiotic resistance among *V. parahaemolyticus*

Antibiotics	Shellfish	Sediment	Water	p - value
Ak	36	62	57	0.000
Amc	36	38	57	0.004
A	100	100	71	0.000
Cx	64	15	29	0.000
Cpd	100	92	86	0.001
Ctx	46	46	72	0.000
C	0	0	29	0.000
Cip	36	54	57	0.006
Cot	27	31	43	0.045
Do	0	0	0	--
Gen	46	69	43	0.000
Na	9	31	43	0.000
Nit	36	46	57	0.012
S	36	69	57	0.000
Te	11	8	14	0.399
Tr	18	31	43	0.001
Total	38	43	47	0.000

Table 2.3 Chi-square test to study sample-wise association of antibiotic resistance among enterococci

Antibiotics	Shellfish	Sediment	Water	p - value
Ak	87	100	57	0.000
Amc	39	43	29	0.107
A	100	100	100	--
Cep	96	100	100	0.017
C	35	43	43	0.412
Cip	83	100	100	0.000
Cot	87	86	100	0.001
Do	22	29	0	0.000
E	83	71	86	0.019
HLG	9	14	0	0.001
HLS	13	21	0	0.000
Lz	17	57	14	0.000
P	91	100	57	0.000
Rif	87	86	86	0.972
Tei	57	57	29	0.000
Te	57	29	14	0.000
Tr	96	100	100	0.017
Va	74	79	86	0.106
Total	63	68	56	0.000

Table 2.4 Chi-square test to study station-wise association of antibiotic resistance among *E-coli*

Antibiotics	Station 1	Station 2	Station 3	Station 4	Station 5	Station 6	Station 7	Station 8	p - value
Ak	19	14	43	28	39	22	0	60	0.000
Amc	62	29	43	14	23	57	30	40	0.000
A	80	100	100	90	92	74	70	40	0.000
Ctx	38	71	71	69	92	65	20	100	0.000
Cx	48	43	100	97	77	52	30	100	0.000
Cpd	95	100	100	100	92	96	100	100	0.000
C	10	0	0	14	8	35	30	0	0.000
Cip	38	14	43	14	31	22	10	0	0.000
Cl	67	43	100	86	92	83	70	100	0.000
Cot	5	14	29	14	8	17	30	20	0.000
Do	24	57	86	45	69	48	80	60	0.000
Gen	43	43	0	10	31	30	0	20	0.000
Ipm	86	71	100	86	85	87	90	80	0.000
Mrp	19	0	43	28	46	17	20	40	0.000
Na	33	29	57	55	39	70	70	60	0.000
Nit	38	71	86	66	62	35	90	80	0.000
S	33	29	86	62	62	30	70	40	0.000
Te	38	43	43	48	85	52	90	40	0.000
Tr	14	14	29	10	23	22	30	20	0.002
Total	42	41	61	49	56	48	49	53	0.000

Table 2.5 Chi-square test to study station-wise association of antibiotic resistance among enterococci

Antibiotics	Station 1	Station 2	Station 3	Station 4	Station 5	Station 6	Station 7	Station 8	p - value
Ak	100	100	60	78	75	90	80	100	0.000
Amc	40	40	20	33	17	70	0	40	0.000
A	100	100	100	100	100	100	100	100	--
Cep	100	100	100	100	100	90	100	100	0.000
C	60	60	40	22	33	50	0	20	0.000
Cip	100	100	60	100	75	90	80	100	0.000
Cot	90	80	100	89	83	100	80	80	0.000
Do	20	0	20	33	25	10	0	20	0.000
E	100	80	60	67	58	90	60	80	0.000
HLG	10	0	0	22	0	10	0	0	0.000
HLS	20	0	20	22	0	20	0	0	0.000
Lz	40	20	40	11	0	60	20	0	0.000
P	100	100	80	78	75	100	100	80	0.000
Rif	90	80	80	89	83	90	80	80	0.099
Tei	80	80	40	56	17	80	0	80	0.000
Te	70	40	20	33	8	70	20	20	0.000
Tr	100	100	100	100	92	100	100	100	0.000
Va	90	100	40	78	58	80	80	80	0.000
Total	73	66	54	62	50	72	50	60	0.000

Table 2.6 Chi-square test to study station-wise association of antibiotic resistance among *V. parahaemolyticus*

Antibiotics	Station 1	Station 2	Station 3	Station 4	Station 5	Station 6	Station 7	Station 8	p - value
Ak	29	67	100	33	50	22	67	67	0.000
Amc	49	67	100	0	0	22	67	67	0.000
A	86	100	100	100	100	89	67	67	0.000
Cx	57	67	100	0	0	44	0	0	0.000
Cpd	100	100	100	67	100	89	67	67	0.000
Ctx	49	67	100	0	0	0	0	0	0.000
C	14	0	50	0	0	0	0	0	0.000
Cip	29	67	100	0	25	22	67	67	0.000
Cot	49	33	100	33	0	11	0	0	0.000
Do	0	0	0	0	0	0	0	0	--
Gen	29	67	50	67	75	33	67	67	0.000
Na	14	67	100	0	25	11	0	0	0.000
Nit	29	67	100	33	25	33	0	0	0.000
S	29	67	100	67	75	22	67	67	0.000
Te	29	0	50	0	0	0	0	0	0.000
Tr	49	33	100	33	0	0	0	0	0.000
Total	40	54	84	27	30	25	29	29	0.000

APPENDIX 3

Table 3.1a: ANOVA for survival of *Escherichia coli* at different temperatures

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.256	2	1.128	2.034	.156
Within Groups	11.651	21	.555		
Total	13.907	23			

Table 3.1b: ANOVA for survival of *V. parahaemolyticus* at different temperatures

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.245	2	1.123	.815	.456
Within Groups	28.936	21	1.378		
Total	31.181	23			

Table 3.1c: ANOVA for survival of *Enterococcus faecalis* at different temperatures

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	28.561	2	14.280	1.685	.209
Within Groups	177.926	21	8.473		
Total	206.487	23			

Table 3.2a ANOVA for relative survival of *E. coli*, *V. parahaemolyticus* and *E. faecalis* at 25 °C

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10.24034	2	5.120168	2.641704	0.094
Within Groups	40.70233	21	1.938206		
Total	50.94267	23			

Table 3.2b ANOVA for relative survival of *E. coli*, *V. parahaemolyticus* and *E. faecalis* at 30 °C

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	44.8103	2	22.40515	5.193469	
Within Groups	90.59613	21	4.314101		0.014
Total	135.4064	23			

Table 3.2c ANOVA for relative survival of *E. coli*, *V. parahaemolyticus* and *E. faecalis* at 35 °C

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	57.74473	2	28.87237	6.592802	0.005
Within Groups	91.96692	21	4.379377		
Total	149.7116	23			

Table 3.3a: ANOVA for survival of *Escherichia coli* in different salinities

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.492	2	1.246	2.681	.092
Within Groups	9.760	21	.465		
Total	12.252	23			

Table 3.3b: ANOVA for survival of *V.parahaemolyticus* in different salinities

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.685	2	1.343	2.069	.151
Within Groups	13.629	21	.649		
Total	16.314	23			

Table 3.3c: ANOVA for survival of *Enterococcus faecalis* in different salinities

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.310	2	.155	.075	.928
Within Groups	43.181	21	2.056		
Total	43.490	23			

Table 3.4a ANOVA for relative survival of *E. coli*, *V. parahaemolyticus* and *E. faecalis* at 0 ppt salinity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.605811	2	3.802906	2.86661	0.079
Within Groups	27.85905	21	1.326621		
Total	35.46486	23			

Table 3.4b ANOVA for relative survival of *E. coli*, *V. parahaemolyticus* and *E. faecalis* at 15 ppt salinity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.155972	2	2.577986	2.317568	0.123
Within Groups	23.3597	21	1.112367		
Total	28.51567	23			

Table 3.4c ANOVA for relative survival of *E. coli*, *V. parahaemolyticus* and *E. faecalis* at 30 ppt salinity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.487734	2	4.243867	4.511818	
Within Groups	19.75284	21	0.940611		0.023
Total	28.24057	23			

Table 3.5a Student's t-test for comparison of survival of *Escherichia coli* in autoclaved and raw estuarine sediment

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Survival of *Escherichia coli* in autoclaved and raw estuarine sediment	Equal variances assumed	1.568	.231	4.881	14	.000	2.61295	.53532	1.46480	3.76111
	Equal variances not assumed			4.881	12.100	.000	2.61295	.53532	1.44765	3.77826

Table 3.5b Student's t-test for comparison of survival of *V. parahaemolyticus* in autoclaved and raw estuarine sediment

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Survival of *V.parahaemolyticus* in autoclaved and raw estuarine sediment	Equal variances assumed	1.568	.231	4.881	14	.000	2.61295	.53532	1.46480	3.76111
	Equal variances not assumed			4.881	12.100	.000	2.61295	.53532	1.44765	3.77826

Table 3.5c Student's t-test for comparison of survival of *Enterococcus faecalis* in autoclaved and raw estuarine sediment

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Survival of *Enterococcus faecalis* in autoclaved and raw estuarine sediments	Equal variances assumed	15.393	.002	-2.644	14	.019	-2.60313	.98458	-4.71484	-.49142
	Equal variances not assumed			-2.644	7.540	.031	-2.60313	.98458	-4.89791	-.30835

Table 3.6a Student's t-test for comparison of survival of *Escherichia coli* in autoclaved and raw estuarine water

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Survival of *Escherichia coli* in autoclaved and raw estuarine waters	Equal variances assumed	5.522	.034	3.593	14	.003	3.24171	.90235	1.30635	5.17707
	Equal variances not assumed			3.593	10.897	.004	3.24171	.90235	1.25336	5.23007

Table 3.6b Student's t-test for comparison of survival of *V. parahaemolyticus* in autoclaved and raw estuarine water

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Survival of *V. parahaemolyticus* in autoclaved and raw estuarine waters	Equal variances assumed	5.522	.034	3.593	14	.003	3.24171	.90235	1.30635	5.17707
	Equal variances not assumed			3.593	10.897	.004	3.24171	.90235	1.25336	5.23007

Table 3.6c Student's t-test for comparison of survival of *Enterococcus faecalis* in autoclaved and raw estuarine water

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Survival of *Enterococcus faecalis* in autoclaved and raw estuarine waters	Equal variances assumed	.456	.510	-1.182	14	.257	-1.73933	1.47090	-4.89410	1.41543
	Equal variances not assumed			-1.182	13.915	.257	-1.73933	1.47090	-4.89590	1.41724

Table 3.7a Student's t-test for comparison of survival of *Escherichia coli* in cycloheximide treated and untreated raw sediment

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Survival of *Escherichia coli* in cycloheximide treated and untreated raw sediment	Equal variances assumed	.423	.526	.117	14	.909	.18075	1.54885	-3.14121	3.50271
	Equal variances not assumed			.117	13.822	.909	.18075	1.54885	-3.14523	3.50674

Table 3.7b Student's t-test for comparison of survival of *V. parahaemolyticus* in cycloheximide treated and untreated raw sediment

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Survival of *V.parahaemolyticus* in cycloheximide treated raw water and raw sediment	Equal variances assumed	.052	.823	-.542	14	.596	-.35158	.64872	-1.74293	1.03978
	Equal variances not assumed			-.542	13.966	.596	-.35158	.64872	-1.74325	1.04009

Table 3.7c Student's t-test for comparison of survival of *Enterococcus faecalis* in cycloheximide treated and untreated raw sediment

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Survival of *Enterococcus faecalis* in raw sediment treated and untreated with cycloheximide	Equal variances assumed	.002	.967	-.020	14	.985	-.02701	1.37277	-2.97130	2.91728
	Equal variances not assumed			-.020	13.998	.985	-.02701	1.37277	-2.97135	2.91733

Table 3.8a Student's t-test for comparison of survival of *Escherichia coli* in cycloheximide treated and untreated raw water

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Survival of *Escherichia coli* in cycloheximide treated and untreated raw water	Equal variances assumed	.000	.993	-.005	14	.996	-.00810	1.48765	-3.19879	3.18258
	Equal variances not assumed			-.005	13.996	.996	-.00810	1.48765	-3.19887	3.18267

Table 3.8b Student's t-test for comparison of survival of *V. parahaemolyticus* in cycloheximide treated and untreated raw water

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Survival of *V.parahaemolyticus* in cycloheximide treated and untreated raw water	Equal variances assumed	.481	.499	-.499	14	.625	-.57697	1.15543	-3.05513	1.90118
	Equal variances not assumed			-.499	13.825	.625	-.57697	1.15543	-3.05808	1.90413

Table 3.8c Student's t-test for comparison of survival of *Enterococcus faecalis* in cycloheximide treated and untreated raw water

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Survival of *Enterococcus faecalis* in raw water treated and untreated with cycloheximide	Equal variances assumed	.033	.858	-.305	14	.765	-.46099	1.51279	-3.70559	2.78362
	Equal variances not assumed			-.305	13.995	.765	-.46099	1.51279	-3.70571	2.78373

APPENDIX 4

Table 4.1 ANOVA with Duncans's multiple range test for reduction of TC with time during depuration

Time	Mean	SD	F - value
0	5.041^{A}	0.000	494.5**
6	3.915^{B}	0.218	
12	3.435^{C}	0.047	
24	2.407^{D}	0.047	
48	2.349^{D}	0.023	
72	2.140^{E}	0.087	
96	1.439^{F}	0.066	

** Difference is significant at 0.01 level
Values with same letters showing no significance

Table 4.2 ANOVA with Duncans's multiple range test for reduction of FC with time during depuration

Time	Mean	SD	F - value
0	4.789^{A}	0.218	894.6**
6	3.789^{B}	0.218	
12	3.407^{C}	0.047	
24	2.335^{D}	0.023	
48	0.000^{E}	0.000	
72	0.000^{E}	0.000	
96	0.000^{E}	0.000	

** Difference is significant at 0.01 level
Values with same letters showing no significance

Table 4.3 ANOVA with Duncans's multiple range test for reduction of FS with time during depuration

Time	Mean	SD	F - value
0	4.915^{A}	0.218	722.1**
6	3.529^{B}	0.116	
12	2.862^{C}	0.223	
24	1.511^{D}	0.059	
48	0.000^{E}	0.000	
72	0.000^{E}	0.000	
96	0.000^{E}	0.000	

** Difference is significant at 0.01 level
Values with same letters showing no significance

Table 4.4 ANOVA with Duncans's multiple range test for difference in reduction between TC, FC and FS

Time	TC	FC	FS	F - value
0	5.041 ± 0.000	4.789 ± 0.218	4.915 ± 0.218	
6	3.915 ± 0.218	3.789 ± 0.218	3.529 ± 0.116	
12	3.435 ± 0.047	3.407 ± 0.047	2.862 ± 0.223	
24	2.407 ± 0.047	2.335 ± 0.023	1.511 ± 0.059	73.60**
48	2.349 ± 0.023	0.000 ± 0.000	0.000 ± 0.000	
72	2.140 ± 0.087	0.000 ± 0.000	0.000 ± 0.000	
96	1.439 ± 0.066	0.000 ± 0.000	0.000 ± 0.000	

** Difference is significant at 0.01 level

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