DISTRIBUTION AND ABUNDANCE OF LUMINESCENT BACTERIA IN PONNANI ESTUARY

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By

RAMINA.P.P

Under the guidance of

Dr. RAZIA BEEVI, M

POST GRADUATE DEPARTMENT OF AQUACULTURE AND FISHERY MICROBIOLOGY

MES PONNANI COLLEGE, PONNANI

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Department of aquaculture and Fishery Microbiology
(Research centre under university of Calicut)
MES Ponnani College, Ponnani South, Malappuram

Dr. Razia Beevi, M
HOD, Associate Professor

November 27th 2018

CERTIFICATE

This is to certify that the thesis entitled **"Distribution and Abundance of Luminescent Bacteria in Ponnani Estuary"** is an authentic record of research work carried out by Mrs. Ramina.P.P, Reg. order No. DoR/B3/3213/Ph.D.2012 (other centers),under my supervision and guidance in the Department of Aquaculture and Fishery Microbiology, MES Ponnani College, University of Calicut, Kerala, for the award of the Degree of Doctor of Philosophy in Aquaculture and Microbiology under the Faculty of Science, University of Calicut. I further certify that the research work is original and no part therefore has been presented for the award of any other degree, diploma or associateship in any University or Institution. All the relevant corrections and modification suggested by the audience during the pre submission seminar and recommended by the Doctoral Committee have been incorporated in the thesis.

Dr. Razia Beevi, M
(Supervising Guide)

DECLARATION

I, Ramina. P.P do hereby declare that the thesis entitled **"Distribution and Abundance of Luminescent Bacteria in Ponnani Estuary"** is an authentic record of research work carried out by me under the guidance of Dr. Razia Beevi, M, Associate Professor, Department of Aquaculture and Fishery Microbiology, MES Ponnani College, Ponnani, in partial fulfillment of the requirements for the award of Degree of Doctor of Philosophy in Aquaculture and Microbiology under the Faculty of Science, University of Calicut, Kerala, and no part of the thesis has been submitted in part or in full for the award of any other degree or diploma from any University or Institution.

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Ramina.P.P

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LIST OF ABBREVIATIONS

APHA	:	American Public Health Association
DO	:	Dissolved Oxygen
BOD	:	Biological Oxygen Demand
BLAST	:	Basic Local Alignment Search Tool
bp	:	base pair
DNA	:	Deoxyribo Nucleic Acid
rRNA	:	Ribosomal ribo nucleic acid
cfu/ml	:	Colony forming unit per millilitre
cfu/gm	:	Colony forming unit per gram
%	:	Percentage
NCBI	:	National Centre for Biotechnology Information
ppt	:	Parts per thousand
PCR	:	Polymerase Chain Reaction
$NaHCO_3$	:	Sodium bicarbonate
KC1	:	Potassium chloride
$MgSO_4$	:	Magnesium sulphate
°C	:	Degree celcius
MDR	:	Multiple drug resistance
ZnPT	:	Zinc pyrithione
µg/L	:	Microgram per litre
Hg	:	Mercury
Co	:	Cobalt
Cu	:	Copper
Pb	:	Lead
Hg	:	Mercury

TCBS	:	Thiosulphate citrate bile salts sucrose
TVLO	:	Total *Vibrio* like organism
BLB	:	Bioluminescent bacteria
SWCA	:	Sea water complex agar
THB	:	Total heterotrophic bacteria
gm	:	Gram
ml	:	Milli litre
MR	:	Methyl red
VP	:	Voges -proskauer
TSI	:	Triple sugar iron
ONPG	:	Ortho-nitrophenyl beta-D-galactopyranoside
CV	:	Crytal violet
H_2S	:	Hydrogen sulphide
µl	:	Micro litre
ATP	:	Adenosine triphosphate
GTP	:	Guanosine triphosphate
CTP	:	Citidine triphosphate
TTP	:	Thymidine triphosphate
SAP	:	Shimp Alkaline Phosphate
BLR	:	Bioluminescent ratio
MIC	:	Minimum inhibitory concentration
ANOVA	:	Analysis of variance
SPSS	:	Statistical package for social science
SE	:	Standard error
Prob.	:	Prabability
MEGA	:	Molecular evolutionary genetic analysis

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CHAPTER 1

INTRODUCTION

Estuaries and their surrounding wetlands are water bodies usually found where rivers meet the sea, which are home to unique plant and animal communities that have adapted to brackish water and are among the most productive ecosystems in the world (McLusky and Elliott, 2004). They maintain water quality through natural filtration of water draining from the land which includes sediments, nutrients and other pollutants. Estuarine water display seasonal variations in its physico-chemical parameters and nutrient content depending on physical and biological processes (Velsamy *et al*., 2013). Many animals rely on this delicate ecosystem for food, breeding and as migration stopovers. Dual challenges of estuarine life are the variability in salinity and sedimentation. Countless species of fish and invertebrates have diverse methods to control the shifts in salt concentrations enabling them to be osmoregulators. Many animals also burrow to avoid predation and to live in the more stable sediment rich environment.

In spite of pollution tendency of man, estuaries remained an interesting insight into the natural world where energy is transformed from sunlight to plant and finally to rich supply of food for birds and fishes through food chain (McLusky and Elliott, 2011).Health of estuaries and its inhabiting organisms can be adversely affected by agricultural activities which comprise of runoff water and chemicals from agricultural land. Industries like fishing and oyster farming can impact the water quality movement in estuaries. Over-fishing and habitat loss reduces the amount of fish in the ocean and disrupts the food chain.Impacts on limnic and estuarine systems may differ in land-borne waste materials and associated pollutants which undergo complex physico-chemical particle-water reactions throughout estuarine mixing because of changes in pH, salinity, biological production, and degradation (Ben *etal* .,2003 ;Turner and Miliward,2002). Since estuary plays a vital role in fishery industry, climate change and environmental factors like pollution, eutrophication, red tides and

hypoxia, it remain as a research focus of a wide range of international programs like Joint Global Ocean Flux Study, Global Ocean Ecosystems Dynamics and Land–Ocean Interactions in the Coastal Zone (Li *et al.*, 2002; Li and Daler, 2004, Dang *et al.*, 2008).

Study on the distribution of luminescent bacteria along Indian estuaries are limited to Vellar estuary whereinqualitative and quantitative distribution and seasonal variation of luminescent bacteria in the sediments had been focused (Ramesh *et al.*, 1989), and recorded an indirect relationship between the salinity of estuarine water and luminous bacterial counts in the sediment.

1.1 Bioluminescent bacteria

Bioluminescence is an enchanting phenomenon of production and emission of light from a living organism that is documented over 700 genera of metazoans across various life forms with huge majority living in ocean (Grzegorz *et al*., 2002; Haddock *et al*, 2010; Margarita *et al*., 2012). The biological significance of bioluminescence is to impart interspecies signaling, startling predators, tempting prey, and camouflaging (Haddock *et al.*, 2010; Widder, 2010; Wilson and Hastings, 2013). Luminescent bacteria are prevailing in terms of abundance and other luminescent organisms are overriding in terms of biomass (Ramesh and Mohanraju, 2017).Luminescent bacteria are gram negative, facultative anaerobes except *Shewanella hanedai* and *S. woodyi* (aerobic) recognized to reside in several niches of diverse environments. Bioluminescent bacteria exist either as free-living in seawater or as symbiotic associations with marine organisms (Dworkin *et al*., 2006).Luminescent bacteria are classified into three major genera which include *Photobacterium, Vibrio*, and *Photorhabdus* where *Photobacterium* and *Vibrio* occupy marine habitats and *Photorhabdus* occupy terrestrial environment (Leo and Edward, 2001; Paul and Henryk, 2013).

Vibrio harveyi is a marine gram-negative, motile, facultative anaerobic luminous organism with a prerequisite for sodium chloride and it also named as *Lucibacterium harveyi*, and *Beneckea harveyi* and finally reached at the current taxonomic position as *Vibrio harveyi* (Farmer *et al.*, 2005). *Vibrio harveyi* occurs as free-swimming in tropical marine waters, commensally in the gut micro flora of marine animals, and as both a primary and opportunistic pathogen of marine animals, including gorgonian corals, oysters, prawns, lobsters, milkfish and seahorses (Owens *et al*., 2006). *Vibrio harveyi* is a serious pathogen for a wide range of marine animals and are the main causative agent of luminous Vibriosis (Austin and Zhang, 2006).

Vibrio fisheri is an oxidase-positive, gram-negative Vibrionaceae member established either as free living or associated with a eukaryotic host (Ruby *et al*., 2005; Hoi *et al*., 2007). *V. fisheri* exists in symbiotic association with Hawaiian squid is a best example of specific cooperativity during the growth of both organisms (Geszvain and Visick, 2006). Bioluminescence of *V. fisheri* and *V. harveyi* led to the discovery of autoinduction, or quorum sensing, in Gram-negative bacteria in the 1970s (Nealson *et al*., 1970; Miller and Bassler, 2001). Quorum sensing discloses how light production was controlled in a density-dependent manner with eukaryotic organisms. *Agrobacterium, Aeromonas, Burkholderia, Chromobacterium, Citrobacter, Enterobacter, Erwinia, Hafnia, Nitrosomonas, Obesumbacterium, Pantoea, Pseudomonas, Rahnella, Ralstonia, Rhodobacter, Rhizobium*, *Serratia* and *Yersiniaalso* exhibit quorum sensing in addition to marine Vibrios (Bassler, 2002).

Photobacterium leiognathi , member of many species within the genus *Photobacterium*(Nijvipakul *et al*., 2008) and the family of vibrionaceae is a gram-negative, coccobacillus, flagellated, chemoorganothrophic and facultative anaerobic bacterium that appears white or colorless colonies which exhibits luminescent properties(Medvedeva *et al*., 2006).*P. leiognathi* found as

free-living organism in warm tropical marine environments within the mesopelagic zone (200-1000m depth) or in relationship with fish. *P. leiognathi* grows in water at temperature between 18-30°C they were mainly studied for their symbiotic association with the ponyfish where the bacteria lives in the light organ. Also found as a neutral entity on the surface of fish or acting as a decomposer of dead fishes (Medvedeva *et al*., 2006).

Bacterial luciferaseis a flavin-dependent monooxygenase composed of alpha and beta subunit that catalyzes light-emitting reaction by using substrates like reduced flavin, long chain aldehyde, and oxygen yielding oxidized flavin, carboxylic acid, and water as products with concomitant emission of blue-green light around 485-490 nm (Tinikul and Chaiyen,2016). Bioluminescence occurs when molecular oxygen combines with aldehyde and flavinmononucleotide in the presence of luciferase enzyme which results in the synthesis of long chain fatty acid and FMN as shown below

$$FMNH_2 + RCHO + 0_2 \xrightarrow{\text{Luciferase}} FMN + RCOOH + H_2O + \text{Blue green light (Amax = 490 nm)}$$

The reactive reaction center is located in the α-subunit, whereas the β-subunit is required for maintaining the active conformation of the α-subunit (Tinikul and Chaiyen,2016).Bacterial luciferase (*lux*) gene consists of five genes (*luxCDABE*) whose protein products synergistically generate bioluminescent light signals exclusive of supplementary substrate additions or exogenous manipulations (Close *et al*., 2010). *lux*AB genes codes for luciferase,while *lux*CDE genes codes for fatty acid reductase complex, and are accountable for producing the fatty aldehyde substrate for the luminescence reaction (Stabb, 2005 and Nijvipakul *etal*., 2008).Relevance of *luxCDABE*-based bioreporters emphasizes on-line ecotoxicological and bioavailability monitoring (Daunert *et al*., 2000;Kohler *et al*., 2000; D'souza, 2001), but latest towards biocomputing(Simpson *et al*., 2001), gene arrays (Van Dyk *et al*.,

2001) and non-invasive invivo diagnostic imaging (Connell *et al*., 2002). In the Lux operon of *Photobacterium*, an extra gene which code for a nonfluorescent flavoprotein is present in between luxB and luxE in which the role of LuxF protein is to scavenge an inhibitory side product of the luciferase reaction and is not essential for light production (Kaeding *et al*., 2007). LuxR protein is encoded by lux *R* gene which has a dual role, act as autoinducer (Al) recepter and luxoperon transcriptional activator (Dekievit and Iglewski, 2000).

1.2 Influence of Physico-chemical factors

Environmental factors influence the growth and distribution of bioluminescent bacteria. Major factors include temperature, salinity, nutrient concentration, pH level and solar radiation (Soto *et al*., 2009). Numerous contamination inputs had interpreted in relation with the temporal and spatial variations in physicochemical parameters in estuaries from Indian coast (Sankar *et al.*, 2010; Brintha and Mathevanpillai, 2013).

Extreme spatial variability exists in salinity, with a range of near zero at the tidal limit of the tributary river(s) to 3.4% at the estuary mouth. At some point, the salinity varies significantly over time and seasons, making it a harsh environment for organisms (Kaiser, 2005). Entire aquatic life, including organisms responsible for the self-purification processes require oxygen for their growth. Variations in dissolved oxygen occur seasonally or over a 24 hour period, in relation to temperature and biological activity. Dissolved oxygen concentration was reduced during biological respiration (Unimke *et al*., 2014).Dissolved oxygen measurement used to indicate the degree of pollution by organic matter, destruction of organic substances, level of self-purification of the water and for measurement of biochemical oxygen demand (BOD) (Eniola, 2005; Okonko *et al*., 2008).

Bioluminescent bacteria *Alivibrio fischeri* grows satisfactorily in environments with a temperature range between 5 and 30°C and a pHless than 6.8.Investigations on members of *Vibrionaceae*revealed dependencies of temperature and salinity in governing *Vibrio* population dynamics (Farmer *et al*., 2001; Urakawa and Rivera, 2006) and physiological stress responses. As an example, brackish, coastal, and pelagic waters are all distinctively populated by distinct *Vibrio* populations (Urakawa and Rivera, 2006).Marine microorganisms are unique in their ability to survive at very low temperature and high salinity and those exhibiting the above characteristics are referred to as psychrophiles and halophiles respectively. Variability in temperature and salinity in environments can determine the fitness of unique *Vibrio* population. *Vibrio* species found in freshwater are outstanding since they possess a low Na^+ requisite for growth and starvation survival e.g., *Vibrio cholerae* and *Vibrio mimicus* (Urakawa and Rivera, 2006). Universally, the genus *Vibrio and Photobacterium* appeared to be particularly adapted engaging pathogenic and benign host-microbe interactions, arising independently numerous times during the evolution of vibrionaceae (Nishiguchi and Nair, 2003). Earlier studies investigating the microbial ecology of luminous bacteria recommends species composition of a precise environment determined by patterns of temperature, salinity, nutrient concentration, solar radiation, and other abiotic factors (Edward and Kyu, 1998). *V. fischeri* is typically a temperate-water species found in hosts inhabiting both tropical and temperate waters (Nishiguchi *et al*., 2004).

1.3 Bioluminescent bacteria as indicators of pollution

Environment hold the waste from households and industry leading to transformation on environmental quality of water, soil and air that also affect on the flora, fauna and microorganisms. Type and number of microorganisms within the environment is predisposed by environment characteristic and waste

that flow into the environment which either inhibits or stimulates the growth of microorganisms (Oksfriani *et al*., 2014)**.** Water pollution caused by the release of harmful substances into rivers, lakes and seas and it may harmfully affect the life or causing potential health hazard to living organism.

Anthropogenic activity continuously affect environment at various levels by the introduction of greater amounts of known and new kinds of pollutants,assessed using chemical assays or evaluating physical parameters (Girotti *et al*.,2002). Bacteria, algae, nematodes, amphiphods, fishes and plants used as bioindicators and biomonitors in air, soil and water pollution (Wolterbeek, 2002). Quality of the environment was evaluated by using bacterial indicator of household waste water pollution in tropical estuaries and reclamation water (Costán *et al*., 2008; Nagvenkar and Ramaiah, 2009). Heterotrophic bacteria, coliform, *Streptococcus* sp., and *Pseudomonas aeruginosa* were found as an indicator of wastewater contamination (Oksfriani *et al*.,2014).*Salmonella* sp. and *Streptococcus* sp. can be used as an indicator of bacterial contamination with wastewater in tropical estuaries (Wéry *et al*.,2008; Nagvenkar and Ramaiah., 2009).

Assessment of anthropogenic contamination on estuarine ecosystemcarried out by bioluminescent ratio (BLR) determination, showed a decrease in BLR in the presence of contaminants like diesel fuel, mercury, and polychlorinated biphenyls (PCBs) (Frischer *et al*., 2005). Luminescence based bioassays for the ecotoxicological assessments of environmental pollutants weredone with *V. fischeri*, *V. harveyi* and *P. fluorescens* (Girotti *et al*., 2008). Bioluminescent bacteria have been used for assessing water pollution, because of their easy cultivable and detectable nature and can be applied in salty and non salty waters (Wolterbeek, 2002).

Bioluminescent bacterial acute Toxicity Test and Algal Growth Inhibitory Test (on sediments only) were used to control effluent water deriving

from purification plants and marine sediments (Grande *et al*., 2007). Bioassay using *P.leiognathi* was employed to detect different toxicants, heavy metals, pesticides, PCBs, polycyclic aromatic hydrocarbons, and fuel traces in water (Ulitzur *et al*., 2002). Pollution level of groundwater samples were monitored by chemical analysis and biological toxicity tests by *V. fischeri* (Kuczynska *et al*., 2006). Bacterial luminescence bioassay and a microtox test were applied to evaluate the seawater quality in areas close to wastewater pipe discharges of small-rate municipal sewers (Samaras *et al*., 2001).

1.4Antibiotic susceptibility pattern of bioluminescent bacteria

Estuarine ecosystem provides an interdependent opportunity for the marine bacteria to encounter washouts containing residues of antibiotics from farmlands and hospitals creating estuaries the mainly dynamic natural habitat to complement events of the nearby lands (Mariya and Gerard, 2010). Antimicrobial resistance among pathogenic bacteria has come forth as a major public health concern leading to an intensification of discussion on prudent use of antimicrobial agents, mainly in veterinary medicine, nutrition and agriculture (Caprioli *et al*., 2000). In treating bacterial infections, widely used antibiotics include tetracycline, doxycycline, erythromycin and streptomycin (Lima, 2001). Antibiotic resistance gene prevent over use of antibiotics and the first direct evidence was reported after analysis of DNA sequences recovered from Late Pleistocene Permafrost sediments to indicate that antibiotic resistance is an ancient, naturally occurring phenomenon detectable in the environment (D'Costa *et al*., 2011). Even though the pathogenic mechanism of *V. harveyi* is not fully understood, several factors contribute to their virulence include proteases, cytotoxins, hemolysins, phospholipases, siderophores, proteases, cytotoxins, lipopolysaccharide and presence of a bacteriophage (Austin and Zhang, 2006).

1.5 Heavy metal resistance

Heterotrophic marine bacteria together with bioluminescent bacteria are found in all oceans, semi closed seas and estuaries responds quickly to environmental alteration and their abundance may provide an indication of the breathing of oceans (Bacci *et al.,* 1994). The major cause for polluted water bodies is the presence of industrial wastes that are typically collected and treated by waste water treatment plants (WWTP). Divalent cations or oxyanionsy regulation system permit the entry of heavy metals into the bacterial cell by two kinds of resistance mechanism. The first mechanism is fast, non specific and is based on proton-motive force. The second mechanism is specific, inducible and is activated in the presence of a metal and usually requires ATP hydrolysis.

Marine luminous bacteria act as a proper candidate for detecting nano or pico molar concentrations of impurities in water bodies, pharmaceuticals and food industries (Ramaiah and Chandramohan, 1993). The inducible bioluminescent bacteria are used to detect specifically a pollutant and their toxic effects. Bioluminescent action is induced by pollutants and hence, sensitivity to metals justifies the choice of an inducible system to build a sensitive strain. The sensitivity depends on the source of the genetic system and the host strain.In order to obtain an interesting sensitivity, the resistance systems from less resistant bacteria such as *Escherichia coli* should be preferred. Metal salts like mercury chloride, copper sulfate, lead nitrate, cadmium acetate and cobalt nitrate were commonly used in studies.

▪ Mercuric chloride

Mercury is a heavy metal belonging to the transition element series and it exists in nature in elemental, inorganic and organic forms with each having its own profile of toxicity (Clarkson *et al*., 2003). Mercury is a prevalent

environmental toxicant and pollutant which causes rigorous alterations in the body tissues and causes a wide range of adverse health effects (Sarkar and Bhan, 2005). Elemental mercury vapor (Hg0), inorganic mercurous (Hg_{+1}), mercuric (Hg_{+2}), and the organic mercury compounds are the major chemical forms of mercury that exist in the environment and are exposed to humans and animals (Zahir *et al*., 2005).Ubiquitous nature of mercury made its continuous exposure to humans, plants and animals (Holmes *et al*., 2009) through accidents, environmental pollution, food contamination, dental care, preventive medical practices, industrial and agricultural operations, and occupational operations (Sarkar and Bhan,2005). Continuous exposure to water- soluble forms of mercury (such as mercuric chloride or methyl mercury) by inhalation of mercury vapor or by ingesting any form of mercury results in mercury poisoning. Mercury chloride ($HgCl_2$) is a chemical compound of mercury and chlorine and is no longer used for medicinal purpose because of mercury toxicity and the availability of superior treatments.

- **Copper sulfate**

The inorganic compound, copper sulfate ($CuSo_4$) is a crucial mineral soluble in water which is toxic to algae, bacteria, and fungi that was first registered in United States in 1956 and has been used since the 1700s, whose registration was completed by Environmental Protection Agency (EPA) in 2009. Copper is present in food, water, and environment which is used in agriculture and non-agricultural settings. Copper ions effects humans and animals byprotein denaturation, producing cell damage and leakage by binding to functional groups of protein molecules (National Pesticide Information Center, 2012). It has been used extensively for the control of algal blooms.

- **Lead nitrate**

Inorganic compound lead nitrate ($Pb\ (No_3)_2$) usually occurs as a colorless crystal or white powder and, unlike most other lead (II) salts, is soluble

in water. Lead nitrate is toxic and are considered as carcinogenic to humans by the International Agency for Research on Cancer. Exposure to lead occurs mainly via inhalation of lead-contaminated dust particles or aerosols and ingestion of lead-contaminated food, water, and paints (ATSDR, 1999).Several microorganisms emerge mechanisms to survive lead exposure despite of its high toxicity.

- **Cobalt nitrate**

Cobalt nitrate is the inorganic cobalt (II) salt of nitric acid, generally found as a hexahydrate essential to the metabolism of all animals and are an important constituent of cobalamin.Ruminant animals convert cobalt salts in to vitamin B_{12} with the help of gut bacteria and are a micronutrient for bacteria, algae, and fungi in inorganic form. Outbreaks of cardiomyopathy happened among heavy consumers of cobalt fortified beer in which poor nutrition and ethanol played a synergistic role. Toxic manifestations had been reported following inhalation of cobalt containing dusts in industry and the vital target organs affected are skin and respiratory tract which might also be responsible for allergic dermatitis, rhinitis and asthma (Lauwerys and Lison, 1994)

- **Cadmium acetate**

Cadmium acetate is a colorless, coordination polymer, featuring acetate ligands interconnecting cadmium centers. The compound exists in anhydrous form and as dehydrate which is formed by treating cadmium oxide with acetic acid. Inhalation to cadmium and cadmium compounds may result in headache, chest pains, muscular weakness, pulmonary edema, and death (USAF, 1990).

1.6 Symbiotic association

Bioluminescent symbiotic associations were shown mainly by four species of luminescent bacteria like *V.fischeri*, *V.logei*, *P. leiognathi* and *P. phosphoreum* (Dunlap and Tsukamoto, 2006). The symbiosis between marine

bioluminescent *V. fischeri* and their sepiolid squid hosts has revolutionized the study of animal–bacteria interactions, serving as a model for the last two decades, because both of them can be maintained independently in the laboratory (Nishiguchi, 2000). Squid hatchlings come out from their eggs with gnotobiotic light organs and afterward colonized within hours by free-living planktonic *V. fischeri* from seawater (Nyholm and Nishiguchi, 2008). Squid utilize the bacterial bioluminescence for counter-illumination camouflage during its nocturnal activity (Jones and Nishiguchi, 2004), and squid assist the bacteria by offering a microhabitat rich in nutrients (Soto *et al*., 2009). *Vibrio* symbionts native to *Euprymna* species are host specialists and exhibit competitive dominance, outcompeting allochthonous isolates (Soto *et al*., 2012).

V. fischeri forms light organ mutualisms with monocentrid fishes (Nishiguchi, 2002). *V. fischeri* is efficient enough for persisting as a native part of marine sediment and sand biofilm microflora, skin and gut commensals of marine animals, and attached to floating debris, particles, zooplankton, phytoplankton, and carrion (Jones and Nishiguchi, 2004; Soto *et al*., 2010). High levels of symbiont biodiversity had been revealed during spatial population genetics studies with *V. fischeri* in allopatric and sympatric squid host population (Kimbell*et al*., 2002; Jones *et al*., 2006; Zamborsky and Nishiguchi, 2011).Biological benefits are gained by both members in the symbiotic relationship between bacteria and host. *P. phosphoreum* colonizes the light organs of the host and plays a role via the emission of light in communication, prey attraction and predator avoidance (Thomson *et al*., 2004).

Bioluminescent *Photobacterium* species such as *P.kishitanii, P. leiognathi* and *P. mandapamensis* were seen in association with the light organs of marine fish and squid, and are provided with nutrients and oxygen for reproduction recompensing by providing bioluminescence to their hosts

(Urbanczyk *et al.,* 2011*). P. leiognathi* exist in symbiotic relationship with the ponyfish which had developed a special compartment (light organ) where the bacteria live, thrive and survive (Herring and Widder, 2001; Medvedeva *et al*., 2006; Malave *et al*., 2010). Symbiotic specificity of *P. leiognathi* and leiognathid fish is maintained at the species level rather than individual level (Dunlap *et al*., 2004).

1.7 Significance of the present study

Studies on the distribution of luminescent bacteria along Indian estuaries are limited to Vellar estuary wherein their qualitative and quantitative distribution and seasonal variation of luminescent bacteria in the sediments had been focused (Ramesh *et al.*, 1989). Even though studies regarding different bacterial population from various estuaries in Kerala were abundant (Ouseph *etal*., 2009; Sudhanandh *et al*., 2012; Ammini *et al*., 2015), no such studies related to bioluminescent bacteria have been reported. Ponnani estuarine region is located between 10^{o} 46' N and 10^{o} 48'N and 75^{o} 54'E to 75^{o} 56'E, the estuary is open all through the year and is adjacent to the major fishing harbor in the Malappuram district. The estuarine system is exposed to tides from the Arabian Sea and hence the water is brackish throughout the year. Ponnani estuaries hold a very rich Ichthyofauna, but are subject to ecological deprivation due to ever growing human impedemence. The estuarine system supports livelihood for many fisherfolks along Ponnani coastal belt and the region received little attention from researchers. Hence the present study has been undertaken to elucidate the distribution and abundance of bioluminescent bacteria in Ponnani estuary to study the pollution status due to anthropogenic activities. The study also focuses on antibiotic and heavy metal susceptibility of isolated strains.

1.8 Objectives of the study

- Isolation and molecular characterization of different strains of luminescent bacteria from water, sediment, fish and invertebrates.
- Determination of the pollution status through BLR (bioluminescent ratio).
- To correlate the growth and abundance of isolated bioluminescent bacteria with selected physico-chemical parameters.
- To study the symbiotic relationship between bioluminescent bacteria and its host to examine ecological and physiological interactions.
- To study antibiotic and heavy metal susceptibility of isolated strains

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CHAPTER 2
REVIEW OF LITERATURE

Bioluminescence is a phenomenon of light emission wherein luciferin reacts with oxygen in the presence of the enzyme luciferase (Welsh and Kay, 2005). This aspect can be noticed in bacteria, dinoflagellates, radiolarians, ctenophores, molluscs, cnidarians, annelids, crustaceans, tunicates, echinoderms, fish and worms (Steven *et al*., 2009). Bioluminescent bacteria can flourish in different hosts like planktonic free-living organisms, saprophytic organisms, pathogenic parasites, gut or light organ symbionts and is usually in the genus of *Vibrio, photobacterium, photorhabdus, and shewanella* (Wannamaker, 2013). Bioluminescent bacteria were employed in pollution monitoring especially metals (Neha and Arti, 2015). The present study was undertaken to assess the abundance and distribution of bioluminescent bacteria in Ponnani estuary with a view to focus on the potential use of bioluminescent isolates in monitoring pollution by BLR ratio.

2.1 Bioluminescent bacteria

Bioluminescence is a fascinating chemical reaction in which obvious visible light will be emitted by several bioluminescent organisms (Ramesh and Mohanraju, 2017). The bioluminescence emission of diverse luminous organisms ranges from 400 nm to 700 nm (Widder, 2010) withemission colors like green, yellow, red, blue and pink (Therese and Woodland, 2013). Bacterial species reported to possess bioluminescence belongs to the families Vibrionaceae, Shewanellaceae or Enterobacteriaceae, and they belong to class Gammaproteobacteria (Eugene *et al*., 2017).

Table 2.1: List of bioluminescent bacterial species with their respective family

Family	**Genus**	**Species**
Enterobacteriaceae	*Photorhabdus*	*Photorhabdus asymbiotica* *Photorhabdus luminescens* *Photorhabdus temperata*
Shewanellaceae	*Shewanella*	*Shewanella woodyi* *Shewanella hanedai*
Vibrionaceae	*Alivibrio*	*Alivibrio fischeri* *Alivibrio logei* *Alivibrio salmonicida* *Alivibrio sifiae* *Alivibrio "thori"* *Alivibrio wodansis*
	Photobacterium	*Photobacterium aquimaris* *Photobacterium damselae* *Photobacterium kishitanii* *Photobacterium leiognathi* *Photobacterium mandapamensis* *Photobacterium phosphoreum*
	Vibrio	*Vibrio azureus* *Vibrio"beijerinckii"* *Vibrio campbellii* *Vibrio chagasii* *Vibrio cholera* *Vibrioharveyi* *Vibrio mediterranea* *Vibrio orientalis* *Vibrio segamienisis* *Vibrio splendidus* *Vibrio vulnicus*
	"Candidatus Photodesmus"	*"Candidatus Photodesmus katoptron"*

(List from Dunlap and Henryk (2013), "Luminous bacteria", The Prokaryotes)

Earlier studies on the distribution of bioluminescent bacteria are reported from Orndorff and Colwell. (1980) where they focused on the distribution of *V. fischeri* and *Lucibacterium harveyi*, *P. leiognathi* and *P. phosphoreum* from Sargasso Sea surface waters. Kathleen *et al*. (1984) reported the distribution of bioluminescent bacteria of Mystic river estuary and observed maximum Population during winter and lowest in summer.

Luminescent bacterial strains resided in whole chum salmon, *Oncorhynchus kisutch*, harvested in the Yukon River, Alaska were isolated by plating on seawater complex agar. Isolated bioluminescent colonies were identified by biochemical characterization and molecular identification as *P. phosphoreum (Budsberg et al.,* 2003).Gallardo *etal*. (2004) disclosed the incidence of *Vibrio* (20%) and *Photobacterium* (2%) groups in the marine algae.Quantitative assessment of luminous bacteria by De Luca (2006) demonstrated the influence of *P. phosphoreum* (87%), and intermittent incidence of *Vibrio* and *Shewanella* species in the Strait of Sicily and Ionian Sea. Brackish, coastal, and pelagic waters are uniquely inhabited by distinct *Vibrio* populations (Urakawa and Rivera, 2006).

Luminous bacteria in cold-dwelling marine fishes were isolated from the light organs and skin of different fish species. Identification of luminescent bacteria by 16S rRNA gene sequence analysis placed these bacteria in the genus *Photobacterium*. The isolated strains were closely related to *P.phosphoreum* and *P.iliopiscarium*. From datas of DNA-DNA hybridization and biochemical tests (arginine dihydrolase and lysine decarboxylase) differentiates these strains from *P. phosphoreum* and *P. indicum*. Hence, the isolated bacteria were confirmed as *Photobacterium kishitanii*, a luminous symbiont isolated from the light organ of the deep-water fish *Physiculus japonicas* (Ast *et al*., 2007).

Gentile *et al*. (2009) examined plentiful distribution of *P.phosphoreum* and *P.kishitanii* in seawater sample in Tyrrhenian Sea. Malave *et al*. (2010)

identified bioluminescent *V. fischeri* from marine environments of Puerto Rico by 16S rRNA sequence analysis to test their potential use as genetic tools and bioindicators. Identification of *V. harveyi* by 16S r RNA gene sequencing and species-specific PCR as a causative bacterium for a tail rot disease of sea bream *Sparus aurata* from research hatchery in Malta showed first report of disease caused by *V. harveyi* and their systematic study in the sea bream hatchery from Malta(Haldar *et al*., 2010).

Aisha and Nuzhat, (2011) identified bioluminescent *V.harveyi* from sea water samples from the shore of Arabian Sea by 16s rRNA sequencing. Uzma *et al*. (2012) charecterized and screened the luminescent bacterial strain from sea water and the organisms collected from Clifton, Hawksbay, Pakisthan were identified by 16s rRNA analysis as *V.chigassi*.

General microbiological techniques have been used to isolate bioluminescent bacteria from water samples and squid of Malaysia. The characterization of bioluminescent isolates was conducted and further analyzed at molecular level using 16srRNA sequencing identified as *P. leiognathi* and *Vibrio* species. Lux gene from isolated bioluminescent bacteria could be explored as it can be used as reporter gene in the development of biosensor (Noor *et al*., 2014). Jabalameli *et al*. (2015) isolated and identified bioluminescent *Vibrio* species for the production of biofilm from Chah Bahar Port of Iran.

Amplified fragment length polymorphism (AFLP) method was used to demonstrate the phylogenetic position of *Photobacterium* species collected from salmon products. By using this method, three species of *Photobacterium*, *P. phosphoreum*, *P. iliopiscarium* and *P. kishitanii* were discriminated. GyrB-gene sequencing and luxA-gene PCR amplification were employed to measure accuracy of the method. This method was excellent tool for genotyping bacterial isolates (Jerome *et al*., 2016).

Complete genome sequence of *V. fischeri* ES114 that exists in symbiotic association in the light organ of the bobtail squid, *Euprymna scolopes* revealed surprising parallels with *V. cholerae* and other pathogens.V. *fischeri* belongs to the Vibrionaceae, family of marine γ-proteobacteria that includes several species known to engage in a diversity of beneficial or pathogenic interactions with animal tissue (Ruby *et al*., 2005).

Bioluminescent bacteria were investigated for its ability to convert chemical energy into light energy source by bioluminescent reaction. Genomic extraction of the bioluminescent bacteria were done to amplify the Lux-AB gene cassette by using primer 611R (reverse) and 66F (forward). The Lux-AB gene presents a PCR product which contained Lux-AB gene proven to be transmitted the light when it's catalyzed by the enzyme called luciferase (Rozila *et al*., 2018).

Genome sequencing and genetic approaches were combined in order to characterize the regulation of luminescence in *P. leiognathi strain KNH6*. Insertions in genes for the luciferase reaction (*lux*, *lum*, and *rib* operons) were identified using transposon mutagenesis and screening for decreased luminescence. Using targeted gene insertional disruptions, demonstrated that under the growth conditions tested, luminescence levels do not be controlled through canonical pheromone signaling systems using targeted gene insertional disruptions (Anne *et al*., 2015).

Recent reports on bioluminescent bacteria focused mainly on biochemistry and genetic studies (Close *et al*., 2012; Nikolakakis *et al*., 2015; Vanmaele *et al*., 2015; Alejandro *et al*., 2018; Gregor *et al*., 2018, Hittu *et al*., 2018).

Qualitative and quantitative distribution and seasonal variation of luminescent bacteria in the sediments of Vellar estuary were studied and

recorded an indirect relationship between the salinity of estuarine water and luminous bacterial counts in the sediment (Ramesh *et al.*, 1989).

The strain isolated from Diu beach, Goa when subjected to 16S rRNA gene sequencing exposed a 1423 bp rDNA gene sequence and culture was identified as *V. rotiferianus* by BLAST analysis (Neha and Arti, 2015). Balan *et al*. (2013), isolated bioluminescent bacteria from various samples from Karaikal coast of Bay of Bengal and identified the organism as *Shewanella henadai*

Arulmoorthy, (2004) isolated and identifiedthe bioluminescent bacterial strains from seawater and sediments of the Roach Park, coastal area of Tuticorin to screen for production of bioactive metabolites. Kannahi and Sivasankari, 2014, while studying bioluminescent bacteria of Nagapattanam sea shore isolated and identified two bioluminescent bacteria, *Vibrio* species and *Pseudomonas* species. Srinivasan. (2014) investigated the distribution of bioluminescent bacteria in water and sediment of Palk Strait region, India and observed maximum bioluminescent load in summer.

Ramesh and Mohanraju. (2017) investigated the genetic diversity and distribution of bioluminescent bacteria in distinct marine niches of Andaman Islands that revealed the prevalence of harveyi clade members including *V.campbellii*, *V. harveyi*, *V. rotiferianus*, *V. alginolyticus* and *V. owensii* and to a lesser extent *P. damselae* and *P. leiognathi.* Bioluminescent Vibrio species were isolated from fish samples of coastal regions of South Gujarat (Neha *et al*., 2017).

2.2 Influence of Physico-chemical factors

Physico-chemical properties of water undergoes variation within seasons due to anthropogenic activities such as agriculture, urbanization, domestic sewage resulting in the deterioration of water quality (Verma *et al*., 2012). Temperature, turbidity, nutrients, hardness, alkalinity and dissolved

oxygen are major factors playing vital role for growth of living organisms in water. Since hydrological properties like physical, chemical and biological characters indicate its relationship with water quality; its assessment reflects the biotic and abiotic status of ecosystem (Smitha and Shivashankar, 2013). Dissolved oxygen in water gives direct and indirect information regarding bacterial activity (Premlata, 2009). Corrosive nature of water was determined by pH measurement since they are inversely related and are directly related with electrical conductance and total alkalinity (Gupta *et al*., 2009).

Abiotic factors such as temperature and salinity have differential effects between free-living and symbiotic strains of *V. fischeri*, which may transform colonization efficiency prior to infection (Soto *et al.,* 2009). *V. fischeri* itself is usually a temperate-water species but can be found in hosts inhabiting both tropical and temperate waters (Nishiguchi *et al*., 2004). Physicochemical and microbiological quality of aquaculture farms of Chellanam Panchayath showed homogenous content of DO, hardness, iron, alkalinity, ammonia, nitrite, nitrate, total plate count (TPC) and coliforms in all farms (Ginson *et al*., 2017).

Bioluminescent *V. fischeri* assays in the assessment of seasonal and spatial patterns in toxicity of contaminated river sediments, total organic carbon (TOC) was found to be highly correlated with toxic effects. Toxicity from sites with direct industrial and agricultural water inputs also correlated with concentrations of metals, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) (Sergio *et al*., 2016).

The influence of seasonal variation on the microbiological and physicochemical parameters of Imo River Estuary of the Niger Delta Mangrove Ecosystem revealed the variation in physicochemical parameters during wet and dry season. Temperature ranges for both surface and subsurface waterwere observed to be in mesophilic limits and also revealed useful information

regarding periodic and seasonal variations in microbiological and physicochemical gradients that occur in Imo river estuary (Unimke *et al*., 2014).

Variation in bioluminescence field intensity at night in the western part of the Black Sea resulted in a difference in the dynamics of the field intensity in the upper (0–35 m) and deep (>35 m) layers and elucidated the presence of bioluminescent species under influence of several biotic and abiotic factors. When compared to abiotic factors, biotic factors exhibited the greatest effect on the periodicity of the bioluminescence field intensity increase and decrease at night (Melnikova and Lymina, 2014).

Bioluminescent bacteria sheet were employed for the measurement of oxygen concentration. *P.kishitanii* sheets were prepared using sodium alginate, and a biofilm of *P.aeruginosa* was dropped over it. The relationship between the dissolved oxygen (DO) concentration in the biofilm and luminescence from the sheet was examined. As a conclusion, the alginate-gel film method was effective in fabricating dissolved oxygen monitoring thin film (Sasaki *et al*., 2013).

Effects of magnesium sulphate on the luminescence of *V.fischeri* under nutrient-starved condition were studied.When *V. fischeri* was cultured in artificial seawater medium, the luminescence intensity was low relative to that observed under normal growth condition. Under MgSO4 - starved conditions, luminescence was not fully induced and decreased afterwards. In contrast, induction of luminescence occurred under $MgSO_4$ - supplemented condition, butMgso4 alone was sufficient to induce luminescence, and required $NaHCO_3$ or KCl. These results suggest that the luminescence of *V. fischeri* is controlled by an exogenous sulfur sourse under nutrient-starved condition. In adition, they indicate the induction of sulfur-dependent luminescence is regulated bythe $NaHCO_3$ or KC1 concentration (Tabei *et al*., 2011).

Leena, (2007) examined physico-chemical features of Veli estuary. Thasneem *et al*., 2018 focused on the water quality status of Cochin estuary where in the water temperature showed an increasing trend and organic pollution and nutrient enrichment attributed to high BOD values. Ranjith *et al*. (2017) examined physico-chemical parameters of Vembanad backwaters.

2.3 Bioluminescent bacteria as indicators of pollution

The past few decades had witnessed vast increase in landscape and water quality deterioration of estuaries all over the world (Howarth *et al*., 2000). Rapid industrialization and constant anthropogenic pressures accounts for pollution of estuaries (Kennish, 2002). The toxic pollutants are extensively distributed on terrestrial and marine environment and their early identification is essential to prevent or to control the damages to humans and environment (Stefano *et al.,* 2008)**.**

Concentration of marine heterotrophic bacteria and their luminescent fraction expressed as percentage of luminous bacteria (LB %) in coastal waters along Campania shoreline used for pollution monitoring. Lower ratio indicates polluted areas and higher value results from unimpacted waters. LB% should be used in a frame of site dependent approach (Sardo *et al*., 2008). Bioluminescent bacteria mainly *V. fischeri* and their correlated detection technologies were used for assessment of water pollution because of their easy cultivable and detectable nature (Perego *et al*., 2002).

The assessment of pollution status of Skidaway river estuary was evaluated by using BLR that could be used as a simple and reliable indicator on chemical contamination of estuarine ecosystem resulting from anthropogenic impact.Bioluminescent ratio inhibition observed after exposure to contaminants suggesting physiological response. Lower ratio indicates pollution and higher ratio obtained from non-polluted waters (Fricsher *et al*., 2005).

Two bioluminescent bioassays based on lyophilized marine luminous bacteria, the Microbiosensor-B17-677F, and genetically modified luminous strain of *E. coli*, the Microbiosensor-ECK, are used to disclose areas of impaired water quality in the river and sewage waters of different regions of Siberia, showing the similar dependence on the concentration of the toxicants (Kuznetsov *et al*., 2002).

Bioluminescence is applied in the construction of biosensors for detection of contaminants, measurement of pollutant toxicity, and monitoring of genetically engineered bacteria released into environment. Biosensors were also used for the detection of pathogen and as the indicator of cellular metabolic activity (Vania and Norma, 2003). Bioluminescent bacteria based bioassays and biosensors offer a crucial way for the estimation of water toxicity and persistently exceed other known bioassays in speed, accuracy, sensitivity and simplicity (Malave *et al.*, 2010).

Freeze-dried cells have been used in Mutatox test which uses the dark variant of *V. fischeri*, which yield bioluminescence after incubation at 27 °C for 16-24 hours in the latency of genotoxic agents (Arfsten *et al*., 1994).The bioassays exploiting luminescence have been used for several decades, and *V.fischeri*, *V.harveyi* and *P.fluorescens* are used as the test organisms among which *V. fisheri* is widely employed because of its cheap and reproducible nature (Girotti *et al*., 2001). The presence of toxic substances, as pesticides, heavy metals or organic compounds can be evaluated by using bioluminescent bacteria as they emit light in the absence of toxic substances and their intensity decreases in its presence (Girotti *et al*., 2002). The luminescent bacterial system applied both as short- and long-term test and used for both water and solid samples (El-Alawi *et al*., 2002). The toxicity test based on bioluminescence developed in the 1970s (Trott *et al*., 2007). Bioanalytical tool like heterotrophic bacterial bioluminescent reporter system responds quantitatively to

bioavailable iron concentrations. These whole-cell biosensors are a powerful tool for environmental monitoring, providing both qualitative and quantitative insights on iron biogeochemistry (Mioni, 2004).

Luminous bacteria like *V.fischeri, V.harveyi and P.leiognathi* isolated from marine fishes like *Sardinella longiceps* used as a biosensor for monitoring heavy metals in marine water.From heavy metal toxicity analysis, it was observed that zinc induces the growth of luminous bacteria where as cadmium inhibited the growth which suggests the importance of bioluminescent bacteria as a biosensor for the detection of heavy metal pollution in marine water (Balachandar *et al*., 2010)

Bioluminecsent bacteria used as bio sensor for mercury toxicity. A bioassay system for detecting heavy metals in water using bioluminescent bacteria, *V.harveyi* and *V.fischeri* has been developed, which offers the adventages of simplicity and rapidity for screening heavy metals in water sources. Mercury, zinc and copper showed definite microbial toxicity and inhibiton of biolumincsence (Seema *et al*., 2014). Detoxification assessment of inorganic mercury can be carried out by biolumincsence of *V. fischeri.* Toxic effects of inorganic mercury ($HgCl_2$) on *V. fischeri* have been determined and evaluated by the detoxification of $HgCl_2$ in fluidized bed reactor using immobilized and free bacteria.*Escherichia coli strain R100* by the luminescence of *V. fischeri* (Abdel *et al.,* 2017)

2.4 Antibiotic susceptibility of bioluminescent bacteria

The widespread discharge of antibiotics in the water, have adverse effects on microorganisms in the aquatic environment. Ciprofloxacin is an example of widely used, broad-spectrum antibiotics and this made it imperative in an environmental risk assessment perspective (Naslund *et al.* 2008).

Antimicrobial resistance and presence of antibiotic resistant genes in different Vibrio species (*V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. harveyi, V. mimicus and V. cholerae*) showed multiple drug resistance to ampicillin, gentamycin and penicillin respectively. Also showed resistance to streptomycin, tetracycline and erythromycin and gene encoding their resistance were present (Raissy *et al*., 2012).Usage of oxytetracycline has resulted in elevated bacterial resistance in shrimp farms (VolumNash *et al.,* 1992). Chloramphenicol resistance had emerged as a result of antibiotic misuse in shrimp hatcheries in Ecuador and Philippines (Baticados and Paclibare, 1992).Most frequently used antibiotics in shrimp farms in Mexico are oxytetracycline, florfenicol, trimethoprim- sulfamethoxazole and more nowadays, sarafloxacin and enrofloxacin were administered by mixing with balanced feed or applied directly in water (Roque *et al.,* 2001).

In a comparative study regarding prevalence and antimicrobial susceptibility of *Vibrio* species in the black tiger shrimp (*Penaeus monodon Fabricius)* culture environment and market samples the highest antimicrobial resistance to penicillin and cephalexin and also displayed multidrug resistance (MDR). The main cause of antibiotic resistance could be the random application of antibiotics in shrimp farming and release of shrimp pond effluent to estuarine ecosystems or post harvest contamination of shrimps with the antibiotic resistant bacteria through the environment and human handling (Hossain *et al*., 2012).

Comparison of antibiotic resistance in bacterial isolates from pond water, pond sediment and cultured shrimp disclosed the incidence of resistance to oxytetracycline was the greatest followed by furazolidone, oxolinic acid and chloramphenicol (Tendenica and dela, 2001). Five common antibiotics, ampicillin, cephalothin, erythromycin, kanamycin and streptomycin, predominantly contributed to AMR including multi-drug resistance (MDR) in

the molluscan *Vibrio* spp. sourced to Canadian estuaries in 2006. A prospective follow-up analysis of these drugs showed a declining trend in the frequency of MDR/AMR-*Vibrio* species in subsequent years until 2012. The observed decline appears to be influenced by the specific downturn in resistance to the aminoglycosides, kanamycin and streptomycin (Swapan and Jeffrey, 2018).

Antimicrobial resistance of *V. parahaemolyticus* and *V. alginolyticus* strains isolated from farmed fish in Korea showed highest resistance to ampicillin (57.8%) followed by rifampicin (11.9%), streptomycin (8.7%) and trimethoprim (6.4%) (Oh *et al*., 2011). *V. harveyi* and *V. alginolyticus* showed multiple resistances to the tested drugs, ampicillin, amoxicillin, erythromycin and slfadiazine in performing the antibiotic susceptibility of *Vibrio* species

The antibiotic resistance profile and its correlation with transposons were investigated in *V. cholera. V.parahaemolyticus, V.vunificus, V.fischeri, V.proteolyticus* and *V.mimicus* isolated from Narmada River. Greater than 50% isolates displayed resistance against five commonly used antibiotics like ampicillin, ceftadizime, erythromycin, chloramphenicol, cefuroxime (Sharma *et al*., 2009).Reyhanath and Ranjeet, (2014) reported the occurrence of multidrug resistant *V. parahaemolyticus* from different samples along Ponnani coast.

2.5 Heavy metal resistance

The metal exists in soil as an outcome of atmospheric deposition and mineral weathering originating from anthropogenic and natural sources (Hookoom and Puchooa, 2013). In case of metal pollution, physicochemical parameters like pH, hardness, interactive effects and presence of natural organic matter plays a major role in solubility, bioavailability and toxicity of heavy metals (Tsiridis *et al*., 2005). Iron can drastically reduce luminous bacterial growth and wide spread occurrence of heavy metal resistance in bacteria were

reported by Ramaiah and Chandramohan,(1993).Copper ions cause protein denaturation, producing cell damage and leakage by binding to functional groups of protein molecules (National Pesticide Information Center, 2012). Zinc showed significant toxicity to bacteria and inhibition and inactivation of cell growth has been reported as antibacterial effect of zinc (Yanping *et al*., 2011).

Zinc pyrithione is membrane active which is indicated by inhibition of uptake of several unrelated substrates in both bacteria and fungi (Dinning *et al*. 1998). Detected concentration of ZnPT in the aquatic environment is between 1.9 – 32 µg/L (Woldegiorgis *et al*., 2007). Triclosan is a synthetic, broad-spectrum antimicrobial agent that has been used in a wide variety of household and personal care products and is found in the environment, food, plasma and human breast milk (Farre *et al*., 2007). The undesirable effect of triclosan is on aquatic organisms such as bacteria and algal communities and toxicity effect higher for bacteria than algae and triclosan of surface waters can degrade in the presence of sun light and it leads to harmful products in the environment. (Farre *et al*., 2007).

Bioluminescent bacterial strains (*Vibrio harveyi*) isolated and charecterised from marine niches of Goa demonstrated multiple metal resistances in terms of growth with altered luminescence with variable metal concentration. This bioluminescent bacterial species can be explored in future for constructing luminescence based biosensor (Neha and Arthi, 2015).

A bioassay system for detecting heavy metals in water by using bioluminescent bacteria like *V. harveyi* and *V. fischeri* was developed that offers simplicity and rapidity for screening heavy metals in water sources. Mercury, zinc and copper showed definite microbial toxicity and bioluminescent inhibition. The inhibition range for each strain was standardized and reproducibility verified (Seema *et al*., 2005).

Metal chemical speciation and toxicity in marine environment with three bioassays based on cellular bioluminescence, using a marine bacterium *P.phosphoreum* were focused on the fact that difference exist among toxicity assessment in bioassays because of differences in the physicochemical characteristics of the medium and different susceptibility pattern of metals towards the cells(Deheyn *et al*., 2004). Twenty five heavy metal resistance strains were isolated from the Alzahra Hospital of Isfahan in Iran in which most of the isolated bacteria showed multiple resistances to four heavy metals (Hg, Cu, Ph, and Cd) and almost all of the 25 isolated strains showed plasmid of different molecular size (Vajiheh *et al*., 2003).

V. harveyi, V. fischeri and *P. phosphoreum* were examined for its effect on different heavy metals. Checker board assay was used for the detection of the natural metal tolerance level of large number of bioluminescent bacteria. This study exposed the incidence of the occurrence of plasmids in heavy metal resistant bacteria and figured out that plasmids are highly ubiquitous and predominant in most heavy metal resistant bacteria (Ranjitha and Karthy, 2012). Bioluminescent bacteria isolated from *Nemipterus japonicas*, *Restrelliger kanagurta* and *Engraulis japonicas* were evaluated for the presence of Chromium in Water. Isolated *Vibrio* species were used for checking toxicity of chemical pollutant chromium in water, since chromium inhibits the growth of bioluminescent bacteria and effects luminous intensity. This study provided ecofriendly and cost effective examination of chromium (Varsha and Bagyashree, 2005).

2.6 Symbiotic association

Bioluminescence symbioses are highly significant due to many aspects and an important aspect of bioluminescent symbiosis is that it is present in a small minority of species (Dunlap and Kita Tsukamoto, 2006). In most bacteria-animal symbiotic associations, the host animal benefits from the relationship

through acquiring of nutrients (e.g. through nitrogen fixation by the bacteria) which are utilized for their growth purposes by the host (Claes and Dunlap, 2000). In the case of some fish like *Monocentris japonicus* development of the light organ occurs after the acquiring of symbionts (*V. fischeri*). Squid and fish acquire their luminescent symbionts by horizontal transfer from the surrounding seawater (Dunlap and Kita-Tsukamoto, 2006).

Genomic polymorphism in symbiotic populations of *P.leiognathi* forms a bioluminescent symbiosis with leiognathid fishes. Colonization of the leiognathid light organ to initiate the symbiosis is likely to be oliogoclonal and specificity of the *Photobacterium leiognathi* and leiognathid fish symbiosis is maintained at the bacterial species level rather than individual level, genotypically defined strain types (Dunlap *et al*., 2004). *V.fischeri* exists as monospecific symbiont of the Hawaiian bobtail squid, *Euprymna scolopes*, which requires signaling pathways and transcriptional responses between them. Early stages of the squid-vibrio symbiosis, and the transcriptional responses underlying the activities of marine microbes during host colonization had been discussed (Thompson *et al*., 2017). Bioluminescent bacteria were present in the gut part of Philippine marine fishes that exist in symbiotic association. Hence, Philippines, the center of marine diversity is a home of bioluminescent bacteria from marine fishes (Leonil *et al.,* 2015).

In the host-microbe symbiosis for the squid-Vibrio association which is a naturally occurring, experimental model of animal/bacterial partnership, the association between the Hawaiian sepiolid squid *Euprymnascolopes* and the marine luminous bacterium *V. fisheri* is highlighted. This symbiosis offers a relatively simple, naturally occurring, association that can be experimentally manipulated. This system provides insight into the precise mechanisms by which a beneficial animal-bacterial symbiosis can be established and maintained (McFall-Ngai, 2008).

Evolutionary perspectives in a mutualism of sepiolid squid and bioluminescent bacteria with combined usage of microbial experimental evolution and temporal population genetics were studied. Any observation regarding symbiont anagenic evolution within squid was present, as competitive dominance does not get rid of *V. fischeri* genetic diversity through time. Instead, abiotic factors affecting profusion of *V. fischeri* variants in the planktonic phase maintain temporal symbiont diversity *(*Soto *et al*., 2012)

Phylogenetic diversity and cosymbiosis in the bioluminescent symbioses of "*P. mandapamensis*"that are closely related to *P. leiognathi* was not the exclusive light organ symbiont of leiognathid fishes. Host species ranges of *P. mandapamensis* and *P. leiognathi* are substantially distinct underscores possible differences in the environmental distributions and physiologies of these two bacterial species (Kaeding *et al.,* 2007). During the study of genomic polymorphism in symbiotic populations of *P. leiognathi*, genomic profiling demonstrated difference in host species harboring genetically distinct strains of the bacterium by 16S rDNA sequence analysis were not the members of *P. leiognathi*, and revealed a genomotypically oligoclonal structure for the populations of *P. leiognathi* of individual fishes (Dunlap *et al*., 2004).

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CHAPTER 3

MATERIALS AND METHODS

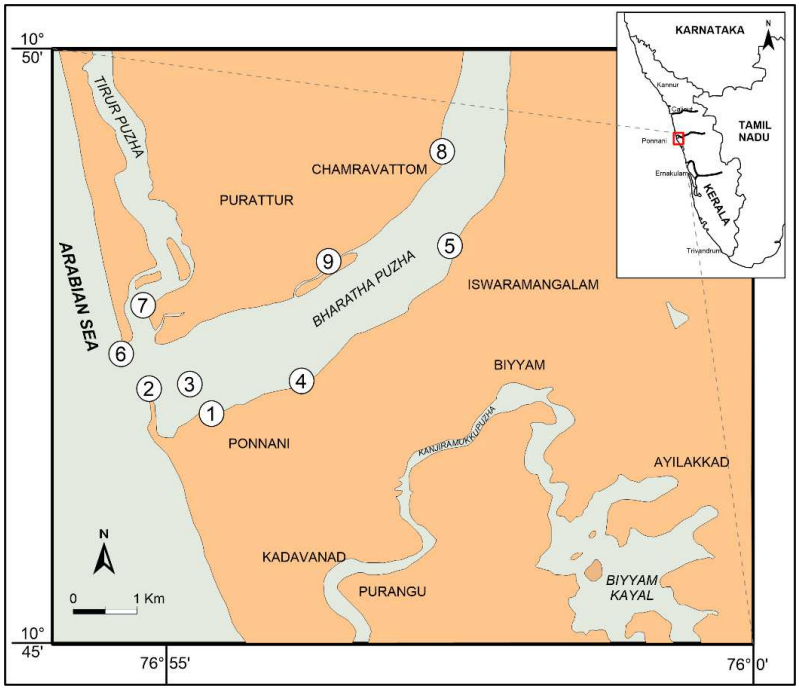

Figure 3.1: Location map of study area

3.1 Study area.

Ponnani estuary is an open estuary lying adjacent to Ponnani fishing harbour in the Malappuram district and is a region where Bharatappuzha meets the Arabian Sea. The study area lies between 10º 46'Nto 10º 48'N and 75º 54'E to 75º 56E. Even though Ponnani estuary supports a wealthy biological diversity; it has been subjected to ecological degradation due to ever increasing human intervention. The increasing levels of siltation, pollution and over exploitation of resources are the major problems. This necessitates frequent monitoring of estuarine resources and estimation of sustainable utilization

No	Stations	No	Stations
1	Jangar	6	Purathoor
2	Pallikkadav	7	Kootai
3	Chamravottom kadav	8	Harbour
4	Kalur	9	Chandappadi
5	Ishwaramangalam		

Figure 3.2: Jangar

Figure 3.3: Pallikadav

Figure 3.4: Chamravottom kadav

Figure 3.5:Kalur

Figure 3.6: Ishwaramangalam

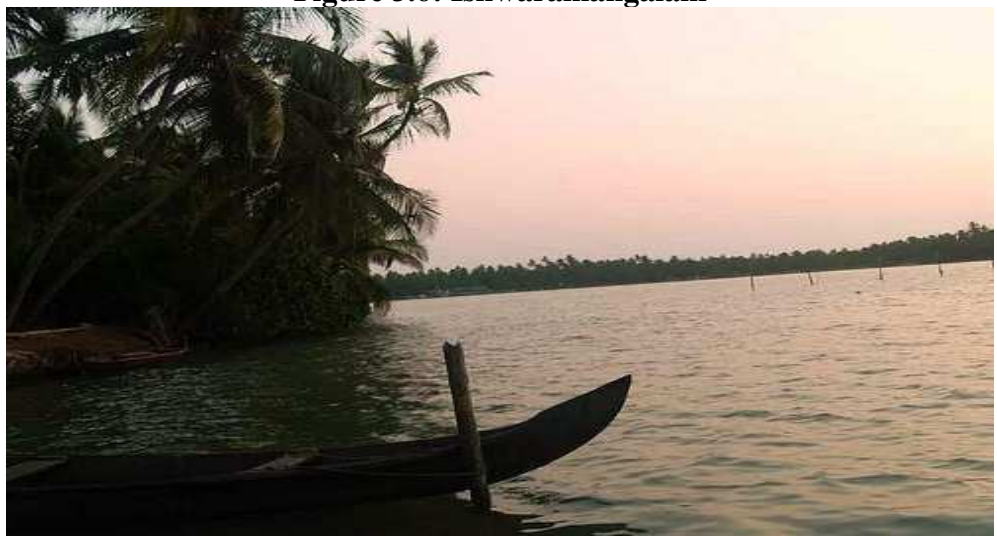

Figure 3.7:Purathur

Figure 3.8:Kootai

Figure 3.9:Harbour

Figure 3.10: Chandappadi

3.2 Sampling

Water, sediment, squid (*Uroteuthis duvaucelii*), Shrimp (*Metapenaeus dobsoni*) and Leiognathid fish (*Secutor ruconius*) samples were taken for the present investigation. Even though many species of invertebrates and fishes emit bioluminescence, widespread accessibility of *S. ruconius*, *U.duvaucelii* and *M. dobsoni* persuaded to select them as samples. These samples were collected from 9 stations (Jangar, Pallikkadav, Chamravottomkadav, Kalur, Ishwaramangalam, Purathur, Kootai, Harbour and Chandappadi) for a period of 24 months (February 2014 to January 2016). Water samples were collected in sterilized capped bottles and brought to the laboratory shortly for processing. Bottom drilled centrifuge tubes were used to collect sediment samples. The samples were quickly transferred to a sterile glass jar and carried to the laboratory for plating. Samples of *S. ruconius*, *U.duvaucelii* and *M. dobsoni* were transferred to sterile jars (250 ml) and were dissected aseptically in laboratory within 1 to 3 hour for bacterial isolation.

Figure 3.11: ***Secutor ruconius***

Figure 3.12: ***Uroteuthis lduvaucelii***

Figure 3.13: ***Metapenaeus dobsoni***

3.3 Analysis of physico-chemical parameters of water samples

The estuarine water samples collected from different stations of Ponnani estuary were evaluated for parameters pH, temperature, salinity, dissolved oxygen (DO) and biological oxygen demand (BOD) following the standard

procedures of APHA (2012) and Grasshoff (1999).Summary of the various methods used for water analysis were furnished in Table 3.3.

Table 3.3: Various methods used for water analysis

No	Parameter	Methodology/Instrument	Reference
1	pH	Electrometric method Digital pH meter (Systronics-335)	APHA 2012
2	Salinity	Argentometric method	Grasshoff (1999)
3	DO(mg /L)	Modified Winkler's method	APHA (2012)
4	BOD (mg/ L)	Unseeded dilution technique and incubation at 20± 1 °C for 5 days	APHA (2012)
5	Temperature(°C)	Thermometer	

3.4 Bacterial isolation and enumeration.

Three different media -Nutrient agar, Thiosulphate Citrate Bile salts Sucrose (TCBS) Agar and Sea water complex Agar (SWCA) were used in the present study. Nutrient Agar were employed in order to obtain total heterotrophic bacterial count where as TCBS agar were used to know the presence of bioluminescent Vibrios and Sea water complex agar were employed to isolate bioluminescent bacteria.

3.4.1 Enumeration of total heterotrophic count.

Total heterotrophic bacterial count of different samples (Water, sediment, *S. ruconius*, *U.duvaucelii* and *M. dobsoni*) were done by pour plating (appropriate dilution of samples) on nutrient agar and incubated at 37°C for 24 hours. *S. ruconius*, *U.duvaucelii* and *M. dobsoni* samples (1 gm) wereweighed and homogenized for 1 min in Waringblender containing 9 ml sterile distilled water.

3.4.2 Enumeration of total Vibrio like organisms.

Total Vibrio count from different samples (Water, Sediment, *S. ruconius*, *U. duvaucelii* and *M. dobsoni*) were done by pourplating (appropriate

dilution of samples) on TCBS Agar and incubated at 37^0C for 24 hours. Initially samples were enriched in alkaline peptone water by incubation at 37ºC for 6-8 hours. After incubation, 1 ml from the pellicle of each tube (Enrichment broth -APW) were then pour plated on to Thiosulfate citrate bile salts sucrose (TCBS) agar plates by taking appropriate dilution and incubated at 37º C for 24 hours.

3.4.3 Enumeration of bioluminescent bacteria

The media used for isolation and maintenance of bioluminescent bacteria were SWCAgar and sea water complex broth. This media contains Nacl, peptone, gycerol, yeast extract and agar prepared in sea water. Pour plate method was the primary procedure for determining the number of bacteria in water, sediment, invertebrates (*U.duvaucelii* and *M. dobsoni*) and alimentary canal of *S. ruconius*. Sterile distilled water was used to make appropriate dilutions. All samples were plated in triplicate.

Water samples were directly plated on seawater complex agar plates and incubated at 37ºC for 24 hours. Sediment, *S. ruconius*, *U.duvaucelii* and *M. dobsoni* samples were weighed (1 gm) and homogenized for 1 min in a Waringblender containing 9ml sterile distilled water. Appropriate dilutions were prepared by taking the prepared suspension as first dilution. From appropriate dilutions, samples were pour plated on seawater complex agar and kept for incubation for 24 hours at 37ºC and observed in dark for luminescence.

3.5 Identification and characterization of luminescent bacteria

Bacterial characterization was preliminarilycarriedout focusing on cellular and microscopic morphology and biochemical tests following the procedures of Cowan and Steel, (1993). Gram's staining was done as general differentiating technique.Motility tests were carried out to understand flagellar movement. Biochemical tests used in this study includes indole, Methyl Red, Voges-Proskauer, Simmon's citrate, nitrate reduction, gelatinase, urease, oxidase, catalase, TSI (Triple sugar iron), ONPG, lysine decarboxylase,

haemolysis and Vibrio disc0129. Growth on TCBS (Thiosulfate-citrate-bile salts-sucrose) agar medium was also tested to gather the nutritional requirements of *Vibrio* spp., as an important genus of light producing bacteria.

3.5.1 Gram's staining

Primary stain Crystal Violet (CV) was added to a heat-fixed smear of bacterial culture. Gram's Iodine was added at 1 minute interval and rinsed with water. Decolorized with 95% ethyl alcohol and counter stained with safranin and the air dried specimens were observed under microscope.

3.5.2 Test for motility (Hanging Drop technique)

A drop of medium containing cells to be focused was allowed to hang in the cavity of slide in this technique and is the generalized method used to observe the motility of bacteria.

3.5.3Indole Test

Bacterial culture is inoculated into tryptone broth in which the amino acid tryptophan is abundant. Tryptophanase, an enzyme that cleaves tryptophan into indole and other product is produced by positive bacteria. When Kovac's reagent (p-dimethylaminobenzaldehyde) was added to a broth, it develops pink color after 48 hours of incubation.

3.5.4 The Methyl Red and Voges-Proskauer Tests

The methyl red (MR) and Voges-Proskauer (VP) tests were read from a single inoculated tube of MR-VP broth. After 24-48 hours of incubation the MR-VP broth was split into two tubes. One tube is used for the MR test; the other is used for the VP test. MR-VP media contains glucose and peptone. All enteric oxidize glucose for energy; however, the end products vary depending on bacterial enzymes. When the pH indicator methyl red was added to acidic broth it will be cherry red (a positive MR test). In this neutral pH the growth of

the bacteria is not inhibited. The bacteria thus start to attack the peptone in the broth, causing the pH to rise above 6.2. At this pH, methyl red indicator is a yellow colour (a negative MR test). The Voges-Proskauer test (VP) cultures were incubated at 30°C for 24-48 hours. Add 1 ml of Barritt Reagent B (R030 - 40% potassium hydroxide) and 3 ml of Barritt Reagent A (R029 - 5% alpha-naphthol in absolute ethanol) to 5 ml culture. Positive test is indicated by eosin pink colour within 2-5 minutes.

3.5.5 Citrate Utilization Test

Simmon's citrate is the medium used to find out if a bacterium can grow utilizing citrate as its sole carbon and energy source. Simmon's media contains bromthymol blue, a pH indicator with a range of 6.0 to 7.6. Bromthymol blue is yellow at acidic pH (around 6), and progressively changes to blue at more alkaline pH (around 7.6). Uninoculated simmon's citrate agar has a pH of 6.9 having an intermediate green colour. Growth of bacteria in the media leads to development of a prussian blue colour (positive citrate).

3.5.6 Triple Sugar Iron agar test

Triple sugar iron (TSI) agar is used for the identification of gram-negative enteric bacilli on the basis of dextrose, lactose and sucrose fermentation and hydrogen sulphide production. Phenol red is the pH indicator.

3.5.7 Urease test

Rapid urease test broth is recommended for rapid detection of urease production. Urease activity can be described as the splitting of urea via hydrolysis by a urease enzyme. The end products from this reaction yield ammonium carbonate and ammonia, which are alkaline in nature.The consequent rise in the pH of the medium, is detected by phenol red indicator.

3.5.8 Oxidase test

Oxidase discs are used for detection of oxidase production by microorganisms. In the oxidase test, a colourless dye such as N, N-dimethy-p-phenylenediamine serves as an artificial electron acceptor for the enzyme oxidase. The dye is oxidized to form indophenol blue, a coloured compound. Oxidase discs are sterile filter paper discs impregnated with N, N-dimethyl-p-phenylenediamine oxalate, ascorbic acid and a-naphthol. In a positive reaction the enzyme cytochrome oxidase combines with N, N-dimethyl-phenylenediamine oxalate and alpha-naphthol to form the dye, indophenols blue.

3.5.9 ONPG test

ONPG discs were used for the rapid detection of beta-galactosidase activity in microorganisms, especially to identify late lactose fermenters quickly. ONPG (Ortho-nitrophenyl Beta-D-galactopyranoside) is a synthetic colourless compound (galactoside) structurally similar to lactose. Beta-galactosidase cleaves ONPG to galactose and O-nitrophenyl, a yellow compound. ONPG discs are sterile filter paper discs impregnated with ONPG and is similar in structure to lactose. The presence of two enzymes is required to demonstrate lactose fermentation in a conventional test.

3.5.10 Catalase test

The catalase test is a test for the presence of the catalase enzyme. Most organisms possess this enzyme capable of breaking down hydrogen peroxide. Organisms containing the catalase enzyme will form oxygen bubbles when exposed to hydrogen peroxide.

3.5.11 H_2S production test

H_2S is produced when sulphur containing amino acids were decomposed. When bacterial culture was added to the medium blackening of the medium was observed after incubation at 35-37^oC overnight in positive result.

3.5.12 Nutrient gelatin test

Nutrient gelatin is recommended for detection of gelatin liquefaction by proteolytic microorganisms. Peptic digest of animal tissue and beef extract supply nutrients for the growth of non-fastidious organisms. Gelatin is the substrate for the determination of the ability of an organism to produce gelatinase, a proteolytic enzyme active in the liquefaction of gelatin.

3.5.13 Amylase

Test samples were streaked over the plate containing amylase medium. The plates were then incubated overnight and observed for zone formation in the positive plates.

3.5.14 Lipase

Bacterial isolates were streaked over the plate containing Zobel 2216E modified media. The plates were then incubated at 30^oC for 10 days and observed for zone formation in the positive plates.

3.5.15 Lysine decarboxylase test

Lysine decarboxylase broth is especially suited to study the decarboxylase reactions for microorganisms. During the initial stages of incubation, following inoculation, fermentation of dextrose by the organisms leads to acid production, which causes a subsequent colour change of the bromocresol purple indicator to yellow. Dextrose non-utilizers will not show any change in the medium colour. Light inoculums were usually used and do

not read the tests after 24 hours of incubation, as some organisms require longer incubation time of up to 4 days.

3.5.16 Nitrate reduction test

Heavy inoculum of test bacteriainoculated in nitrate broth were incubated for 24-48 hours, after the incubation, sulfanilic acid and α-naphtylamine (nitrate disc) were added and observed the colour change.

3.5.17 Haemolysis

To detect pathogenicity of organisms on blood agar, pure, fresh culture of the test organism from sample were streaked on a non-selective blood agar plate (containing 0.5% NaCl) and later incubated at 35 – 37°C for 24 hours and observed for zones of inhibition.

3.5.18 Vibrio disc 0129

For differentiation of *Vibrio* species based on sensitivity to Vibrio static agent O129, with a sterile swab, pure, fresh culture of the test organism from samples were straked on a non-selective blood agar plates (containing 0.5% NaCl). Aseptically both Vibrio 0129 differential discs [10 mcg, (DD047) and 150 mcg (DD048)] were placed on the swabbed plates. The plates were later incubated at 35 – 37°C for 24 hours and observed for zones of inhibition.

3.6 Molecular identification

3.6.1 Sequencing of 16S rRNA Region using Universal Primers

3.6.1.1 *Genomic DNA Isolation*

NucleoSpin® Tissue Kit (Macherey-Nagel) was used to isolate genomic DNA following manufacturer's instructions.

A part of culture was taken in a micro centrifuge tube to which 180 µl of T1 buffer and 25 µl of proteinase K was added and incubated at 56 ºC in a water bath until it were completely lysed. After lysis, 5 µl of RNase A (100

mg/ml) was added and incubated at room temperature for 5 minutes. After 5 minutes of incubation, 200 µl of B3 buffer was added and incubated at 70°C for 10 minutes and later 210 µl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into NucleoSpin® Tissue column placed in a 2 ml collection tube and centrifuged at 11000 x g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2 ml tube and washed with 500 µl of BW buffer. Wash step was repeated using 600 µl of B5 buffer. After washing the NucleoSpin® Tissue column was placed in a clean 1.5 ml tube and DNA was eluted out using 50 µl of BE buffer.

3.6.1.2 *Agarose Gel Electrophoresis for DNA Quality and Quantity check*

Agarose gel electrophoresis was used to check the quality of isolated DNA. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front had migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.6.1.3 *PCR Analysis*

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM Tris HCl, pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5pM of forward and reverse primers and template DNA.

Primers used

Target	Primer Name	Direction	Sequence (5' → 3')
16S rRNA	16S-RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

PCR amplification profile

16S rRNA

95 ºC - 5.00 min

95 ºC - 30 sec
60 ºC - 40 sec } 35 cycles
72 ºC - 60 sec

72 ºC - 7.00 min

4 ºC - ∞

3.6.1.4 *Agarose Gel electrophoresis of PCR products*

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.6.1.5 *ExoSAP-IT Treatment*

ExoSAP-IT (USB) consists of two hydrolytic enzymes, exonuclease I and shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for

the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product was mixed with 2 µl of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 80°C for 15 minutes.

3.6.1.6 *Sequencing using BigDye Terminator v3.1*

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

The PCR mix consisted of the following components

PCR Product(ExoSAP treated)	-	10-20 ng
Primer	-	3.2 pM (either Forward or Reverse)
Sequencing Mix	-	0.28 µl
Reaction buffer	-	1.86 µl
Sterile distilled water	-	make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96°C for 2 minutes followed by 30 cycles at 96°C for 30 secs, 50°C for 40 secs and 60°C for 4 minutes.

3.6.1.7 *Post Sequencing PCR Clean up*

1. Master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction and master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol were prepared.
2. 12µl of master mix I was added to each reaction containing 10µl of reaction contents and was properly mixed.
3. 52 µl of master mix II was added to each reaction.

4. Contents were mixed by inverting and incubated at room temperature for 30 minutes
5. Spinned at 14,000 rpm for 30 minutes
6. Decanted the supernatant and added 100 µl of 70% ethanol
7. Spun at 14,000 rpm for 20 minutes.
8. Decanted the supernatant and repeated 70% ethanol wash
9. Decanted the supernatant and air dried the pellet.

The cleaned up air dried product was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems).

3.6.1.8 *Sequence Analysis*

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6 (Drummond et al., 2012).The sequence analysis was carried out using bioinformatics tool BLAST of NCBI. Based on maximum identity score first few sequences were selected and aligned using multiple sequence alignment software ClustalW2. Dendrogram was constructed

3.7 Effect of different parameters on the luminescence of bioluminescent bacteria

Growth and light emission of bioluminescent bacteria were optimized by altering different parameters like pH, salinity, incubation temperature and glycerol concentration following the procedures of Alex (2010).

3.7.1 Effect of salinity (Varying concentrations of NaCl)

SWCA medium was prepared by adding varying concentration of NaCl such as 0%, 3%, 6%, 9% and 12%. The medium was poured into petri plates

and allowed to settle. Bacterial isolates were streaked and the plates were incubated at 37°C for 24 hours.

3.7.2 Effect of pH

SWCA medium was prepared with four different pH values such as 5, 7, 9 and 11 by adding acid or base. Isolated bacteria were streaked on presetted media and the plates were incubated at 37°C for 24 hours and the intensity of luminescence was recorded.

3.7.3 Effect of glycerol concentration

Different concentration of glycerol such as 0.1%, 0.3%, 0.6%, 0.9% and 1.2% were employed for SWC agar media preparation. Isolated bioluminescent bacteria were streak plated on SWCA and the plates were incubated at 37°Cfor 24 hours.

3.7.4 Effect of temperature

Isolated bioluminescent bacteria were streaked on SWCA plates. The plates were incubated for 24 hoursat different temperatures (15°C, 20°C, 25°C, 30°C, 35°C) and observed for their luminescence.

3.8 Pollution status of Ponnani estuary through determination of BLR (Bioluminescent ratio)

Ratio of bioluminescent colony forming unit (cfu) to total cfu was determined following the procedures of Ramaiah and Chandramohan (1993). Samples were filtered and pour plated on sea water complex agar and incubated at 37^0C for 24 hours and luminescent colonies were counted in a dark room for obtaining bioluminescent cfu. Total colony forming units were counted by pour plating the water samples on nutrient agar and incubated at 37°C for 24 hours. The BLR was calculated as the ratio of bioluminescent cfu

3.9 Antibiotic susceptibility of bioluminescent bacteria

Bioluminescent bacteria isolated from estuarine samples were subjected to antibiotic sensitivity testing against commonly used antibiotics following Agar disc diffusion method of Baur *et al*. (1966). Commercially available antibiotic discs of chloramphenicol, ciprofloxacin, gentamycin, cefotaxime, tetracycline, aztreonam and polymixin B were placed over the plates swabbed with isolated strains in muller hinton agar medium and incubated at 37^{o}C for 24 hours and the zone of inhibition were measured.

3.10 Heavy metal susceptibility of bioluminescent bacteria

The sensitivity or resistance nature of isolates towards different dilutions of 1mg/ml concentration of various heavy metals (Hg, Cu, Pb, Cd and Co) was tested by agar disc diffusion method following the procedures of Alex (2010). After overnight incubation, plates were observed for the zone of inhibition. Minimum Inhibitory Concentration (MIC) of the heavymetals against isolated bacteria was determined by gradually increasing the heavy metal concentration. The lowest concentration of metal that completely prevented growth was termed the MIC.

Procedure:

- Bacteria were transferred aseptically to 5ml sea water broth and incubated overnight at 37°C.
- Sterile filter paper discs, (Whatman No. 1 disc) 6mm in diameter were soaked in solutions of the appropriate heavy metal salts(Hg, Cu, Pb, Co, Cd) with different concentration dilutions.
- The filter paper discs were impregnated with different concentrations of the heavy metal salts and allowed to dry.

- Sterile cotton swabs were immersed to the overnight microbial suspension, and then isolated and compressed against the wall of the test tube so as to expel the excess fluid.
- The surface of the sea water agar plate was inoculated with the swab to ensure uniform and confluent growth.
- The filter paper discs were then placed on the surface of the plates. Each plate contained nine dilutions of the heavy metals.
- The plates were then incubated at 37°C for 24 hours.
- After incubation, the zone of inhibition was measured. MIC was determined from the plates.

3.11 Statistical Analysis

3.11.1 ANOVA

Two-way analysis of variance (ANOVA) was conducted to determine any significant difference in the value of each parameter between station and between seasons using StatistiXL-data analysis package.

3.11.2 Pearson's correlation coefficient

The relationship between the different environmental parameters and abundance of bioluminescent bacteria were calculated using Pearson's correlation coefficient.

3.11.3 Cluster analysis

Cluster analysis was done for drawing antibiogram which will help to find out which of the antibiotics are having similar response using SPSS Software.

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CHAPTER 4
RESULTS

The present study was conducted to explore the distribution and abundance of bioluminescent bacteria in relation to the physico-chemical parameters from Ponnani estuary. Isolation, identification and molecular characterization of bioluminescent bacteria were done and the study also focused on antibiotic and heavy metal susceptibility of the isolated strains. The pollution status of the estuary was also assessed based on bioluminescent ratio.

4.1 Physico-chemical parameters of estuarine water samples

Analysis of physico-chemical parameters (pH, salinity, temperature, dissolved oxygen (DO) and biological oxygen demand (BOD) for water samples from different stations of Ponnani estuary were evaluated over two years (February 2014-January 2016) using standard methods (APHA, 2012 and Grasshoff, 1999).

4.1.1 Seasonal variations of physico-chemical parameters in Jangar

In Jangar station the pH ranged between 7.6-8.4 with an average of 8.1. Remarkable variation in salinity was observed in Jangar and the values ranged between 12-32ppt with an average of 22ppt. Temperature ranged between 29-32.5^{o}C with an average of 31^{o}C. Comparatively low level of DO obtained at Jangar and values ranged between 2.02-5.98 mg/L with an average of 3.59mg/L. BOD values ranged between 0.72-2.56mg/L with an average of 1.50mg/L (Table 4.1).

4.1.2 Seasonal variations of physico-chemical parameters in Pallikadav

In Pallikkadav, pH ranged between 7.8-8.5 with an average of 8.2. Notable variation in salinity was observed in Pallikkadav and the values ranged between 12-38ppt with an average of 22ppt. Temperature ranged between 29-32.5^{o}C with an average of 31^{o}C. Relatively low level of DO obtained at Pallikkadav and their values ranged between 3.56-4.98 mg/L with an average of 3.89mg/L. BOD values ranged between 0.2-3.55mg/L with an average of 2.15mg/L (Table 4.2).

Table 4.1: Seasonal variations of Physico-chemical parameters in Jangar

Season	Month	pH	Salinity (ppt)	Temp (°C)	DO (mg/L)	BOD (mg/L)
2014-15						
Pre- Monsoon	February	8	32	30	4.26	2.13
	March	8.5	32	32.5	3.56	1.42
	April	8.5	32	32	2.85	0.72
	May	8.4	29	32.5	4.98	2.13
Monsoon	June	8.1	16	29	2.02	0.98
	July	7.6	16	30	2.02	0.98
	August	7.8	12	31	2.56	1.02
	September	7.8	13	31	3.56	1.42
Post- Monsoon	October	8.1	15	30	3.26	1.24
	November	8	15	30	3.56	1.42
	December	8.2	18	29	4.28	2.16
	January	8.4	28	30	4.96	2.13
2015-16						
Pre- Monsoon	February	8.1	28	29	4.98	2.26
	March	8.6	30	32	2.56	1.02
	April	8.4	32	32.5	2.56	1.02
	May	8.2	35	32.5	4.56	2.12
Monsoon	June	8	18	30	2.58	0.75
	July	7.6	18	30	2.58	0.75
	August	7.8	16	31	3.86	1.02
	September	8	12	31	3.92	1.15
Post-Monsoon	October	8	16	30	4.26	2.24
	November	8.1	16	31	4.26	2.24
	December	8.3	20	29	4.98	2.56
	January	8.2	25	30	3.13	1.08

Table 4.2: Seasonal variations of physico-chemical parameters in Pallikadav

Season	Month	pH	Salinity (ppt)	Temp (°C)	DO (mg/L)	BOD (mg/L)
2014-15						
Pre- monsoon	February	8.5	26	32.5	4.26	2.13
	March	8.3	30	32.5	4.26	3.55
	April	8.4	32	32.5	3.56	1.43
	May	8.3	23	32.5	4.26	1.51
Monsoon	June	8	12	29	4.28	2.32
	July	8	12	29	3.96	2.98
	August	8.2	15	31	4.98	2.78
	September	8.1	18	30	2.56	3.01
Post-monsoon	October	8.1	18	30	2.56	3.01
	November	8	20	31	4.56	1.98
	December	7.8	20	30	3.56	0.2
	January	8.3	21	29	3.58	0.98
2015-16						
Pre- monsoon	February	8.3	29	29	3.16	0.76
	March	8.2	32	32.5	3.92	1.56
	April	8.5	37	32.5	4.26	2.98
	May	8.5	38	32.5	4.26	2.56
Monsoon	June	8.1	16	31	4.16	2.02
	July	8.1	16	29	3.13	2.56
	August	7.9	20	31	4.28	2.12
	September	8	22	31	4.16	3.02
Post-monsoon	October	8	22	29	3.98	2.98
	November	7.9	10	30	3.56	2.56
	December	8.2	22	29	3.46	1.02
	January	8.1	22	31	4.56	1.56

4.1.3 Seasonal variations of physico-chemical parameters in Chamravottom kadav

In the station Chamravottom kadav, pH ranged between 7.5-8.5 with an average of 8.1. Prominent variation in salinity was observed in Chamravottom kadav where salinity values were not detected during monsoon and post monsoon and the values ranged between 4-27ppt with an average of 7ppt. Temperature ranged between 28-32.5^{o}C with an average of 30^{o}C. Relatively low level of DO obtained at Chamravottom kadav and their values ranged between 2.13-5.69 mg/L with an average of 3.7mg/L. BOD values ranged between 0.56-2.85mg/L with an average of 1.31mg/L (Table 4.3).

4.1.4 Seasonal variations of physico-chemical parameters in Kalur

In Kalur, pH ranged between 7.4-8.5 with an average of 8.1. Salinity values were not detected during monsoon and post monsoon seasons and their values ranged between 2-30ppt with an average of 9 ppt. Temperature ranged between 28-32.5^{o}C with an average of 3^{o}C. Fairly low level of DO obtained at Kalur and their values ranged between 1.82-4.98 mg/L with an average of 3.21mg/L. BOD values ranged between 0.68-2.85mg/L with an average of 1.50 mg/L (Table 4.4).

4.1.5 Seasonal variations of physico-chemical parameters in Ishwaramangalam

In Ishwaramangalam, pH ranged between 7.3-8.5 with an average of 8.1. Salinity values were not detected during monsoon and their values ranged between 2-36ppt with an average of 10ppt. Temperature ranged between 28-32.5^{o}C with an average of 30^{o}C.DO values ranged between 2.85-5.69mg/L with an average of 4.00mg/L. BOD values ranged between 0.7-2.98mg/L with an average of 1.98mg/L (Table 4.5).

Table 4.3: Seasonal variations of physico-chemical parameters in Chamravottom kadav

Season	Month	pH	Salinity (ppt)	Temp (°C)	DO (mg/L)	BOD (mg/L)
2014-15						
Pre-monsoon	February	8.2	7	30	4.26	1.51
	March	8.3	14	32	5.69	2.85
	April	8.4	27	32.5	3.56	1.43
	May	8.2	20	32.5	2.13	1.42
Monsoon	June	7.6	nd	28	3.56	1.21
	July	7.5	nd	26	4.26	1.23
	August	8	nd	29	4.98	1.34
	September	7.8	nd	28	4.26	1.44
Post-monsoon	October	8.2	nd	28	3.56	1.56
	November	8.2	nd	29	4.26	0.98
	December	8.4	nd	28	4.26	0.72
	January	7.8	5	30	3.56	0.96
2015-16						
Pre-monsoon	February	8.2	5	29	4.26	0.86
	March	8.3	15	28	4.26	2.24
	April	8.5	27	32.5	3.56	2.66
	May	8.4	25	32.5	2.13	1.98
Monsoon	June	8.1	nd	29	2.82	1.02
	July	7.9	nd	27	3.12	0.98
	August	7.9	nd	30	3.26	0.62
	September	8.1	nd	29	4.12	0.81
Post-monsoon	October	8	nd	29	4.42	1.09
	November	8.2	nd	30	3.12	1.18
	December	8	4	29	2.26	0.56
	January	7.9	6	31	3.12	0.88

nd: not detected

Table 4.4: Seasonal variations of physico-chemical parameters in Kalur

Season	Month	pH	Salinity (ppt)	Temp (°C)	DO (mg/L)	BOD (mg/L)
2014-15						
Pre-monsoon	February	8.5	22	30	3.56	1.43
	March	8.3	25	30	4.98	2.13
	April	8.1	30	32	3.56	1.43
	May	8.3	15	32.5	2.85	1.43
Monsoon	June	7.7	nd	29	3.16	1.32
	July	7.4	nd	27	3.86	1.13
	August	7.9	nd	28	4.28	1.74
	September	7.9	nd	29	3.26	1.14
Post-monsoon	October	8.3	nd	29	2.56	1.76
	November	8.3	nd	30	3.26	1.88
	December	8.1	nd	29	3.98	2.85
	January	7.9	10	31	2.56	1.46
2015-16						
Pre-monsoon	February	8.3	5	30	3.26	1.06
	March	8.4	20	29	4.26	2.14
	April	8.5	30	32.5	2.56	2.16
	May	8.5	28	32.5	2.03	1.28
Monsoon	June	8.2	nd	30	1.82	1.78
	July	8	nd	28	2.92	0.68
	August	8	nd	31	2.86	0.92
	September	8.2	nd	30	3.92	1.21
Post-monsoon	October	8.1	nd	30	4.02	1.59
	November	8.3	2	31	2.82	1.08
	December	8.1	8	30	1.86	1.26
	January	8	10	32	2.92	0.98

Table 4.5: Seasonal variations of physico-chemical parameters in Ishwaramangalam

Season	Month	pH	Salinity (ppt)	Temp (°C)	DO (mg/L)	BOD (mg/L)
2014-15						
Pre- monsoon	February	8.3	26	30	4.98	2.84
	March	8.3	20	29	5.69	2.85
	April	8.4	29	32	2.85	1.43
	May	8.1	15	32.5	3.56	0.71
Monsoon	June	7.6	nd	28	3.98	2.98
	July	7.3	nd	27	3.26	2.76
	August	7.8	nd	28	3.02	2.04
	September	8	nd	28	2.98	2.52
Post- monsoon	October	8.2	nd	28	2.88	1.98
	November	8.5	2	29	3.86	1.52
	December	7.5	2	29	4.26	0.7
	January	8	12	30	4.58	1.98
2015-16						
Pre-monsoon	February	8.2	8	31	4.98	2.54
	March	8.3	22	29	5.96	2.98
	April	8.4	32	30	3.02	1.66
	May	8.5	36	32.5	3.86	1.04
Monsoon	June	8.1	nd	30	4.02	2.12
	July	7.9	nd	29	4.76	2.06
	August	8	nd	30	3.64	2.86
	September	8	2	29	3.02	2.12
Post- monsoon	October	8.1	2	30	3.26	1.81
	November	8.2	4	30	3.98	1.99
	December	8	10	29	4.86	1.02
	January	8.1	12	31	4.81	0.98

4.1.6 Seasonal variations of physico-chemical parameters in Purathoor

In Purathoor, pH ranged between 7.8-8.5 with an average of 8.3. Salinity values ranged between 2-35ppt with an average of 14 ppt. Temperature ranged between 25-32.5^{o}C with an average of 30^{o}C. DO values ranged between 2.13-5.98 mg/L with an average of 3.92mg/L. BOD values ranged between 0.2-2.56mg/L with an average of 1.18mg/L (Table 4.6).

4.1.7 Seasonal variations of physico-chemical parameters in Kootai

In the station Kootai, pH ranged between 6.7-8.5 with an average of 7.7. Salinity values ranged between 12-34ppt with an average of 21 ppt. Temperature ranged between 27-32.5^{o}C with an average of 30^{o}C. DO values ranged between 2.13-5.69 mg/L with an average of 4.07mg/L. BOD values ranged between 0.25-2.64mg/L with an average of 1.40mg/L (Table 4.7).

4.1.8 Seasonal variations of physico-chemical parameters in Harbour

In the station Harbour, pH ranged between 6.8-8.5 with an average of 7.7. Salinity values ranged between 14-36ppt with an average of 23ppt. Temperature ranged between 28-32.5^{o}C with an average of 30^{o}C. DO values ranged between 1.02-4.62 mg/L with an average of 2.66mg/L**.** BOD values ranged between 0.12-2.63mg/L with an average of 1.18mg/L (Table 4.8).

4.1.9 Seasonal variations of physico-chemical parameters in Chandappadi

In the station Chandappadi, pH ranged between 7.8-8.5 with an average of 8.2. Salinity values ranged between 2-36ppt with an average of 15ppt. Temperature ranged between 28-32.5^{o}C with an average of 31^{o}C. DO values ranged between 0.03-3.56 mg/L with an average of 3.43mg/L. BOD values ranged between 0.12-2.63mg/L with an average of 1.37mg/L (Table 4.9).

Table 4.6: Seasonal variations of physico-chemical parameters in Purathoor

Season	Month	pH	Salinity (ppt)	Temp (°C)	DO (mg/L)	BOD (mg/L)
2014-15						
Pre-monsoon	February	8.6	30	32	3.56	2.14
	March	8.4	26	32	3.56	0
	April	8.5	33	32.5	2.85	0.72
	May	8.3	20	32.5	2.85	0.71
Monsoon	June	7.9	8	25	4.26	2.12
	July	8	nd	28	5.69	1.98
	August	8.1	nd	28	4.98	1.82
	September	7.8	5	30	4.26	1.56
Post-monsoon	October	8.2	5	31	3.56	1.02
	November	8.4	6	29	2.85	0.76
	December	8.4	6	29	2.13	0.2
	January	8.5	25	30	4.26	1.76
2015-16						
Pre- monsoon	February	8.6	30	31	3.56	2.56
	March	8.2	26	31	2.85	0.02
	April	8.5	33	32.5	2.85	0.54
	May	8.4	35	32.5	3.12	0.89
Monsoon	June	8	7	26	4.86	1.94
	July	8.1	nd	26	5.98	1.86
	August	8.2	2	28	5.69	1.22
	September	7.9	6	29	4.98	1.07
Post-monsoon	October	8	4	32	4.26	1.15
	November	8.5	7	31	3.12	0.78
	December	8.3	5	30	2.98	0.51
	January	8.6	30	32	3.56	2.14

Table 4.7: Seasonal variations of physico-chemical parameters in Kootai

Season	Month	pH	Salinity (ppt)	Temp (°C)	DO (mg/L)	BOD (mg/L)
2014-15						
Pre-monsoon	February	8.5	32	32	5.69	2.13
	March	8.5	30	32.5	4.98	2.13
	April	8.2	34	32	4.26	1.43
	May	6.2	32	32	4.98	0.72
Monsoon	June	7	14	30	2.13	1.89
	July	6.8	14	29	2.13	1.89
	August	7.2	13	28	3.56	1.34
	September	7.6	12	27	3.56	1.12
Post-monsoon	October	7.8	14	29	4.26	0.65
	November	8	14	30	4.26	0.25
	December	7.3	16	28	4.98	0.7
	January	8	25	29	4.26	1.76
2015-16						
Pre-monsoon	February	8.6	26	32	4.98	2.35
	March	8.5	30	32	3.56	2.64
	April	8.4	32	32.5	3.56	1.98
	May	8.6	33	32.5	4.56	1.08
Monsoon	June	6.9	12	29	2.98	1.22
	July	6.7	14	28	2.98	1.64
	August	7	14	29	3.86	1.86
	September	7.8	15	26	3.12	1.22
Post-monsoon	October	7.9	16	30	4.56	0.98
	November	8.1	16	31	4.86	0.52
	December	7.5	18	29	5.02	0.82
	January	7.9	23	30	4.56	1.34

Table 4.8: Seasonal variations of physico-chemical parameters in Harbour

Season	Month	pH	Salinity (ppt)	Temp (ºC)	DO (mg/L)	BOD (mg/L)
2014-15						
Pre- monsoon	February	8.5	32	32	4.62	2.63
	March	8	33	32.5	4.12	2.48
	April	8.5	33	32.5	3.16	0.43
	May	7.8	33	32	3.98	1.72
Monsoon	June	7.2	18	29	2.42	1.39
	July	7	16	29	2.80	1.29
	August	6.8	14	29	2.98	1.40
	September	7.2	15	28	1.56	0.12
Post-monsoon	October	7.6	15	30	2.16	0.65
	November	7.8	15	31	4.26	0.25
	December	8	18	29	2.66	1.78
	January	7.3	28	30	3.12	1.06
2015-16						
Pre- monsoon	February	8	26	31	2.12	1.18
	March	8.6	32	32.5	3.64	1.78
	April	8.5	34	32.5	4.08	3.24
	May	8.4	36	32.5	2.12	0.62
Monsoon	June	8.6	14	30	2.64	0.75
	July	6.9	16	27	0.98	0.12
	August	6.7	16	29	1.88	0.65
	September	6.2	18	28	2.56	1.34
Post-monsoon	October	7.5	18	29	1.09	0.31
	November	7.2	20	30	1.02	0.58
	December	7.9	24	30	2.08	1.56
	January	8	20	31	1.72	0.92

Table 4.9: Seasonal variations of physico-chemical parameters in Chandappadi

Season	Month	pH	Salinity (ppt)	Temp (°C)	DO (mg/L)	BOD (mg/L)
2014-15						
Pre- monsoon	February	8.4	20	30	4.26	3.55
	March	8.5	31	32.5	3.56	0.71
	April	8.4	31	32.5	3.56	3.56
	May	8.2	24	32.5	3.56	1.43
Monsoon	June	7.8	2	29	4.12	3.09
	July	7.4	2	29	4.98	3.24
	August	7.8	4	30	3.08	0.74
	September	8	10	30	3.44	0.65
Post-monsoon	October	8.2	10	31	3.12	2.51
	November	8.5	12	30	4.36	2.46
	December	8.5	12	30	4.98	0.72
	January	8.1	12	31	4.31	0.88
2015-16						
Pre- monsoon	February	8.2	18	32	3.25	1.01
	March	8.3	22	32.5	3.44	1.93
	April	8.4	32	32.5	2.12	0.23
	May	8.5	36	32.5	2.86	0.46
Monsoon	June	8.1	4	29	3.94	0.62
	July	7.9	4	28	3.56	0.76
	August	8	6	29	4.36	2.78
	September	8	12	30	3.22	0.64
Post-monsoon	October	8.1	12	30	1.78	0.03
	November	8.2	14	31	1.12	0.08
	December	8	10	30	1.76	0.12
	January	8.1	14	29	3.56	0.76

For the ease of presentation, the two year data were pooled by taking its average and the results were recorded (Table 4.10).

Table 4.10: Seasonal variation of physico-chemical parameters in different stations of Ponnani estuary.

Season	pH	Salinity (ppt)	Temp (°C)	DO (mg/L)	BOD (mg/L)
Jankar					
Pre-Monsoon	8.3	31	32	3.78	1.6
Monsoon	7.8	15	30	2.89	1.01
Post-Monsoon	8.2	19	30	4.08	2.44
Pallikkadav					
Pre-Monsoon	8.4	31	32.5	4	2.06
Monsoon	8.1	16	30	3.94	2.6
Post-Monsoon	8.1	19	30	3.73	2.6
Chamravottom kadav					
Pre-Monsoon	8.3	18	32	3.73	1.86
Monsoon	7.9	nd	28	3.79	1.08
Post-Monsoon	8.1	2	29	3.57	0.99
Kalur					
Pre-Monsoon	8.4	22	32	3.38	1.63
Monsoon	8	nd	29	3.26	1.24
Post-Monsoon	8.1	4	30	3	1.61
Ishwaramangalam					
Pre-Monsoon	8.3	24	31	4.36	2.00
Monsoon	7.8	nd	29	3.58	2.43
Post- Monsoon	8	6	30	4.06	1.49
Purathoor					
Pre-Monsoon	8.4	29	32.5	3.15	0.94
Monsoon	8	4	28	5.08	1.69
Post-Monsoon	8.3	10	30	3.51	1.02
Kootai					
Pre-Monsoon	8.2	23	32.5	4.57	1.81
Monsoon	7.1	14	28	3.04	1.52
Post-Monsoon	6.8	18	30	4.6	0.88
Harbour					
Pre-Monsoon	8.3	32	32.5	3.48	1.76
Monsoon	7.1	16	29	2.28	0.88
Post-Monsoon	7.7	20	30	2.26	0.89
Chandappadi					
Pre-Monsoon	8.4	27	32.5	3.33	0.61
Monsoon	7.9	6	29	3.83	1.57
Post-Monsoon	8.2	12	30	3.12	0.95

The pH of the samples ranged between 6.8-8.4 indifferent stations of Ponnani estuary. In pre-monsoon, the highest pH recorded was (8.4) at four stations (Purathoor, Chandappadi, Kalur and Pallikkadav) and the lowest was (8.2) at Kootai. There was not much variation in pH between stations. During monsoon, the highest pH noticed was (8.1) at Pallikkadav and the lowest (7.1) in Kootai and Harbour. In post-monsoon the pH was maximum (8.3) at Purathoor and minimum (6.8) at Kootai. Seasonal changes in pH were recorded in the present study (Figure 4.1). Two-way ANOVA showed a significant difference between stations ($F=12.2$, $P<0.01$) and seasons ($F=77.6$, $P<0.01$) for pH(Table 4.11). Using Tukey test, within stations, Kootai and Harbour showed a variation with all the other stations (Table 4.12) and within season a significant variation was obtained between the three seasons (Table 4.17).

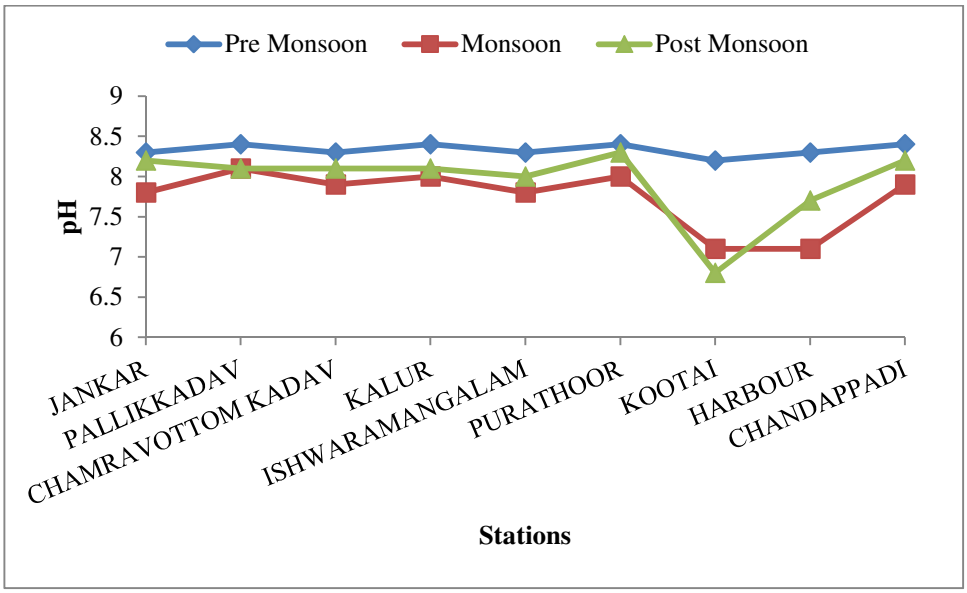


Figure 4.1: Seasonal variation of hydrogen ion concentration in different stations of Ponnani estuary

Salinity ranged between 4-32ppt in the study area. In pre-monsoon, the highest salinity of 32ppt was recorded at Harbour and lowest salinity of 18ppt was recorded at Chamravottom kadav. In monsoon, the highest salinity (16ppt) was recorded at Harbour and Pallikkadav, where as lowest salinity (4ppt) was obtained from Purathoor. In post-monsoon, the maximum salinity (20ppt) was recorded from Harbour and minimum salinity level (2ppt) was obtained from Chamravottom kadav. Two-way ANOVA showed a significant difference between stations ($F=45.2$, $P<0.01$) and seasons ($F=339.9$, $P<0.01$) for saliniy(Table 4.11). Significant difference between various stations (Table 4.13) and seasons (Table 4.17) were obtained in the present study for salinity. The salinity variation in all the stations in different seasons were depicted in the Figure 4.2

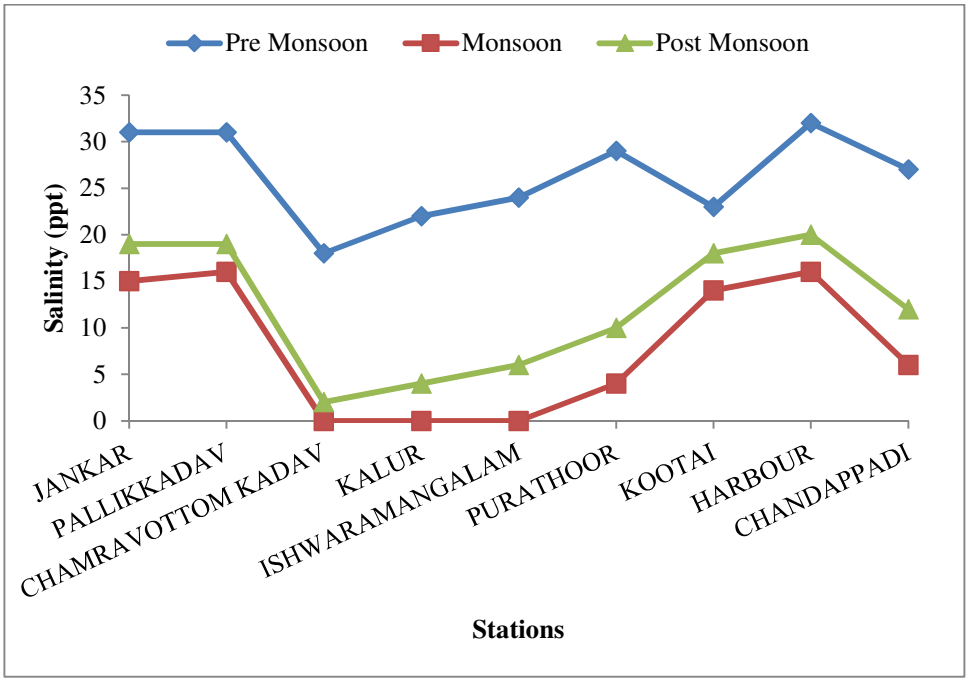


Figure 4.2: Seasonal variation of salinity in different stations of Ponnani estuary

Temperature influences the growth of bioluminescent bacteria. Temperature in different stations of Ponnani estuary ranged between 28^{o}C -

32.5°C. In pre-monsoon, highest temperature (35°C) was recorded at Pallikkadav, Purathoor, Kootai, Harbour and Chandappadi and lowest temperature (31°C) was recorded at Ishwaramangalam. In monsoon, highest temperature (30°C) was recorded at Kootai and Chamravottom kadav while lowest temperature (28°C) was recorded at Kootai. In post-monsoon, all the stations showed a temperature of 30°C except Chamravottom kadav that recorded a temperature of 29°C. Highest temperature ranges were showed during pre-monsoon season followed by post-monsoon and monsoon seasons.Two-way ANOVA showed a significant difference between stations ($F=3.6$, $P<0.01$) and seasons ($F=123$, $P<0.01$) for temperature (Table 4.11). Significant difference between various stations (Table 4.14) and seasons (Table 4.17) were obtained in the present study for temperature. Seasonal variation in temperature recorded in all stations of Ponnani estuary were given in the Figure 4.3

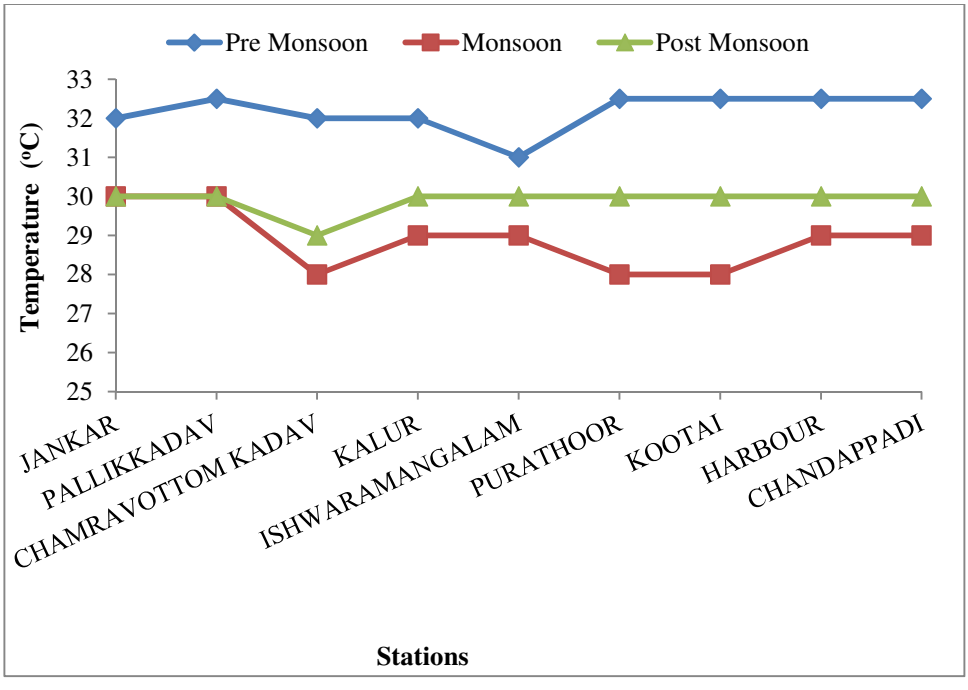


Figure 4.3: Seasonal variation of temperature in different stations of Ponnani estuary

Dissolved oxygen values obtained were comparatively low and ranged between 2.26.-5.08mg/L. In pre-monsoon, highest range of DO (4.57mg/L) was noticed at Kootai and lowest range of DO (3.15mg/L) noticed at Purathoor. In monsoon, highest value of DO (5.08mg/L) was recorded at Purathoor while lowest value (2.28 mg/L) was noticed at Harbour. In post-monsoon, highest value of DO (4.6 mg/L) was recorded at Kootai whereas lowest (2.26mg/L) was observed at Harbour.Even though highest values for DO noticed during monsoon season, pre-monsoon and post-monsoon were almost similar in DO. Two-way ANOVA showed a significant difference between stations only ($F=7.4$, $P<0.01$) and not for seasons in the case of DO(Table 4.11). Significant differences between various stations (Table 4.15) were obtained in the present study for DO using Tukey test. The seasonal changes in DO of Ponnani estuary were given in the Figure 4.4.

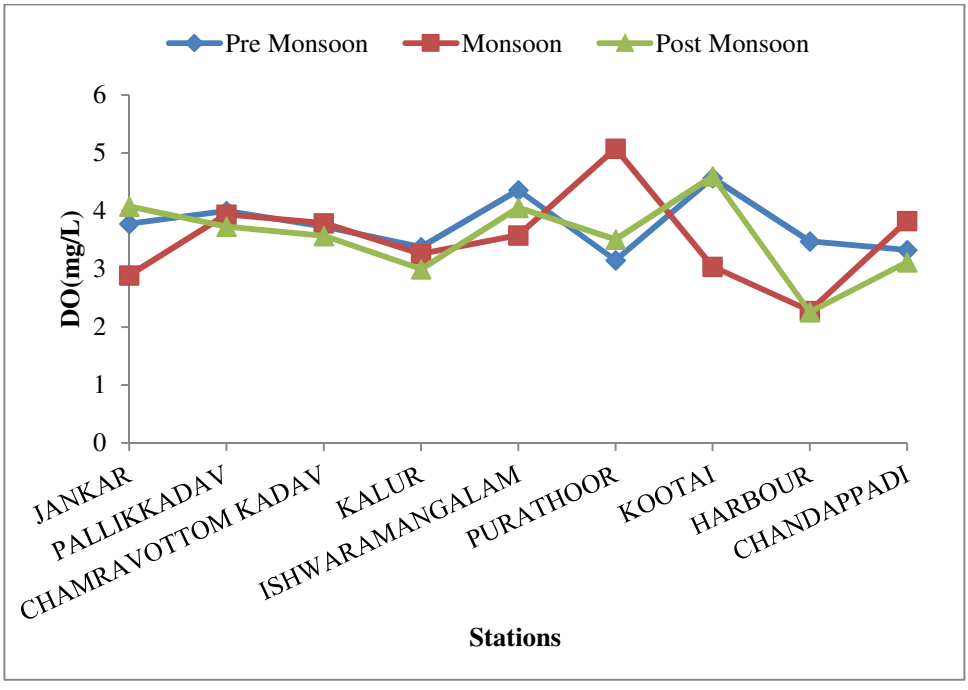


Figure 4.4: Seasonal variation of DO in different stations of Ponnani estuary

Biological oxygen demand (BOD) varied between 0.61-2.6mg/L in nine stations. In pre-monsoon, Highest BOD value (2.06mg/L) was noticed at Pallikkadav where as lowest value (0.61 mg/L) was observed at Chandappadi. In monsoon, maximum level of BOD (2.6 mg/L) was observed at Pallikkadav and minimum level (1.01 mg/L) was observed at Jangar. In post-monsoon, highest value of BOD (2.6mg/L) was noticed at Pallikkadav and lowest value (0.88mg/L) was observed at Kootai. No remarkable variation recorded seasonally in stations. Two-way ANOVA showed a significant difference between stations ($F=5.6$, $P<0.01$) and seasons ($F=7.3$, $P<0.01$) for BOD(Table 4.11). Significant difference between various stations (Table 4.16) and seasons (Table 4.17) were obtained in the present study for BOD. Seasonal BOD variation Ponnani estuary was recorded in the Figure 4.5.

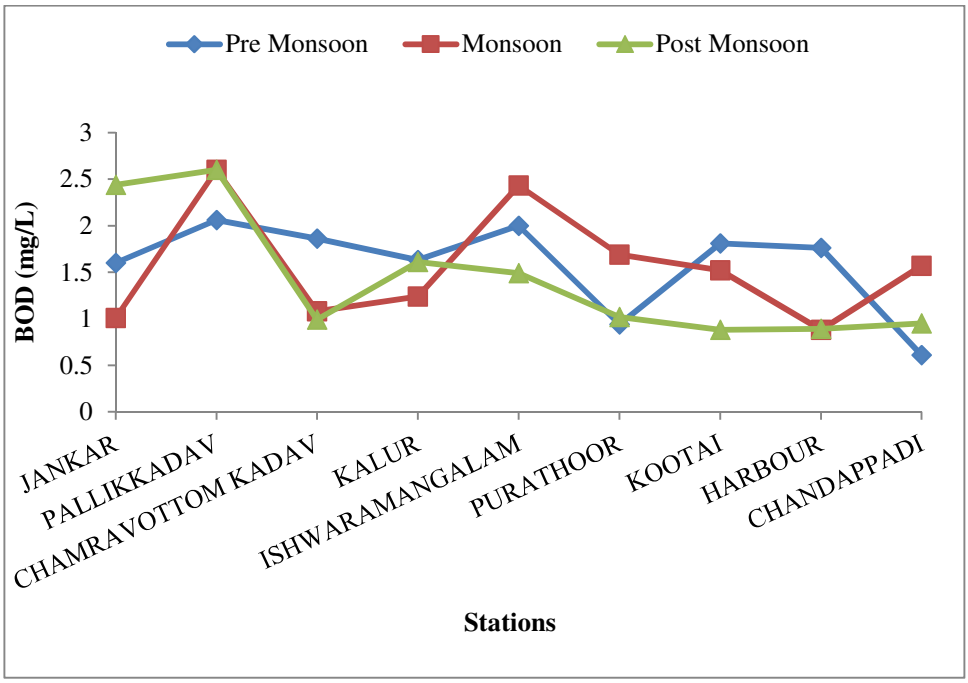


Figure 4.5: Seasonal variation of biological oxygen demand in different stations of Ponnani estuary

Table 4.11: Results of two-way ANOVA for major physico-chemical parameters at the study stations in the Ponnani estuary tests

Parameters	Source	Type III SS	Df	Mean Sq.	F	Prob.
PH	Stations	8.18	8	1.0225	12.19451	5.22E-14 p<0.01
	Seasons	13.01	2	6.506667	77.59962	2.5E-25 p<0.01
Salinity	Stations	7916.83	8	989.6042	45.20898	5.57E-40 p<0.01
	Seasons	14881.44	2	7440.722	339.9212	2.49E-63 p<0.01
Temp	Stations	33.50	8	4.188657	3.58013	0.000684 p<0.01
	Seasons	288.62	2	144.3102	123.3448	5.29E-35 p<0.01
DO	Stations	39.42	8	4.928291	7.364823	1.63E-08 p<0.01
	Seasons	2.36	2	1.182848	1.767644	0.173546 p<0.01
BOD	Stations	21.99	8	2.748829	5.610233	2.18E-06 p<0.01
	Seasons	7.10	2	3.553006	7.251519	0.000924 p<0.01

Table 4.12:Results of tukeys post hoc test for pH at the study stations in the Ponnani estuary

Group 1	Group 2	Mean Diff.	SE	q	Prob.
Jankar	Pallikkadav	-0.046	0.059	0.775	1.000
Jankar	Chamravottom Kadav	0.025	0.059	0.423	1.000
Jankar	Kalur	-0.025	0.059	0.423	1.000
Jankar	Ishwaramangalam	0.038	0.059	0.634	1.000
Jankar	Purthoor	-0.158	0.059	2.679	0.618
Jankar	Kootai	0.404	0.059	6.838	p<0.01
Jankar	Harbour	0.437	0.059	7.402	p<0.01
Jankar	Chandappadi	-0.037	0.059	0.634	1.000
Pallikkadav	Chamravottom Kadav	0.071	0.059	1.198	0.995
Pallikkadav	Kalur	0.021	0.059	0.352	1.000
Pallikkadav	Ishwaramangalam	0.083	0.059	1.410	0.986
Pallikkadav	Purthoor	-0.113	0.059	1.903	0.917
Pallikkadav	Kootai	0.450	0.059	7.613	p<0.01
Pallikkadav	Harbour	0.483	0.059	8.177	p<0.01
Pallikkadav	Chandappadi	0.008	0.059	0.141	1.000
Chamravottom Kadav	Kalur	-0.050	0.059	0.846	1.000
Chamravottom Kadav	Ishwaramangalam	0.013	0.059	0.211	1.000
Chamravottom Kadav	Purthoor	-0.183	0.059	3.102	0.410
Chamravottom Kadav	Kootai	0.379	0.059	6.415	p<0.01
Chamravottom Kadav	Harbour	0.413	0.059	6.979	p<0.01
Chamravottom Kadav	Chandappadi	-0.062	0.059	1.057	0.998
Kalur	Ishwaramangalam	0.063	0.059	1.057	0.998
Kalur	Purthoor	-0.133	0.059	2.256	0.808
Kalur	Kootai	0.429	0.059	7.261	p<0.01
Kalur	Harbour	0.463	0.059	7.825	p<0.01
Kalur	Chandappadi	-0.012	0.059	0.211	1.000
Ishwaramangalam	Purthoor	-0.196	0.059	3.313	0.316
Ishwaramangalam	Kootai	0.367	0.059	6.203	p<0.01
Ishwaramangalam	Harbour	0.400	0.059	6.767	p<0.01
Ishwaramangalam	Chandappadi	-0.075	0.059	1.269	0.993
Purthoor	Kootai	0.562	0.059	9.517	p<0.01
Purthoor	Harbour	0.596	0.059	10.080	p<0.01
Purthoor	Chandappadi	0.121	0.059	2.044	0.880
Kootai	Harbour	0.033	0.059	0.564	1.000
Kootai	Chandappadi	-0.442	0.059	7.472	p<0.01
Harbour	Chandappadi	-0.475	0.059	8.036	p<0.01

Table 4.13:Results of tukeys post hoc test for salinity at the study stations in the Ponnani estuary

Group 1	Group 2	Mean Diff.	SE	q	Prob.
Jankar	Pallikkadav	-0.375	0.955	0.39	1.000
Jankar	Chamravottom Kadav	15.375	0.955	16.0	p<0.01
Jankar	Kalur	13.292	0.955	13.9	p<0.01
Jankar	Ishwaramangalam	12.083	0.955	12.6	p<0.01
Jankar	Purthoor	7.708	0.955	8.07	p<0.01
Jankar	Kootai	1.042	0.955	1.09	0.998
Jankar	Harbour	-0.833	0.955	0.87	1.000
Jankar	Chandappadi	7.083	0.955	7.41	p<0.01
Pallikkadav	Chamravottom Kadav	15.750	0.955	16.4	p<0.01
Pallikkadav	Kalur	13.667	0.955	14.3	p<0.01
Pallikkadav	Ishwaramangalam	12.458	0.955	13.0	p<0.01
Pallikkadav	Purthoor	8.083	0.955	8.46	p<0.01
Pallikkadav	Kootai	1.417	0.955	1.48	0.981
Pallikkadav	Harbour	-0.458	0.955	0.48	1.000
Pallikkadav	Chandappadi	7.458	0.955	7.81	p<0.01
Chamravottom	Kalur	-2.083	0.955	2.18	0.835
Chamravottom	Ishwaramangalam	-3.292	0.955	3.44	0.264
Chamravottom	Purthoor	-7.667	0.955	8.02	p<0.01
Chamravottom	Kootai	-14.33	0.955	15.0	p<0.01
Chamravottom	Harbour	-16.20	0.955	16.9	p<0.01
Chamravottom	Chandappadi	-8.292	0.955	8.68	p<0.01
Kalur	Ishwaramangalam	-1.208	0.955	1.26	0.993
Kalur	Purthoor	-5.583	0.955	5.84	0.001
Kalur	Kootai	-12.25	0.955	12.8	p<0.01
Kalur	Harbour	-14.12	0.955	14.7	p<0.01
Kalur	Chandappadi	-6.208	0.955	6.50	p<0.01
Ishwaramangalam	Purthoor	-4.375	0.955	4.58	0.033
Ishwaramangalam	Kootai	-11.04	0.955	11.5	p<0.01
Ishwaramangalam	Harbour	-12.91	0.955	13.5	p<0.01
Ishwaramangalam	Chandappadi	-5.000	0.955	5.23	p<0.01
Purthoor	Kootai	-6.667	0.955	6.98	p<0.01
Purthoor	Harbour	-8.542	0.955	8.94	p<0.01
Purthoor	Chandappadi	-0.625	0.955	0.65	1.000
Kootai	Harbour	-1.875	0.955	1.96	0.903
Kootai	Chandappadi	6.042	0.955	6.32	p<0.01
Harbour	Chandappadi	7.917	0.955	8.29	p<0.01

Table 4.14: Results of tukeys post hoc test for temperature at the study stations in the Ponnani estuary

Group 1	Group 2	Mean Diff.	SE	q	Prob.
Jankar	Pallikkadav	-0.063	0.221	0.283	1.000
Jankar	Chamravottom	1.083	0.221	4.907	p<0.05
Jankar	Kalur	0.521	0.221	2.359	0.766
Jankar	Ishwaramangalam	1.000	0.221	4.529	p<0.05
Jankar	Purthoor	0.667	0.221	3.019	0.449
Jankar	Kootai	0.646	0.221	2.925	0.495
Jankar	Harbour	0.354	0.221	1.604	0.969
Jankar	Chandappadi	0.083	0.221	0.377	1.000
Pallikkadav	Chamravottom	1.146	0.221	5.190	p<0.01
Pallikkadav	Kalur	0.583	0.221	2.642	0.636
Pallikkadav	Ishwaramangalam	1.063	0.221	4.812	p<0.05
Pallikkadav	Purthoor	0.729	0.221	3.303	0.321
Pallikkadav	Kootai	0.708	0.221	3.208	0.361
Pallikkadav	Harbour	0.417	0.221	1.887	0.921
Pallikkadav	Chandappadi	0.146	0.221	0.661	1.000
Chamravottom	Kalur	-0.563	0.221	2.548	0.681
Chamravottom	Ishwaramangalam	-0.083	0.221	0.377	1.000
Chamravottom	Purthoor	-0.417	0.221	1.887	0.921
Chamravottom	Kootai	-0.438	0.221	1.982	0.898
Chamravottom	Harbour	-0.729	0.221	3.303	0.321
Chamravottom	Chandappadi	-1.000	0.221	4.529	p<0.05
Kalur	Ishwaramangalam	0.479	0.221	2.170	0.839
Kalur	Purthoor	0.146	0.221	0.661	1.000
Kalur	Kootai	0.125	0.221	0.566	1.000
Kalur	Harbour	-0.167	0.221	0.755	1.000
Kalur	Chandappadi	-0.438	0.221	1.982	0.898
Ishwaramangalam	Purthoor	-0.333	0.221	1.510	0.979
Ishwaramangalam	Kootai	-0.354	0.221	1.604	0.969
Ishwaramangalam	Harbour	-0.646	0.221	2.925	0.495
Ishwaramangalam	Chandappadi	-0.917	0.221	4.152	0.080
Purthoor	Kootai	-0.021	0.221	0.094	1.000
Purthoor	Harbour	-0.313	0.221	1.415	0.986
Purthoor	Chandappadi	-0.583	0.221	2.642	0.636
Kootai	Harbour	-0.292	0.221	1.321	0.991
Kootai	Chandappadi	-0.563	0.221	2.548	0.681
Harbour	Chandappadi	-0.271	0.221	1.227	0.995

Table 4.15: Results of tukeys post hoc test for DO at the study stations in the Ponnani estuary

Group 1	Group 2	Mean Diff.	SE	q	Prob.
Jankar	Pallikkadav	-0.299	0.167	1.78	0.941
Jankar	Chamravottom Kadav	-0.112	0.167	0.67	1.000
Jankar	Kalur	0.374	0.167	2.24	0.814
Jankar	Ishwaramangalam	-0.415	0.167	2.48	0.709
Jankar	Purthoor	-0.331	0.167	1.98	0.898
Jankar	Kootai	-0.481	0.167	2.88	0.517
Jankar	Harbour	0.930	0.167	5.57	p<0.01
Jankar	Chandappadi	0.158	0.167	0.94	0.999
Pallikkadav	Chamravottom Kadav	0.187	0.167	1.11	0.997
Pallikkadav	Kalur	0.673	0.167	4.03	0.101
Pallikkadav	Ishwaramangalam	-0.117	0.167	0.69	1.000
Pallikkadav	Purthoor	-0.032	0.167	0.19	1.000
Pallikkadav	Kootai	-0.183	0.167	1.09	0.998
Pallikkadav	Harbour	1.229	0.167	7.36	p<0.01
Pallikkadav	Chandappadi	0.457	0.167	2.73	0.589
Chamravottom	Kalur	0.486	0.167	2.91	0.502
Chamravottom	Ishwaramangalam	-0.303	0.167	1.81	0.936
Chamravottom	Purthoor	-0.219	0.167	1.31	0.992
Chamravottom	Kootai	-0.369	0.167	2.21	0.825
Chamravottom	Harbour	1.043	0.167	6.24	p<0.01
Chamravottom	Chandappadi	0.270	0.167	1.61	0.967
Kalur	Ishwaramangalam	-0.790	0.167	4.72	0.023
Kalur	Purthoor	-0.705	0.167	4.22	0.070
Kalur	Kootai	-0.855	0.167	5.12	p<0.01
Kalur	Harbour	0.556	0.167	3.33	0.309
Kalur	Chandappadi	-0.216	0.167	1.29	0.992
Ishwaramangalam	Purthoor	0.085	0.167	0.50	1.000
Ishwaramangalam	Kootai	-0.066	0.167	0.39	1.000
Ishwaramangalam	Harbour	1.346	0.167	8.06	p<0.01
Ishwaramangalam	Chandappadi	0.574	0.167	3.43	0.268
Purthoor	Kootai	-0.150	0.167	0.90	0.999
Purthoor	Harbour	1.261	0.167	7.55	p<0.01
Purthoor	Chandappadi	0.489	0.167	2.93	0.493
Kootai	Harbour	1.412	0.167	8.45	p<0.01
Kootai	Chandappadi	0.640	0.167	3.83	0.145
Harbour	Chandappadi	-0.772	0.167	4.62	p<0.05

Table 4.16: Results of tukeys post hoc test for BOD at the study stations in the Ponnani estuary

Group 1	Group 2	Mean Diff.	SE	q	Prob.
Jankar	Pallikkadav	-0.651	0.143	4.555	p<0.0
Jankar	Chamravottom	0.185	0.143	1.292	0.992
Jankar	Kalur	0.005	0.143	0.035	1.000
Jankar	Ishwaramangalam	-0.480	0.143	3.362	0.296
Jankar	Purthoor	0.317	0.143	2.219	0.822
Jankar	Kootai	0.096	0.143	0.671	1.000
Jankar	Harbour	0.321	0.143	2.248	0.811
Jankar	Chandappadi	0.125	0.143	0.875	1.000
Pallikkadav	Chamravottom	0.835	0.143	5.847	p<0.0
Pallikkadav	Kalur	0.656	0.143	4.590	p<0.0
Pallikkadav	Ishwaramangalam	0.170	0.143	1.193	0.996
Pallikkadav	Purthoor	0.968	0.143	6.774	p<0.0
Pallikkadav	Kootai	0.747	0.143	5.226	p<0.0
Pallikkadav	Harbour	0.972	0.143	6.803	p<0.0
Pallikkadav	Chandappadi	0.776	0.143	5.430	p<0.0
Chamravottom	Kalur	-0.180	0.143	1.257	0.994
Chamravottom	Ishwaramangalam	-0.665	0.143	4.654	p<0.0
Chamravottom	Purthoor	0.133	0.143	0.927	0.999
Chamravottom	Kootai	-0.089	0.143	0.621	1.000
Chamravottom	Harbour	0.137	0.143	0.956	0.999
Chamravottom	Chandappadi	-0.060	0.143	0.417	1.000
Kalur	Ishwaramangalam	-0.485	0.143	3.397	0.282
Kalur	Purthoor	0.312	0.143	2.184	0.834
Kalur	Kootai	0.091	0.143	0.636	1.000
Kalur	Harbour	0.316	0.143	2.213	0.824
Kalur	Chandappadi	0.120	0.143	0.840	1.000
Ishwaramangalam	Purthoor	0.798	0.143	5.582	p<0.0
Ishwaramangalam	Kootai	0.576	0.143	4.033	0.101
Ishwaramangalam	Harbour	0.802	0.143	5.611	p<0.0
Ishwaramangalam	Chandappadi	0.605	0.143	4.237	0.068
Purthoor	Kootai	-0.221	0.143	1.548	0.975
Purthoor	Harbour	0.004	0.143	0.029	1.000
Purthoor	Chandappadi	-0.192	0.143	1.344	0.990
Kootai	Harbour	0.225	0.143	1.578	0.972
Kootai	Chandappadi	0.029	0.143	0.204	1.000
Harbour	Chandappadi	-0.196	0.143	1.374	0.988

Table 4.17: Results of tukeys post hoc test for physico-chemical parameters at the study stations in the Ponnani estuary

Parameters	Group 1	Group 2	Mean Diff.	SE	q	Prob.
PH	Pre-monsoon	Monsoon	0.600	0.034	17.582	p<0.01
	Pre-monsoon	Post-monsoon	0.267	0.034	7.814	p<0.01
	Monsoon	Post-monsoon	-0.333	0.034	9.768	p<0.01
Salinity	Pre-monsoon	Monsoon	19.361	0.551	35.114	p<0.01
	Pre-monsoon	Post-monsoon	15.056	0.551	27.305	p<0.01
	Monsoon	Post-monsoon	-4.306	0.551	7.809	p<0.01
Temp	Pre-monsoon	Monsoon	2.792	0.127	21.900	p<0.01
	Pre-monsoon	Post-monsoon	1.806	0.127	14.164	p<0.01
	Monsoon	Post-monsoon	-0.986	0.127	7.736	p<0.01
DO	Pre-monsoon	Monsoon	0.236	0.096	2.448	0.194
	Pre-monsoon	Post-monsoon	0.205	0.096	2.124	0.290
	Monsoon	Post-monsoon	-0.031	0.096	0.324	0.971
BOD	Pre-monsoon	Monsoon	0.141	0.082	1.704	0.450
	Pre-monsoon	Post-monsoon	0.435	0.082	5.277	p<0.01
	Monsoon	Post-monsoon	0.295	0.082	3.573	p<0.05

4.2 Distribution of THB, BLB and TVLO in different samples of Ponnani estuary

The total heterotrophic bacterial count (THB) was recorded by observing the number of colonies in nutrient agar. Colonies appeared in TCBS Agar was taken as total number of*Vibrio* like organisms (TVLO). Bioluminescent bacterial load (BLB) were obtained from Sea Water Complex agar plates in dark.

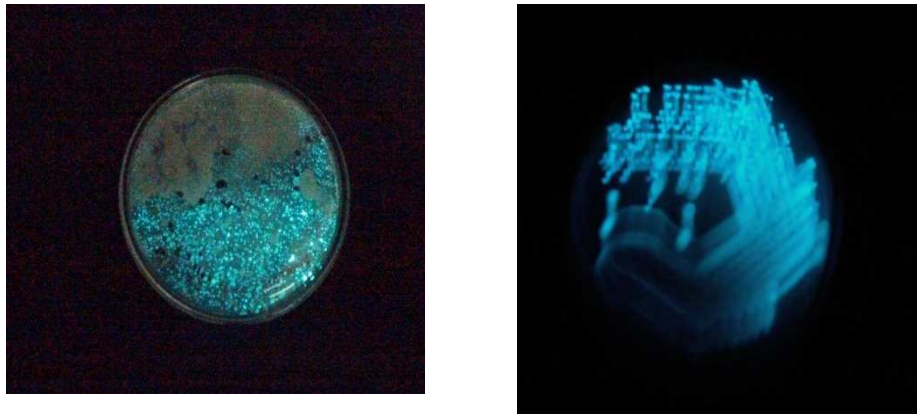


Figure 4.6: Growth of bioluminescent bacteria in sea water complex agar plates

Figure 4.7: Growth of bioluminescent bacteria in sea water complex broth

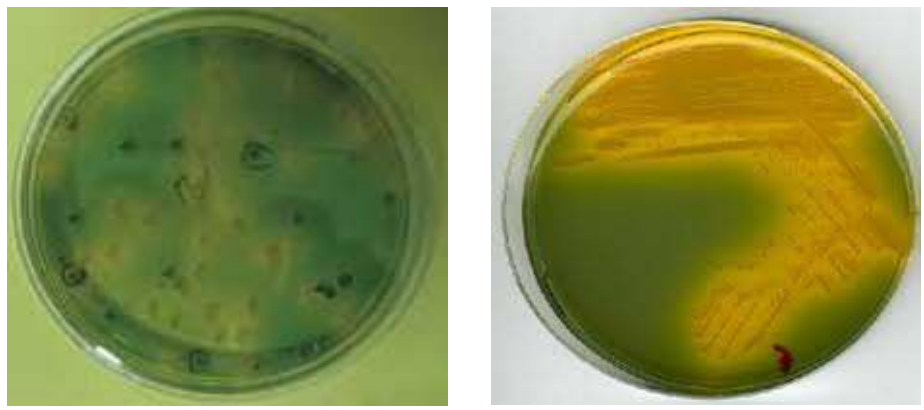


Figure 4.8: Growth of TVLO in TCBS Agar plates.

Due to limited data, further statistical analysis couldn't be conducted and only the mean values were given in the present study.

4.2.1 Distribution of THB, BLB and TVLO in water and sediment samples of Ponnani estuary

In Jangar, mean THB load was 158cfu/ml during pre-monsoon followed by 133cfu/ml (monsoon) and 191cfu/ml (post-monsoon) for water samples. For sediment samples, mean THB load was 238cfu/gm (pre-monsoon) followed by 170cfu/gm (monsoon) and 217 cfu/gm (post monsoon).Mean BLB load was 5cfu/ml (pre-monsoon) followed by 1 cfu/ml for water (monsoon) and 3cfu/ml (post monsoon) for water samples. In the case of sediment samples, mean BLB load was 6cfu/gm (pre-monsoon) followed by 1cfu/gm (monsoon) and 3cfu/gm (post monsoon).Mean TVLO load was 47cfu/ml(pre-monsoon) followed by 26cfu/ml(monsoon) and37cfu/ml(post monsoon) for water samples and 74cfu/gm(pre-monsoon), 37cfu/gm(monsoon)and 70cfu/gm(post-monsoon)

In Pallikkadav, mean THB load was 200cfu/ml during pre-monsoon followed by 104cfu/ml (monsoon) and 191cfu/ml (post-monsoon) for water samples. For sediment samples, mean THB load was 230cfu/gm (pre-monsoon) followed by 182cfu/gm (monsoon) and 244cfu/gm (post monsoon).Mean BLB load was 6cfu/ml (pre-monsoon) and 1cfu/ml (post monsoon) for water samples. During monsoon, BLB was absent for water and sediment samples. In the case of sediment samples, mean BLB load was 3cfu/gm (pre-monsoon) and 1cfu/gm (post monsoon). Mean TVLO load was 108cfu/ml (pre-monsoon) followed by 54cfu/ml (monsoon) and59cfu/ml (post monsoon) for water samples and 123cfu/gm (pre-monsoon), 70cfu/gm (monsoon) and 67 cfu/gm (post-monsoon) for sediments.

In Chamravottom kadav, mean THB load was 247 cfu/ml during pre-monsoon followed by 152 cfu/ml (monsoon) and 142 cfu/ml (post-monsoon) for water samples. For sediment samples, mean THB load was 289 cfu/gm

(pre-monsoon) followed by 216 cfu/gm (monsoon) and 206 cfu/gm (post monsoon). For all the seasons, bioluminescent bacteria were lacking for water and sediment samples. Mean TVLO load was 151 cfu/ml(pre-monsoon) followed by 77 cfu/ml(monsoon) and80 cfu/ml(post monsoon) for water samples and 178 cfu/gm(pre-monsoon), 121 cfu/gm(monsoon) and 103 cfu/gm(post-monsoon) in sediments.

In Kalur, mean THB load was 232 cfu/ml during pre-monsoon followed by 150 cfu/ml (monsoon) and 175 cfu/ml (post-monsoon) for water samples. For sediment samples, mean THB load was 232 cfu/gm (pre-monsoon) followed by 208 cfu/gm (monsoon) and 247 cfu/gm (post monsoon). For all the seasons, bioluminescent bacteria were lacking for water and sediment samples. Mean TVLO load was 100 cfu/ml (pre-monsoon) followed by 68 cfu/ml(monsoon) and 110 cfu/ml(post monsoon) for water samples and in sediment samples, 138 cfu/gmpre-monsoon), 127 cfu/gm(monsoon) and 150 cfu/gm(post-monsoon).

In Ishwaramangalam, mean THB load in water samples were 258 cfu/ml during pre-monsoon followed by 203 cfu/ml (monsoon) and 187 cfu/ml (post-monsoon). For sediment samples, mean THB load were 280 cfu/gm (pre-monsoon) followed by 239 cfu/gm (monsoon) and 236 cfu/gm (post monsoon). For all the seasons, bioluminescent bacteria were lacking for water and sediment samples. Mean TVLO load were 176 cfu/ml (pre-monsoon) followed by 123 cfu/ml (monsoon) and 126 cfu/ml (post monsoon) for water samples and 182 cfu/gm (pre-monsoon), 149 cfu/gm(monsoon) and 158 cfu/gm (post-monsoon) for sediment samples.

In Purathoor, mean THB load was 190 cfu/ml during pre-monsoon followed by 165 cfu/ml (monsoon) and 146 cfu/ml (post-monsoon) for water samples. For sediment samples, mean THB load was 215 cfu/gm (pre-monsoon) followed by 200 cfu/gm (monsoon) and 193 cfu/gm (post monsoon).

Mean BLB during pre-monsoon was 3cfu/ml for water and 5 cfu/gm for sediment. For the other seasons, bioluminescent bacteria were lacking for water and sediment samples. Mean TVLO load was 117 cfu/ml(pre-monsoon) followed by 84 cfu/ml(monsoon) and 80 cfu/ml(post monsoon) for water samples and 128 cfu/gm (pre-monsoon), 109 cfu/gm(monsoon) and 171 cfu/gm(post-monsoon) for sediment samples.

In Kootai, mean THB load was 110 cfu/ml during pre-monsoon followed by 83 cfu/ml (monsoon) and 94 cfu/ml (post-monsoon) for water samples. For sediment samples, mean THB load was 157 cfu/gm (pre-monsoon) followed by 143 cfu/gm (monsoon) and 127 cfu/gm (post monsoon).Mean BLB during pre-monsoon was 6cfu/ml for water and 7cfu/gm for sediment. Monsoon season lack bioluminescent load. Post-monsoon season bears average BLB (1 cfu/ml) for water and the sediment samples do not harbour any bioluminescent load. Mean TVLO load was 48 cfu/ml(pre-monsoon) followed by 37 cfu/ml(monsoon) and 43 cfu/ml(post monsoon) for water samples and 157 cfu/gm(pre-monsoon), 143 cfu/gm(monsoon) and 127 cfu/gm(post-monsoon) for sediments.

In Harbour, mean THB load was 200 cfu/ml during pre-monsoon followed by 136 cfu/ml (monsoon) and 201cfu/ml (post-monsoon) for water samples. For sediment samples, mean THB load was 247 cfu/gm (pre-monsoon) followed by 201 cfu/gm (monsoon) and 252 cfu/gm (post monsoon).Mean BLB during pre-monsoon was 4cfu/ml for water and 5cfu/gm for sediment. Monsoon season lack bioluminescent load. Post-monsoon season bears mean BLB (2 cfu/ml) for water and (1cfu/gm) for sediment sample. Mean TVLO load was 66 cfu/ml(pre-monsoon) followed by 45 cfu/ml(monsoon) and 75 cfu/ml(post monsoon) for water samples and 141 cfu/gm(pre-monsoon), 115 cfu/gm(monsoon) and 160 cfu/gm(post-monsoon) in sediments.

In Chandappadi, mean THB load was 111 cfu/ml during pre-monsoon followed by 77 cfu/ml (monsoon) and 135 cfu/ml (post-monsoon) for water samples. For sediment samples, mean THB load was 172 cfu/gm (pre-monsoon) followed by 127 cfu/gm(monsoon) and 175 cfu/gm(post monsoon).Mean BLB during pre-monsoon was 3 cfu/ml for water and 4 cfu/gm for sediment. Monsoon and Post-monsoon seasons lack bioluminescent load. Mean TVLO load was 54 cfu/ml(pre-monsoon) followed by 34 cfu/ml(monsoon) and 61 cfu/ml(post monsoon) for water samples and 84 cfu/gm(pre-monsoon), 55 cfu/gm(monsoon) and 75 cfu/gm(post-monsoon) for sediment samples. Mean distribution of THB, BLB and TVLO in water and sediment samples of different stations were showed in the table 4.18 and that of *S. ruconius, U. duvaucelii* and *M. dobsoni* were showed in the Table 4.19

Table 4.18: Seasonal distribution of THB, BLB and TVLO of water and sediment samples of Ponnani estuary

Season	Water (cfu/ml)			Sediment (cfu/gm)		
	THB	BLB	TVLO	THB	BLB	TVLO
Jankar						
Pre Monsoon	158	5	47	238	6	74
Monsoon	133	1	26	170	1	37
Post Monsoon	191	3	37	217	3	70
Pallikkadav						
Pre Monsoon	200	6	108	230	3	123
Monsoon	104	0	54	182	0	70
Post Monsoon	191	1	59	244	1	67
Chamravottom kadav						
Pre Monsoon	247	0	151	289	0	178
Monsoon	152	0	77	216	0	121
Post Monsoon	142	0	80	206	0	103
Kalur						
Pre Monsoon	174	0	100	232	0	138
Monsoon	150	0	68	208	0	127
Post Monsoon	175	0	110	247	0	150

Ishwaramangalam						
Pre Monsoon	258	0	176	280	0	182
Monsoon	203	0	123	239	0	149
Post Monsoon	187	0	126	236	0	158
Purathoor						
Pre Monsoon	190	3	117	215	5	128
Monsoon	165	0	84	200	0	109
Post Monsoon	146	0	80	193	0	171
Kootai						
Pre Monsoon	110	6	48	157	7	88
Monsoon	83	0	37	143	0	79
Post Monsoon	94	1	43	127	0	52
Harbour						
Pre Monsoon	200	4	66	247	5	141
Monsoon	136	0	45	201	0	115
Post Monsoon	201	2	75	252	1	160
Chandappadi						
Pre Monsoon	111	3	54	172	4	84
Monsoon	77	0	34	127	0	55
Post Monsoon	135	0	61	175	0	75

4.2.2 Seasonal distribution of THB, BLB and TVLO in *S. ruconius, U. duvaucelii* and *M. dobsoni* samples from Ponnani estuary

From *S. ruconius, U. duvaucelii* and *M. dobsoni*samples of Ponnani estuary, total heterotrophic bacterial count (THB), Total Vibrio like organism (TVLO) and bioluminescent load (BLB) were recorded. THB varied between 122-287cfu/gm for *S. ruconius* with mean value of 237cfu/gm followed by *U. duvaucelii*(100-289 cfu/gm) with a mean value of 243cfu/gm and *M. dobsoni* (211-288cfu/gm) with a mean of 270cfu/gm. Bioluminescent bacterial load varied between 1-91cfu/gm for *S. ruconius*with a mean of 47cfu/gm followed by *U. duvaucelii*(1-97 cfu/gm) with a mean of 41cfu/gm and *M. dobsoni*(32-106 cfu/gm) with a mean of 75cfu/gm.TVLO varied between52-221 cfu/gm for

S. ruconius with a mean value, 115 cfu/gm followed by *U. duvaucelii*(56-235 cfu/gm) with a mean of of 131 cfu/gm and *M. dobsoni*(78-227) with a mean of 154 cfu/gm

Table 4.19: Seasonal distribution of THB, BLB and TVLO in*S. ruconius, U. duvaucelii* and *M. dobsoni* samples fromPonnani estuary

Season	*S. ruconius* (cfu/gm)			*U. duvaucelii* (cfu/gm)			*M. dobsoni* (cfu/gm)		
	THB	BLB	TVLO	THB	BLB	TVLO	THB	BLB	TVLO
Pre Monsoon	269	74	137	278	72	157	287	65	203
Monsoon	217	33	113	206	25	104	255	88	123
Post monsoon	226	34	94	245	25	133	269	72	134

4.3 Seasonal distribution of bioluminescent bacteria from different stations of Ponnani estuary

Of the nine stations, bioluminescent bacterial load of water sample was high in Kootai (7 cfu/ml) during pre-monsoon followed by Jangar and Pallikkadav (6cfu/ml). In Harbour and Chandappadi, relatively low level (4cfu/ml) of bioluminescent load was observed. Bioluminescent bacteria in Chamravottom kadav, Kalur and Ishwaramangalam were found to be absent (Figure 4.9)

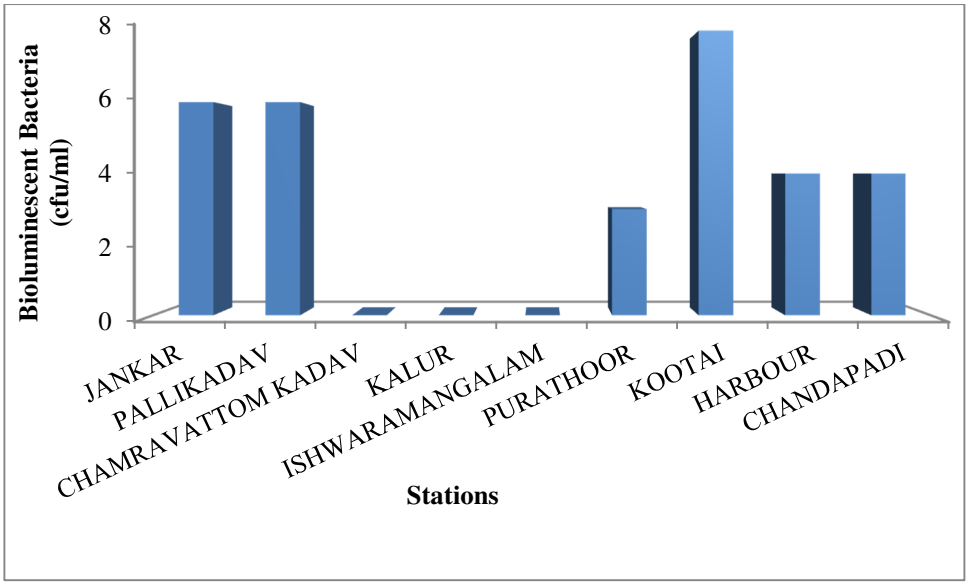


Figure 4.9: Distribution of bioluminescent bacteria of water in different stations of Ponnani estuary during pre-monsoon season

During monsoon, only jangar showed the presence of bioluminescent bacteria in water (1cfu/ml). All the other stations lack bioluminescent load (Figure 4.10).

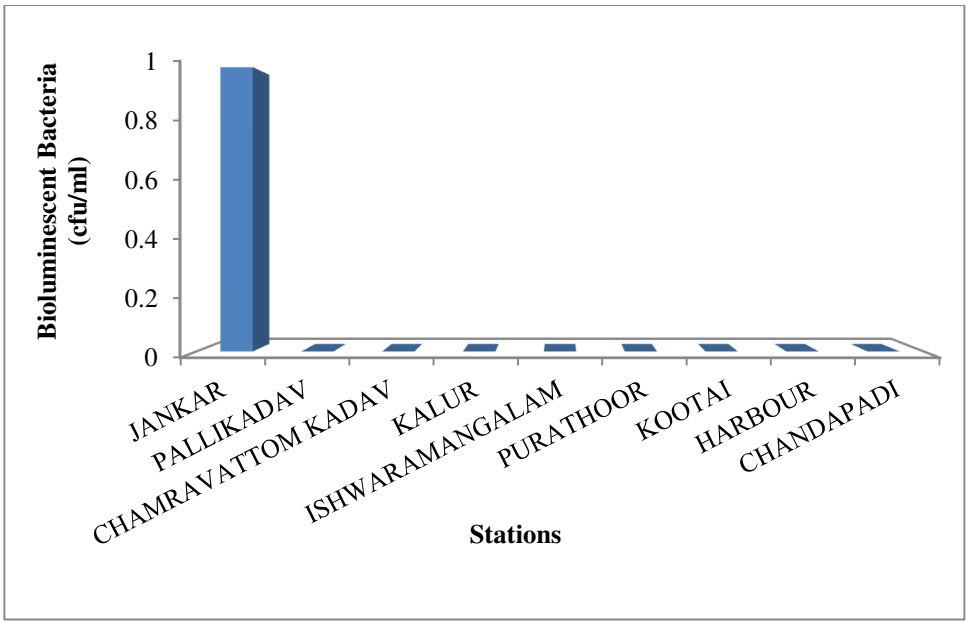


Figure 4.10: Distribution of bioluminescent bacteria of water in different stations of Ponnani estuary during monsoon season

Of the nine stations, bioluminescent bacterial load of water sample was high in Jangar (3cfu/ml) during post-monsoon followed by Harbour (2cfu/ml). Almost similar values were obtained for Pallikkadav and Kootai. In other stations, bioluminescent bacteria were found absent (Figure 4.11).

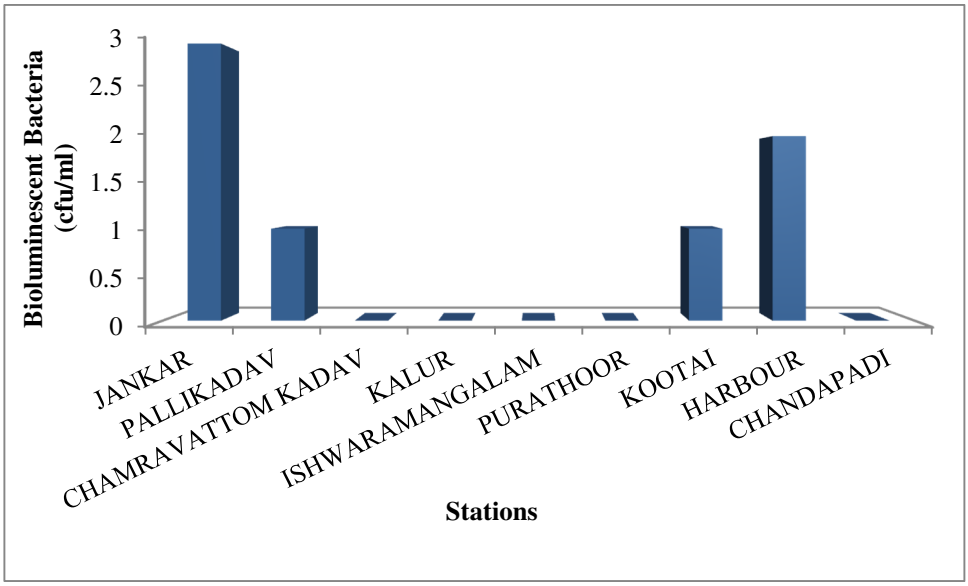


Figure 4.11: Distribution of bioluminescent bacteria of water in different stations of Ponnani estuary during post-monsoon season

The results of the analysis of sediment sample for bioluminescent bacterial load in the nine stations revealed the absence of bioluminescent bacteria in three stations (Chamravottom kadav, Kalur and Ishwaramangalam). The bioluminescent bacterial distribution did not showed much variation among other stations during pre-monsoon (Figure 4.12).

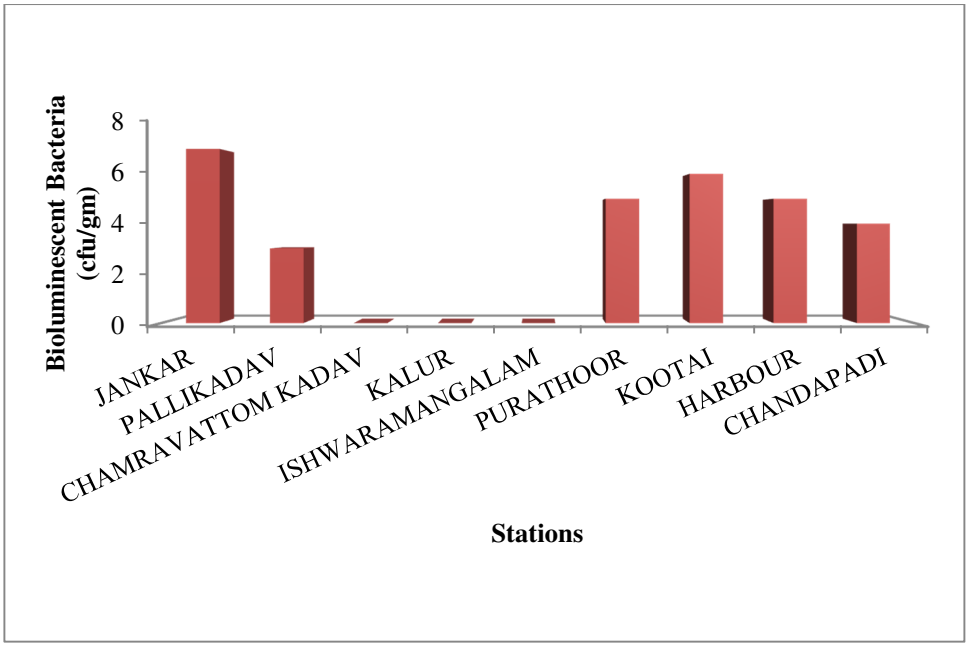


Figure 4.12: Distribution of bioluminescent bacteria of sediment in different stations of Ponnani estuary during pre-monsoon season

During monsoon season, bioluminescent bacteria were observed in the sediment sample taken from Jangar (1cfu/gm) (Figure 4.13).

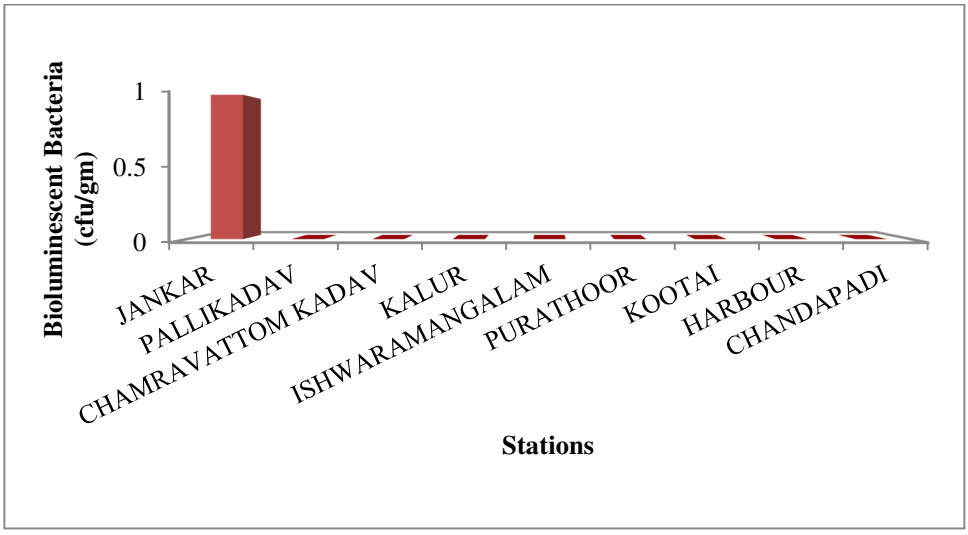


Figure 4.13: Distribution of bioluminescent bacteria of sediment in different stations of Ponnani estuary during monsoon season

During post-monsoon, the bioluminescent bacterial load of sediment samples was obtained from Jangar (4cfu/gm), Pallikkadav (1cfu/gm) and Harbour (2cfu/gm) (Figure 4.14).

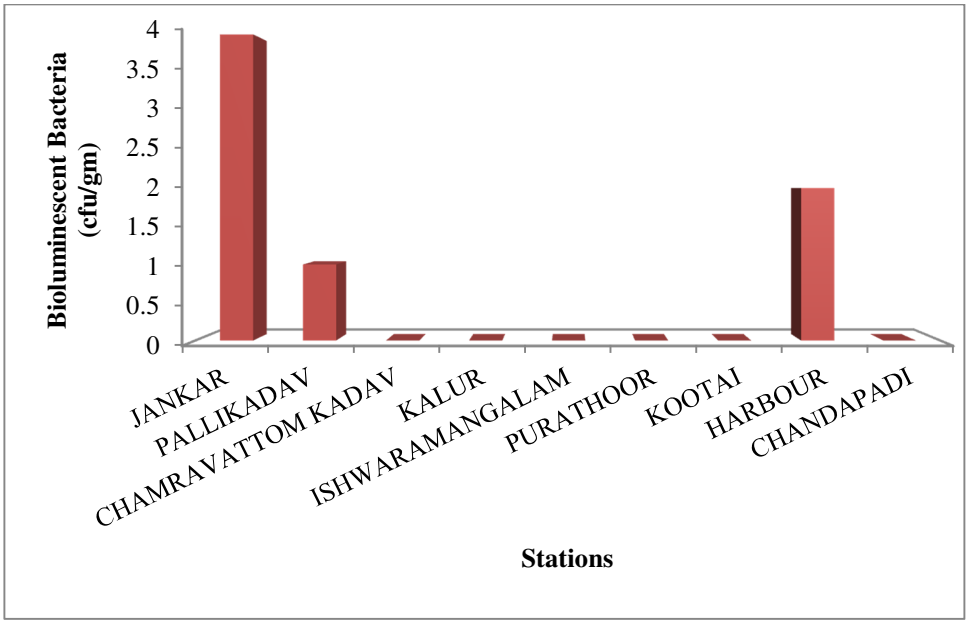


Figure 4.14: Distribution of bioluminescent bacteria of sediment in different stations of Ponnani estuary during Post-monsoon season

During pre-monsoon, bioluminescent bacterial load of *S. ruconius, U. duvaucelii* and *M. dobsoni*did not showed remarkable variation (Figure 4.15). During monsoon, bioluminescent bacterial load was found highest for *M. dobsoni*(88cfu/gm) than *S. ruconius* (33.cfu/gm) and *U. duvaucelii*(25cfu/gm) (Figure 4.16).

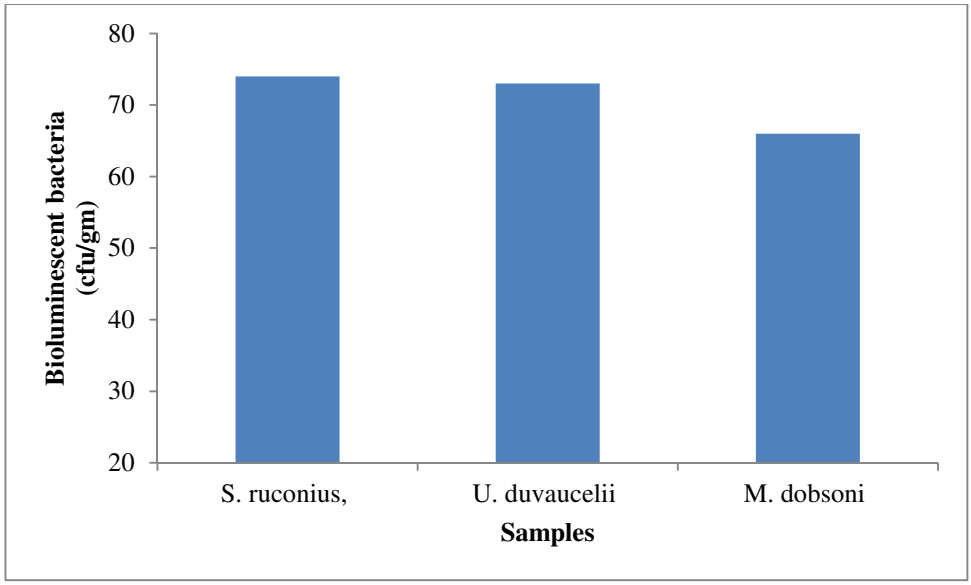


Figure 4.15: Distribution of bioluminescent bacteria of *S. ruconius, U. duvaucelii* and *M. dobsoni* samples of Ponnani estuary during pre-monsoon season.

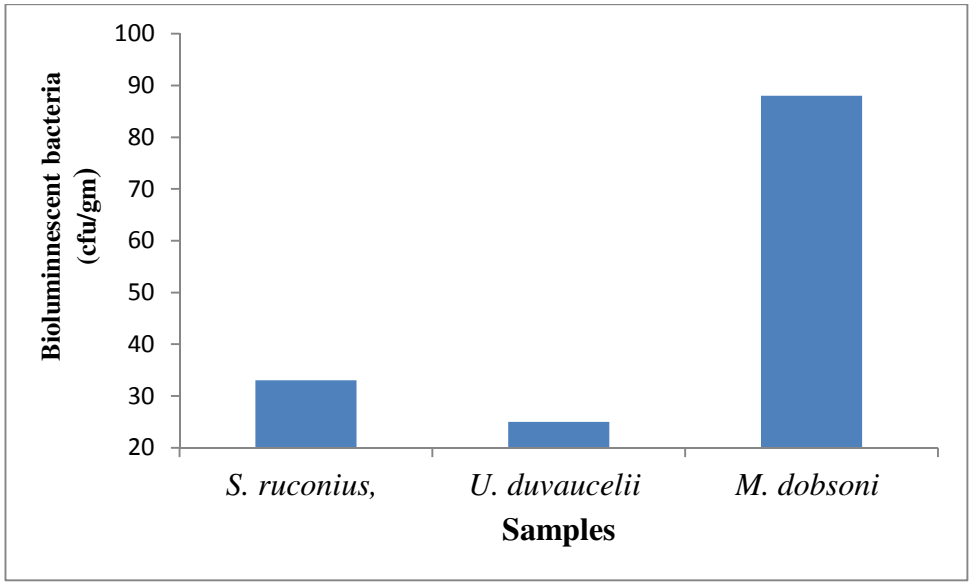


Figure 4.16: Distribution of bioluminescent bacteria of *S. ruconius, U. duvaucelii* and *M. dobsoni* samples of Ponnani estuary during monsoon season.

During post-monsoon, bioluminescent bacterial load was found highest for *M. dobsoni* (72cfu/gm) than *S. ruconius* (34cfu/gm) and *U. duvaucelii* (26cfu/gm) (Figure 4.17).

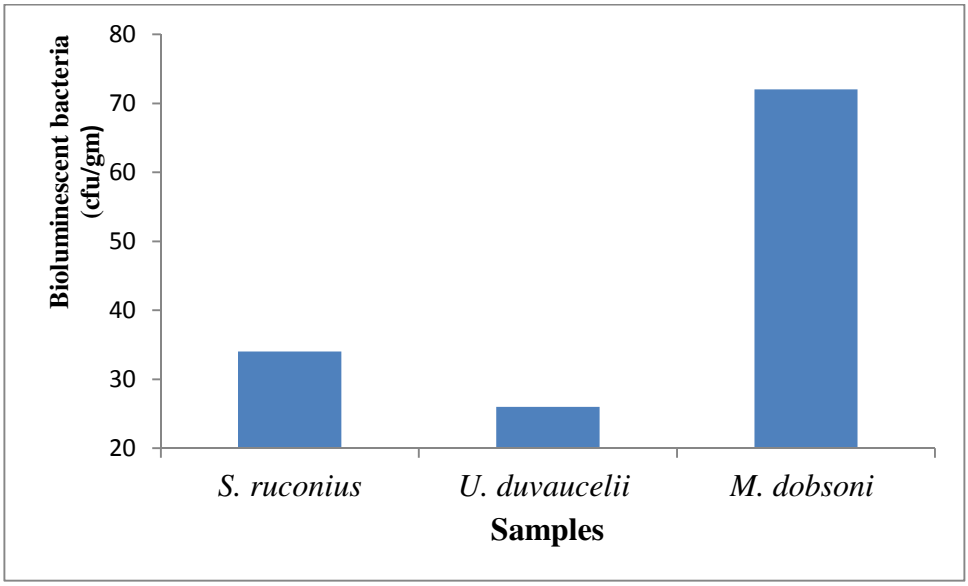


Figure 4.17: Distribution of bioluminescent bacteria of *S. ruconius, U. duvaucelii* and *M. dobsoni* samples of Ponnani estuary during post-monsoon season.

4.4 Relation between Physico-chemical parameters and distribution of bioluminescent bacteria

Among five Physico-chemical parameters (pH, temperature, salinity, DO and BOD), strong correlation exist in temperature and salinity.

4.4.1 Temperature

There is a significant positive correlation between water temperature and bioluminescent bacteria(r=0.7, n=37 and P-value=0.0001).

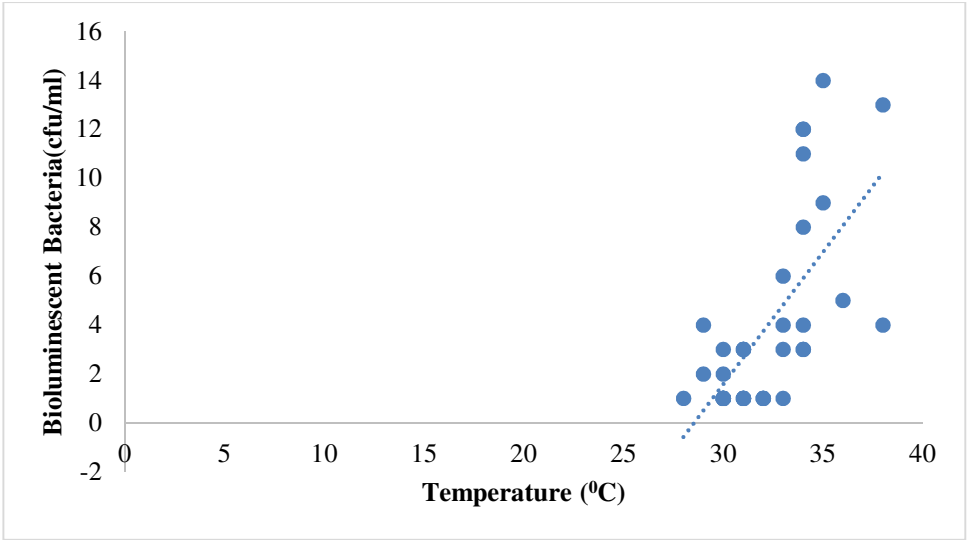


Figure 4.18: Correlation between water temperature and distribution of bioluminescent bacteria

4.4.2 Salinity

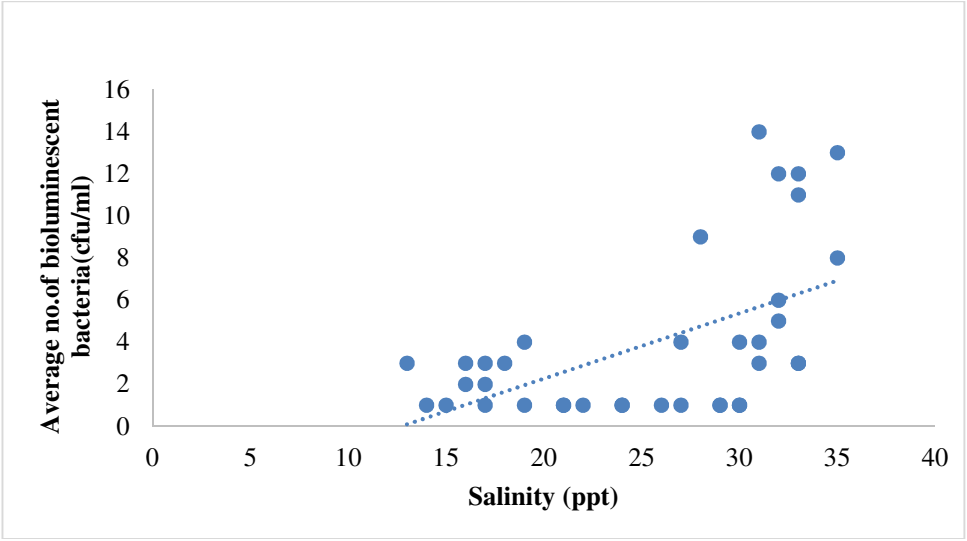


Figure 4.19: Correlation between Salinity and distribution of bioluminescent bacteria

There is a significant positive correlation between salinity and bioluminescent bacteria(r=0.6, n=37 and P-value=0.0005).

4.5 Identification of the bioluminescent bacteria (isolate 1) based on phenotypic characteristics and 16S rRNA sequencing

4.5.1 Phenotypic identification of the Isolate1

Colony Characteristics-Isolate 1 was medium, flat, white colonies with entire margin

4.5.2 Morphological and biochemical characteristics

A set of analysis were conducted for the isolate 1 and the results were given in the Table 4.20

Table 4.20: Morphological and biochemical characteristics of isolate 1

TEST	RESULT	TEST	RESULT
Gram staining	Negative	Catalase	Positive
Motility	Actively motile	H_2S	Positive
Indole	Negative	Lipase	Positive
MR	Positive	Gelatinase	Positive
VP	Positive	Amylase	Negative
Citrate	Positive	Lysine	Positive
TSI	Acid butt and acid slant	Nitrate reduction	Positive
		Hemolysis	Negative
Urease	Negative	Vibrio0129	Negative
Oxidase	Positive	ONPG	Positive

The isolate is a gram negative, motile lipase and gelatinase producing bacteria that are MR –VP and citrate positive.

4.5.3 Molecular identification by 16S rRNA Sequence Analysis

TheDNA extracted from Isolate 1 and the 16S rRNA amplified employing the universal primers. After partially sequencing the PCR reaction product, each nucleic acid sequence was manually edited to correct falsely

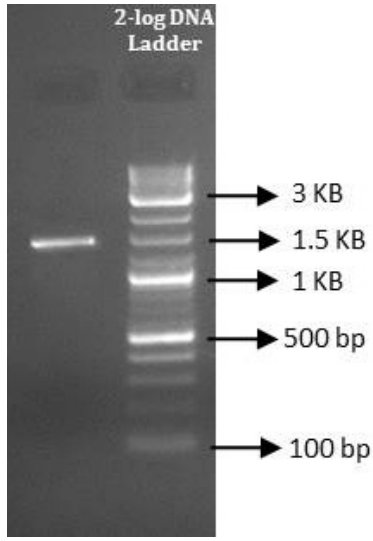
identified bases and trimmed to remove unreadable sequence at the 3^1 and 5^1 ends using the sequence analysis tools.

The edited sequences (16S rDNA) were then employed for similarity searches utilizing BLAST Programme (Basic local Alignment search Tool) in the NCBI Genbank (www.ncbi.nlm.nih.gov) DNA database bacterial strain identification. The isolate showed 98% similarity to the 16s rRNA of the *Photobacterium leiognathi* strain UMTGB206, *Photobacterium leiognathi* subspecies *mandapamensis* strain Mah B7, *Photobacterium leiognathi* strain LC1-113 and *Photobacterium leiognathi* strain LC1-038.

Based on morphological, phylogenetic and molecular characteristics, the Isolate 1 was identified as *Photobacterium leiognathi* strain UMTGB206

Partial nucleotide sequence of 16S rRNA gene (951bp) of the *Photobacterium leiognathi* strain UMTGB206 is given below. The sequence is deposited in genbank with an accession number: MK073017

1CGCAATCGGTTAACTCCTGTTCCCGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTG
61CAGACTCCAATCCGGACTACGACGTACTTTATGGGATTCGCTCACTCTCGCGAGTTGGCA
121GCCCTCTGTATACGCCATTGTAGCACGTGTGTAGCCCTCTCGTAAGGGCCATGATGACTT
181GACGTCGTCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTGGAGTTCCCACCCGA
241AGTGCTGGCAAACAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACA
301ACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAAAGCAT
361CTCTGCTAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAA
421ACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAATCTTGCGA
481CCGTACTCCCCAGGCGGTCTACTTAACGCGTTAGCTCCGAAAGCCACGGCTCAAGGCCAC
541AACCTCCAAGTAGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTC
601CCCACGCTTTCGCATCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTAT
661TCCTTCAGATCTCTACGCATTTCACCGCTACACCTGAAATTCTACCCCCCTCTACAGACT
721CTAGTCTGCCAGTTCAAAATGCTGTTCCGAGGTTGAGCCCCGGGCTTTCACATCTGCTTA
781ACAGACCGCCTGCATGCGCTTTACGCCCAGTAATTCCCGATTAACGCTCGCACCCTCCGT
841ATTACCGCGGCTGCTGGCACGGGAGTTAGCCCGTGCTTCTTCTGTAGGTAACGTCAACAT
901GCGCTATACCGACACTGCATCCTCCCTTACTGAAAGTTACCTTTTACGAC



Lane 1: Molecular weight Markers; Lane 2: Amplified PCR product

Figure 4.20: PCR product of 16S rRNA gene amplified from the DNA extracted from *Photobacterium leiognathi* strain UMTGB206

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacterium 1D707 16S ribosomal RNA gene, partial sequence	2597	2597	100%	0.0	99%	JF411428.1
Photobacterium sp. YL2 16S ribosomal RNA gene, partial sequence	2597	2597	100%	0.0	99%	EF187015.1
Photobacterium leiognathi gene for 16S rRNA, partial sequence, strain: LC1-113	2597	2597	99%	0.0	99%	AB243234.1
Photobacterium leiognathi gene for 16S rRNA, partial sequence, strain: LC1-026	2591	2591	99%	0.0	99%	AB243237.1
Photobacterium leiognathi gene for 16S rRNA, partial sequence, strain: AK7	2591	2591	100%	0.0	99%	AB243233.1
Photobacterium leiognathi subsp. mandapamensis strain DH55 16S ribosomal RNA gene, partial sequence	2590	2590	99%	0.0	99%	FJ240415.1
Photobacterium leiognathi gene for 16S rRNA, partial sequence, strain: LC1-038	2590	2590	99%	0.0	99%	AB243239.1
Photobacterium sp. YL 16S ribosomal RNA gene, partial sequence	2588	2588	100%	0.0	99%	EF017227.1
Photobacterium leiognathi strain Iriyu1.1 16S ribosomal RNA gene, partial sequence	2588	2588	100%	0.0	99%	AY204498.1
Photobacterium leiognathi strain VITJJSM4 16S ribosomal RNA gene, partial sequence	2586	2586	100%	0.0	99%	KX770726.1
Uncultured bacterium clone 1-2 16S ribosomal RNA gene, partial sequence	2586	2586	100%	0.0	99%	KX431265.1
Photobacterium leiognathi partial 16S rRNA gene, isolate M4B61	2586	2586	100%	0.0	99%	LN812996.1
Bacterium 3H201 16S ribosomal RNA gene, partial sequence	2586	2586	100%	0.0	99%	JF411535.1
Photobacterium mandapamensis strain ATCC 33981 16S ribosomal RNA gene, partial sequence	2586	2586	100%	0.0	99%	AY341442.1
Bacterium 3H204 16S ribosomal RNA gene, partial sequence	2584	2584	100%	0.0	99%	JF411538.1
Photobacterium leiognathi subsp. mandapamensis strain DH33 16S ribosomal RNA gene, partial sequence	2584	2584	99%	0.0	99%	FJ240414.1
Photobacterium leiognathi subsp. mandapamensis strain CIFT MFB 10119(3) 16S ribosomal RNA gene, partial sequence	2582	2582	100%	0.0	99%	KP241010.1
Photobacterium leiognathi gene for 16S rRNA, partial sequence, strain: LC1-023	2582	2582	99%	0.0	99%	AB243236.1
Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 16S ribosomal RNA gene, partial sequence	2582	2582	100%	0.0	99%	NR_115206.1
Photobacterium leiognathi strain gachi1.1 16S ribosomal RNA gene, partial sequence	2582	2582	100%	0.0	99%	AY204488.1
Photobacterium leiognathi strain XLum 16S ribosomal RNA gene, partial sequence	2580	2580	100%	0.0	99%	KP843691.1
Photobacterium sp. Rai-7 16S ribosomal RNA gene, partial sequence	2580	2580	100%	0.0	99%	EU807747.1
Photobacterium leiognathi gene for 16S rRNA, partial sequence, strain: LC1-101	2580	2580	100%	0.0	99%	AB243245.1

Figure 4.21: List of identical sequences to *Photobacterium leiognathi* strain UMTGB206

The phylogenetic tree has been constructed for *Photobacterium leiognathi* strain UMTGB206 using MEGA 4 software to show evolutionary relationship with other related genera

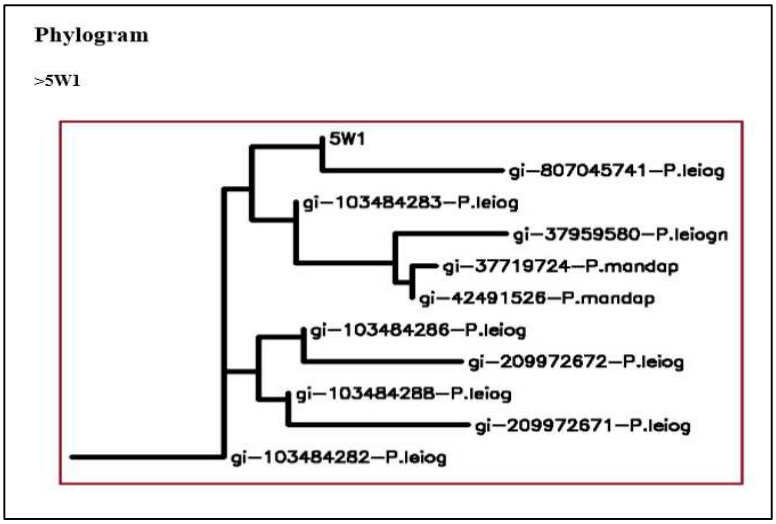


Figure 4.22: Phylogenetic tree of *Photobacterium leiognathi* strain UMTGB206

4.5.4 Phenotypic identification of Isolate 2

Colony Characteristics-Isolate 2 was large, flat, white wrinkled colonies

4.5.5 Morphological and biochemical characteristics

Biochemical characterization for isolate 2 were done and the results were given in the table 4.31

Table 4.21: Morphological and Biochemical characteristics of isolate 2

TEST	RESULT	TEST	RESULT
Gram	Negative	Catalase	Positive
Motility	Actively motile	H_2S	Positive
Indole	Negative	Lipase	Positive
MR	Positive	Gelatinase	Positive
VP	Positive	Amylase	Positive
Citrate	Positive	Lysine	Positive
TSI	Acid butt and alkaline slant	Nitrate reduction	Positive
		Hemolysis	Positive
Urease	Negative	Vibrio0129	Positive
Oxidase	Positive	ONPG	Negative

The isolate is a gram negative, motile lipase and gelatinase producing that are IMViC positive and ONPG negative bacteria

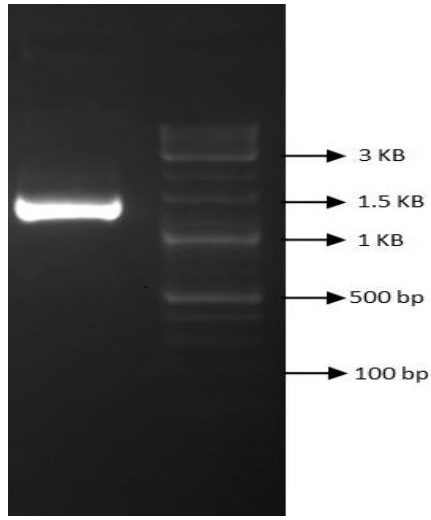
4.5.6 Molecular identification of Isolate 2 by 16S rRNA sequence analysis

The procedure of 16S rRNA sequencing for isolate 2 is similar to that of isolate 1. The sequence obtained showed 100% similarity to *Vibrio fischeri*MJ11, *Allivibrio fischeri* strain IMCC34228, *Vibrio fischeri*ES114 and *Vibrio fischeri* strainET101

Based on morphological, phylogenetic and molecular characteristics, the Isolate 2 was identified as *Vibrio fischeriMJ11*

Partial nucleotide sequence of 16S rRNA gene (1411 bp) of the *Vibrio fischeri*MJ11 is given below. The sequence is deposited in genbank with an accession number: MK114558

1TTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGAAACGACTTAACTGAACCT
61TCGGGGAACGTTAAGGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTGGGAATATGCCTT
121AGTGTGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAATGTCTTCGGACCAAAG
181AGGGGGACCTTCGGGCCTCTCGCGCTAAGATTAGCCCAGGTGAGATTAGCTAGTTGGTGA
241GGTAAGAGCTCACCAAGGCGACGATCTCTAGCTGGTCTGAGAGGATGATCAGCCACACTG
301GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG
361CGAAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTT
421TCAGTAGGGAGGAAGGTGTTGTAGTTAATAGCTGCAGCATTTGACGTTACCTACAGAAGA
481AGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGG
541AATTACTGGGCGTAAAGCGCATGCAGGTGGTTCATTAAGTCAGATGTGAAAGCCCGGGGC
601TCAACCTCGGAACCGCATTTGAAACTGGTGAACTAGAGTGCTGTAGAGGGGGGTAGAATT
661TCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCAGTGGCGAAGGCGGCCCC
721CTGGACAGACACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT
781GGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTTCG
841GAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGA
901ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAA
961CCTTACCTACTCTTGACATCCAGAGAATTCGCTAGAGATAGCTTAGTGCCTTCGGGAACT
1021CTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCC
1081GCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCACGTAATGGTGGGAACTCCAGGGAGA
1141CTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAG
1201TAGGGCTACACACGTGCTACAATGGCGCATACAGAGGGCTGCAAGCTAGCGATAGTGAGC
1261GAATCCCAAAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCG
1321GAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACAC
1381ACCGCCCGTCACACCATGGGAGTGGGCTGCA



Lane 1: Molecular weight Markers; Lane 2: Amplified PCR product

Figure 4.23: PCR product of 16S rRNA gene amplified from the DNA extracted from*Vibrio fischeriMJ11*

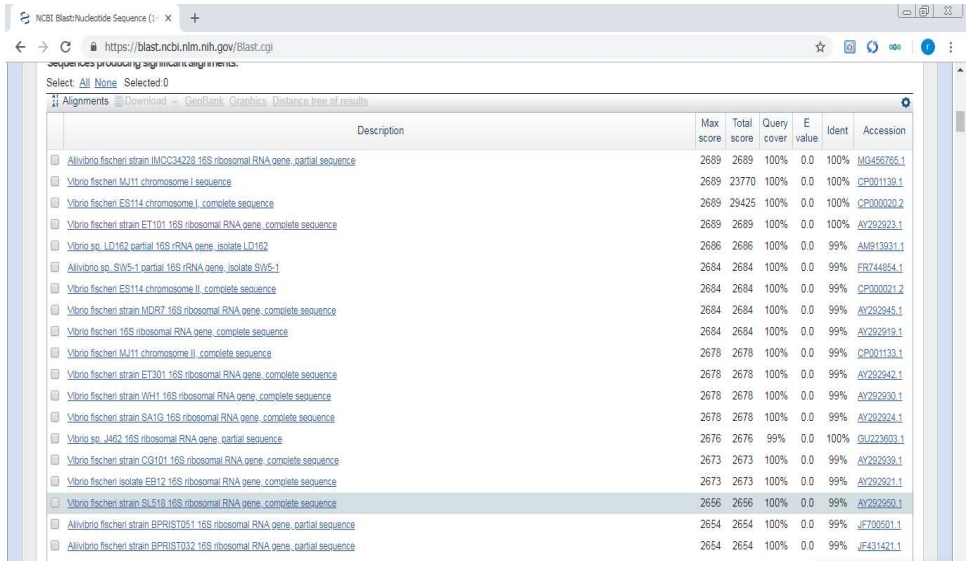


Figure 4.24: List of identical sequences to *Vibrio fischeriMJ11*

The phylogenetic tree has been constructed for *Vibrio fischeri*MJ11 using MEGA SIFT software to show evolutionary relationship with other related genera.

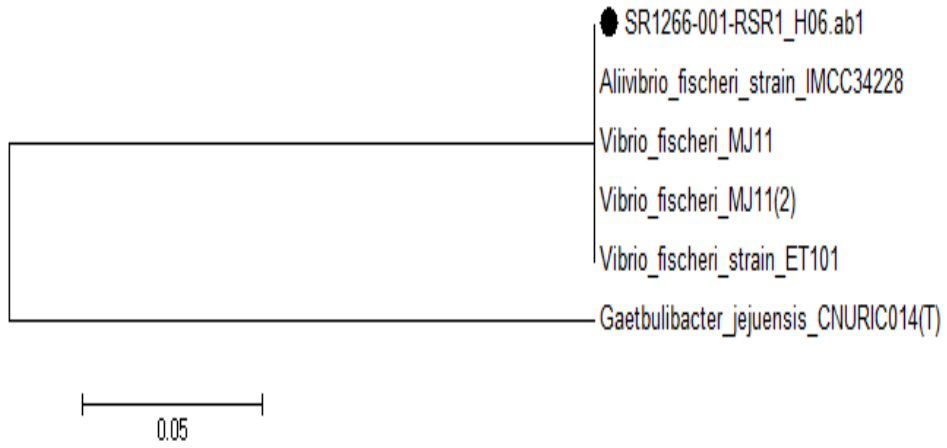


Figure 4.25: Phylogenetic tree of *Vibrio fischeri*MJ11

4.6 Distribution of isolated strainsin different estuarine samples

4.6.1 *P. leiognathi*

The isolated and identified *P. leiognathi* was found more in the fish *S. ruconius* (141 cfu/gm) than the shrimp *M. dobsoni* (94 cfu/gm) and the squid *U. duvaucelii* (52cfu/gm). *P. leiognathi* was distributed in low number in sediment (12 cfu/ml) and water samples (9 cfu/ml) of Ponnani estuary. Fourty six percent of the isolated and identified *P. leiognathi* occurred in the fish *S. ruconius*. 30% in *M. dobsoni* and 17% in the *U. duvaucelii*. The water and sediment samples contained 3 and 4% respectively.

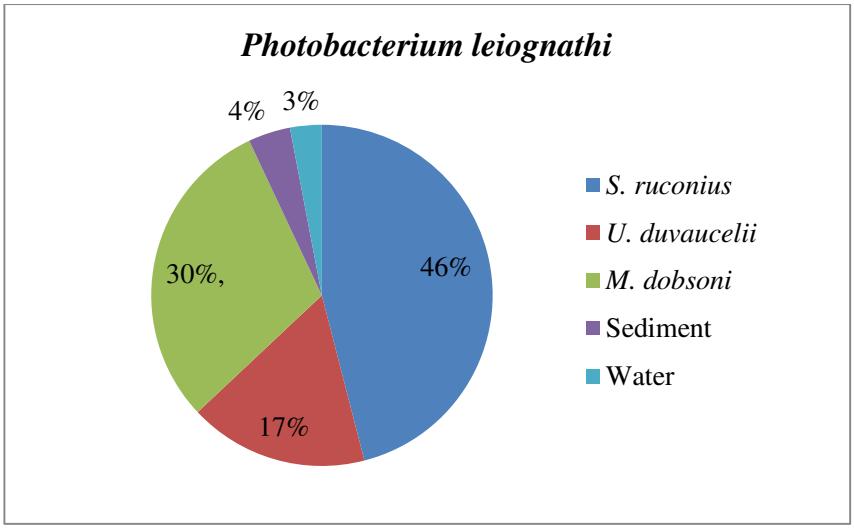


Figure 4.26: Percentage occurence of *Photobacterium leioganthi* in different samples

4.6.2 *V. fischeri*

Highest number of *V. fisheri* inhabited in *M. dobsoni* (132 cfu/ml) and *U. duvaucelii* (72 cfu/ml) followed by water (30 cfu/ml)and sediment (26 cfu/ml). 50% of isolated *V. fischeri* occurred in the shrimp, *M. dobsoni* and 28% in the squid, *U. duvaucelii*. The water and sediment samples contained 12% and 10% respectively.

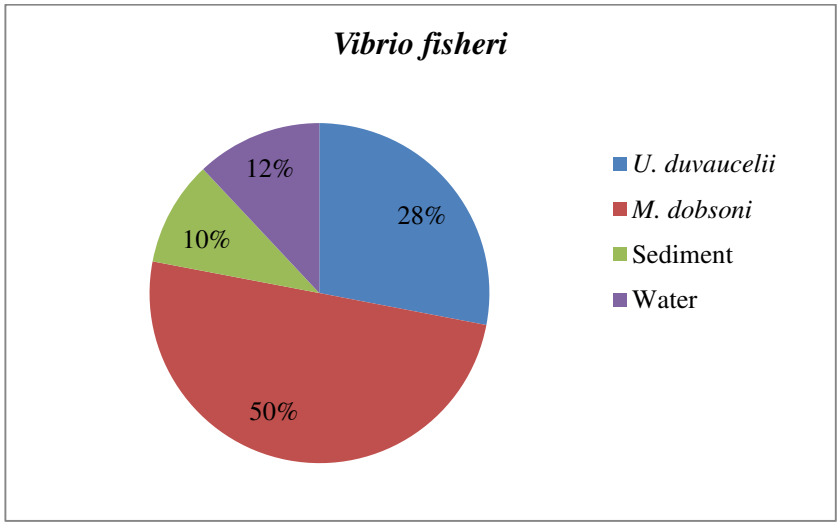


Figure 4.27: Percentage occurrence of *V. fischeri* in different samples

4.7 Effect of different parameters on the luminescence of bioluminescent bacteria

Evaluation of maximum growth and light emission of the isolated bioluminescent bacteria in different concentration of salinity, pH, glycerol concentration and incubation temperature were carried out.

4.7.1 Effect of salinity (Varying concentrations of NaCl)

The growth and intensity of light emission of the bioluminescent bacteria showed a very good luminescence at 3% and 6% of sodium chloride concentration and good luminescence in the sodium chloride level at 9% and 0 and luminescence was absent at 12% NaCl.

4.7.2 Effect of pH

The maximum growth and intensity of bioluminescent light emission was recorded at neutral pH showing very good luminescence. Good luminescence was observed at pH 5 and 9. pH 11 showed negative luminescence.

4.7.3 Effect of glycerol concentration

Very good luminescence was observed at 0.3% glycerol concentration. Good luminescence observed in 0.1% and 0.6% of glycerol concentration and dull luminescence observed with 0.9% and 1.2% of glycerol concentration.

4.7.4 Effect of Temperature

Incubation temperature plays a significant role in the growth of bioluminescent bacteria. Very good luminescence was observed at $30^{O}C$ and good luminescence was observed at $25^{O}C$. At $20^{O}C$ and $35^{O}C$, dull luminescence was observed. Luminescent emission was absent at $15^{O}C$ (Table 4.22).

Table 4.22: Effect of different parameters (pH, salinity, glycerol concentration and temperature) on bioluminescence by visual scoring

No.	Parameter	Visual Scoring	
1	pH	5	++
		7	+++
		9	++
		11	-
2	Salinity (NaCl)	0%	++
		3%	+++
		6%	+++
		9%	++
		12%	-
3	Glycerol	0.1%	++
		0.3%	+++
		0.6%	++
		0.9%	+
		1.2%	+
4	Temperature	15^0C	-
		20^0C	+
		25^0C	++
		30^0C	+++
		35^0C	+

- Negative, + dull luminescence, ++ good luminescence and +++ very good luminescence,

4.8 Symbiotic association

V. fisheri and *P. leiognathi* were the bioluminescent bacteria obtained from Ponnani estuary. *P. leiognathi* occurred in light organ of *S. ruconius and U. duvaucelii*, also isolated from alimentary canal of *M. dobsoni.* But *V. fischeri* was obtained from the alimentary canal of *M. dobsoni* and light organ of *U. duvaucelii*. Fourty six percentage of *Photobacterium leiognathi* was found in *S. ruconius* (141 cfu/gm), thirty percentages was found in *M. dobsoni* (94 cfu/gm) and seventeen percentages in *U. duvaucelii* (52cfu/gm). Hence *P. leiognathi* was most host specific to *S. ruconius.* Fifty percentage of *V. fisheri* inhabited in *M. dobsoni* (132 cfu/mg) and twenty eight percentage of *V. fischeri* were

distributed in *U. duvaucelii* (72 cfu/ml). In this case *V. fischeri* was most host specific to *M. dobsoni* than *U. duvaucelii.*

Eventhough *V. fischeri* and *P. leiognathi* were isolated from M. dobsoni, they do not exist in symbiotic relationship, and instead they may induce pathogenicity in host. Comparing *P. leiognathi* and *V. fischeri*, the result showed that *P. leiognathi* was more host specific than *V. fischeri* since their numbers were high in the host.Symbiotic specificity of *P. leiognathi* and *S. ruconius* was maintained at the species level than individual level. *U. duvaucelii* and *S. ruconius* utilize the bacterial bioluminescence for counter-illumination camouflage during its night-time activity and in return *U. duvaucelii* and *S. ruconius* assist the bacteria by offering a microhabitat rich in nutrients.

4.9 Pollution status of Ponnani estuary through BLR (bioluminescent ratio)

Bioluminescent ratio is the ratio of bioluminescent bacterial count to the total heterotrophic bacterial count. Pollution status of Ponnani estuary was explored from bioluminescent ratio. BLR Ratio of less than 4% indicates polluted area. Though nine stations were sampled for the present study, bioluminescent bacteria was obtained only in six stations, among these BLR of all stations except Kootai during pre-monsoon were low (less than 4%). Hence, Pallikkadav (BLR-0.5%), Jangar (BLR-0.7%) and Harbour (BLR-1%) were the most polluted stations in the present study, since lowest ratio indicates the polluted area. BLR ratios of the other stations (Kootai and Purathoor) were also low since the BLR values were less than four. Hence, Ponnani estuary was becoming polluted due to human influences (Table 4.33).

Table 4.23: Bioluminescent ratio of different stations in Ponnani estuary

Site	Season	Number of colonies in		BLR Ratio A/B	%
		Sea water complex agar (A)	Nutrient agar (B)		
Jangar	Pre Monsoon	6	158	0.038	3.8
	Monsoon	1	134	0.007	0.7
	Post Monsoon	3	221	0.013	1.3
Pallikkadav	Pre Monsoon	6	200	0.03	3.0
	Post Monsoon	1	191	0.005	0.5
Purathur	Pre Monsoon	3	190	0.016	1.6
Kootai	Pre Monsoon	8	111	0.072	7.2
	Post monsoon	1	95	0.011	1.1
Harbour	Pre Monsoon	4	201	0.02	2.0
	Post monsoon	2	202	0.01	1.0
Chandappadi	Pre Monsoon	4	112	0.036	3.6

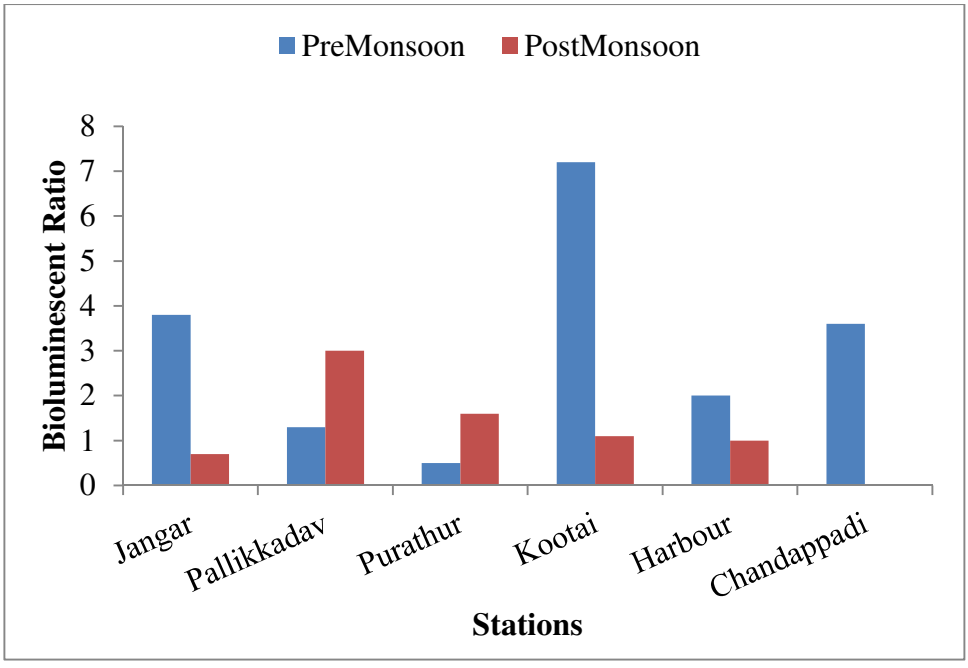


Figure 4.28: Bioluminescent ratio of different stations in Ponnani estuary

4.10 Antibiotic susceptibility pattern

Isolated bioluminescent strains were tested for its antibiotic susceptibility pattern following the procedures of Baur *et al*. (1966). Eleven strains of *V.fischeri* and *P.leiognathi* had been tested to perceive their antibiotic susceptibility pattern. Different antibiotics (chloramphenicol, ciprofloxacin, gentamycin, cefotaxime, tetracycline, aztreonam and polymixin B) had been tested for its susceptibility towards the isolated bioluminescent strains of *P.leiognathi* and *V.fisheri*.

Figure 4.29: Antibiotic zone formation in muller hinton agar plates

4.10.1 Antibiotic susceptibility pattern of *P. leiognathi*

Chloramphenicol showed highest zone for the strain 4 with a zone diameter of 26mm followed by strain 9(25mm) and strain 11(24mm). For strain 5 and 6, zone formation was absent. Gentamycin showed highest zone for strain 2 and 4(28mm) followed by strain 5(23mm) and strain 6 (22mm). For strain 8, 9 and 10, zone formation was absent signifying resistance. In the case of aztreonam, strain 8 showed highest zone formation (27mm) followed by strain 4(26mm) and strain 7(25mm). Cefotaxime showed zone formation only for strain 10(23mm). For all other strains, zone formation was absent showing their resistance. Ciprofloxacin showed highest zone formation for the strain 7 and 11(26mm) followed by strain 9(25mm) and strain 4(22mm). Strain 5 doesn't showed any zone formation. In the case of tetracycline, strain 2 and strain 7 showed maximum zone formation(26mm) followed by strain 6(22mm) and strain 11(20mm). Polymixin B showed highest zone formation for the strain 3(32mm) followed by strain 8(29mm) and strain 9(25mm). For strain 7 and 11, zone formation was absent showing their resistance. Diameter of zone of inhibition (in mm) of *P. leiognathi* strains with different antibiotics discs were showed in the table 4.24

Table 4.24: Diameter of zone of inhibition (in mm) of *P. leiognathi* strains with different antibiotics discs.

Antibiotic Discs	Mean diameter of the zone of inhibition (mm)										
	PLS 1	**PLS 2**	**PLS 3**	**PLS 4**	**PLS 5**	**PLS 6**	**PLS 7**	**PLS 8**	**PLS 9**	**PLS 10**	**PLS 11**
Chloramphenicol (30mcg)	**23**	**21**	**23**	**26**	**0**	**0**	**9**	**22**	**25**	**19**	**24**
Gentamycin (10mcg)	**22**	**28**	**21**	**28**	**23**	**22**	**8**	**0**	**0**	**0**	**11**
Aztrionam (30mcg)	**25**	**20**	**19**	**26**	**23**	**21**	**25**	**27**	**9**	**19**	**23**
Cefotaxime (30mcg)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**23**	**0**
Ciprofloxacin (30mcg)	**21**	**10**	**19**	**22**	**0**	**12**	**26**	**22**	**25**	**22**	**26**
Tetracycline (30mcg)	**10**	**26**	**0**	**0**	**0**	**22**	**26**	**0**	**0**	**9**	**20**
Polymixin (10mcg)	**9**	**10**	**32**	**8**	**24**	**12**	**0**	**29**	**25**	**8**	**0**

PLS- *P. leiognathi* strains

*P. leiognathi*strains were found to be resistant to cefotaxime (91%), followed by tetracycline (45%) and gentamycin (27%). Ninety one percentage of *P. leiognathi* strains were found to be sensitive to aztreonam (91%) followed by chloramphenicol and ciprofloxacin (73%). Percentage of sensitive, intermediate sensitive and resistant strains of *P. leiognathi*were showed in the Figure 4.30.

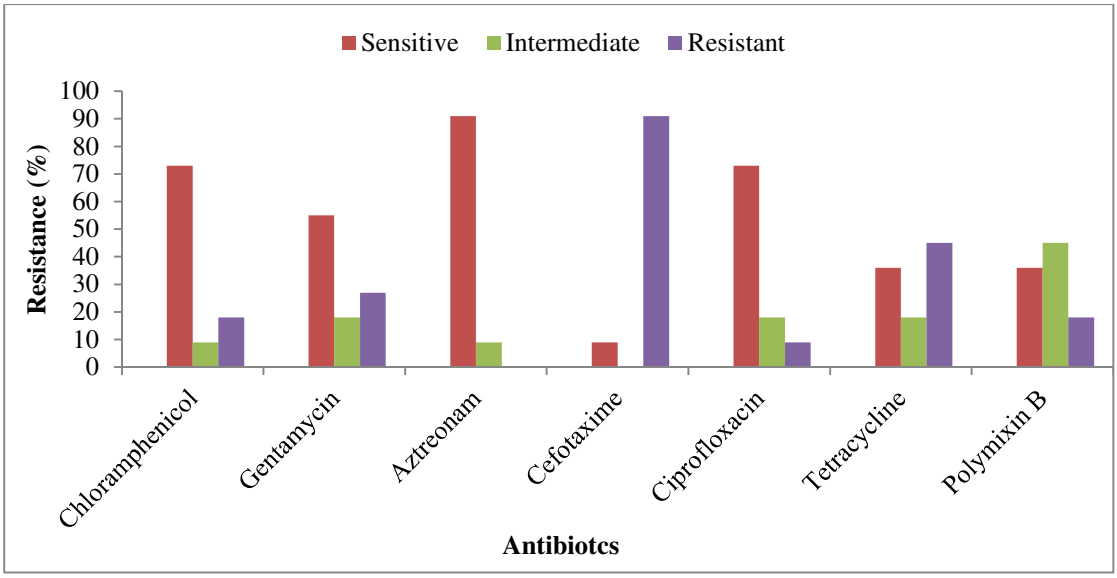


Figure 4.30: Antimicrobial resistance in *P. leiognathi* strains isolated from Ponnani estuary

Antibiotic resistance patterns and multiple drug resistance (more than three antibiotics) observed in selected *P. leiognathi* strains. Among ten strains, one displayed multidrug resistance (MDR) of more than three antibiotics and two strains displayed multidrug resistance of more than two antibiotics. Highest number of resistant antibiotics (CM-CX-CIP-TC) was observed to strain 5 (Table 4.25).

Table 4.25: Antibiotic resistance profiles for *P. leiognathi* strains

No. of resistant antimicrobes	No. of strains	Antimicrobial resistance profiles	%	Resistance classification
1	1	CX	10	NMDR
2	2	CX-TC	20	NMDR
4	1	CM-CX-CIP-TC	10	MDR
1	1	CM-CX	10	NMDR
2	1	CX-PMB	10	NMDR
3	2	GM-CX-TC	20	MDR
1	1	GM	10	NMDR
2	1	CX-PMB	10	NMDR

Cefotaxime-CX, Tetracycline-Tc, Gentamycin-Gm, Chloramphenicol-Cm, ciprofloxacin-Cip, Polymixin B-PmB,
MDR - Multiple drug resistance, NMDR - Non multiple drug resistance.

4.10.1.1 Cluster analysis to show similar response of antibiotics to *P. leiognathi*

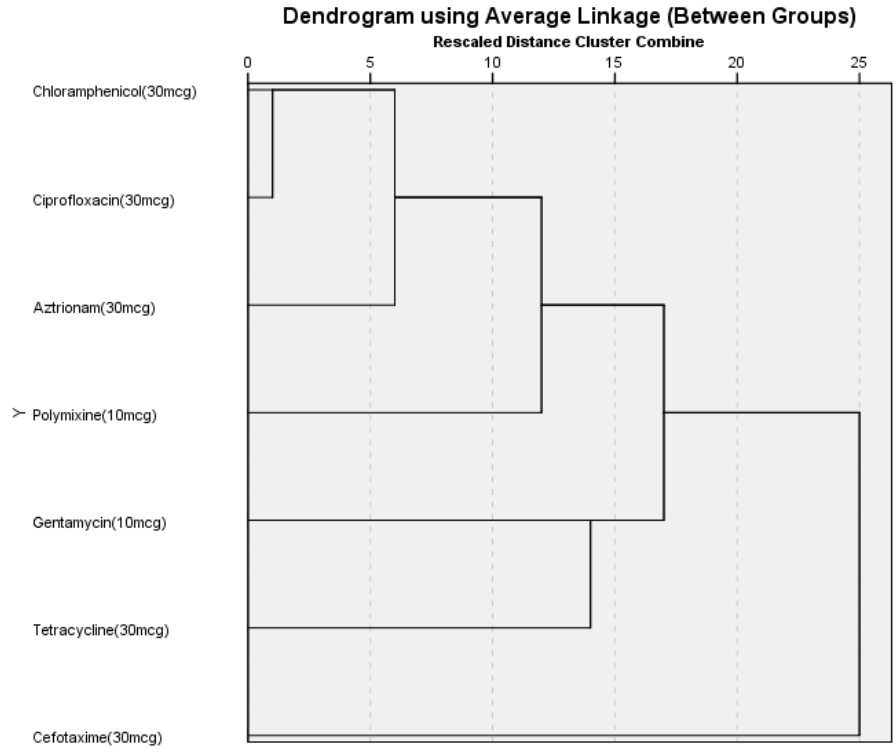


Figure 4.31: Antibiogram showing the antibiotic susceptibility pattern of *P. leiognathi*

Table 4.26: Response of *P. leiognathi* to antibiotics by different strains and cluster membership

Frequency	Resistant	Intermediate	Sensitive	Cluster No
Chloramphenicol(30mcg)	2 (18.2)	1 (9.1)	8 (72.7)	1
Ciprofloxacin(30mcg)	1 (9.1)	2 (18.2)	8 (72.7)	
Aztrionam(30mcg)	1 (9.1)	--	10 (90.9)	2
Polymixin(10mcg)	2 (18.2)	5 (45.5)	4 (36.4)	3
Gentamycin(10mcg)	3 (27.3)	2 (18.2)	6 (54.5)	4
Tetracycline(30mcg)	5 (45.5)	2 (18.2)	4 (36.4)	5
Cefotaxime(30mcg)	10 (90.9)	--	1 (9.1)	6

Values in the brackets are percentages

Antibiogram shows that only chloramphenicol and ciprofloxacin have similar response pattern. Responses of all other antibiotics were significantly different.

4.10.2 Antibiotic susceptibility pattern of *V. fischeri*

Chloramphenicol showed highest zone for the strain 1 with a zone diameter of 32 mm followed by strain 5(26mm) and strain 10(25mm). For strains 4, 7 and 11 zone formation was absent. Gentamycin showed highest zone for strain 4 with a zone diameter of 32 mm followed by strain 5(28 mm) and strain 1(28 mm). For strain 8, zone formation was absent signifying resistance. In the case of aztreonam, strain 6 showed highest zone formation (32 mm) followed by strain 1 and 5(28 mm) and strain 3(26mm).For strain 7 zone formation was absent. Cefotaxime showed maximum zone formation for strain 11(26mm) followed by strain 6(24mm) and strain 5(10 mm). For all other strain, zone formation was absent showing their resistance. Ciprofloxacin showed highest zone formation for the strain 2 (31 mm) followed by strain 8(29mm) and strain 5(27mm). Strain 3 did not showed any zone formation. In the case of tetracycline, strain 2 and strain 9 showed maximum zone formation(26mm) followed by strain 6(21 mm). For all other strains zone formation was absent. Polymixin B showed highest zone formation for the strains 3 and 7 (29mm) followed by strain 6(27mm) and strain 9(25mm). For strains 7 and 11, zone formation was absent showing their resistance. Diameter of zone of inhibition (in mm) of *V. fischeri* strains with different antibiotics discs were showed in the table 4.27

Table 4.27: Diameter of zone of inhibition (in mm) of *V. fischeri* strains with different antibiotics discs.

Antibiotic Discs	Mean diameter of the zone of inhibition (mm)										
	VFS 1	**VFS 2**	**VFS 3**	**VFS 4**	**VFS 5**	**VFS 6**	**VFS 7**	**VFS 8**	**VFS 9**	**VFS 10**	**VFS 11**
Chloramphenicol (30mcg)	32	19	21	0	26	22	0	21	19	25	0
Gentamycin (10mcg)	28	25	22	32	29	21	20	0	24	26	19
Aztrionam (30mcg)	28	22	26	21	28	32	0	9	22	25	23
Cefotaxime (30mcg)	0	0	0	0	10	24	0	0	0	0	26
Ciprofloxacin (30mcg)	8	31	0	24	27	20	21	29	10	9	24
Tetracycline (30mcg)	0	26	0	0	0	21	0	0	26	0	0
PolymixinB (10mcg)	8	22	29	8	9	27	29	21	25	23	8

VFS-*V. fischeri* strains

V. fischeri strains showed the highest resistance of 73% to cefotaxime and tetracycline followed by polymixin B (36%) and chloramphenicol (28%). Ninety one percentage of *V. fischeri* isolates were sensitive to gentamycin followed by aztreonam (82%), ciprofloxacin and Polymixin B (64%). Percentage of sensitive, intermediate sensitive and resistant strains of *V. fischeri* were showed in the figure 4.32

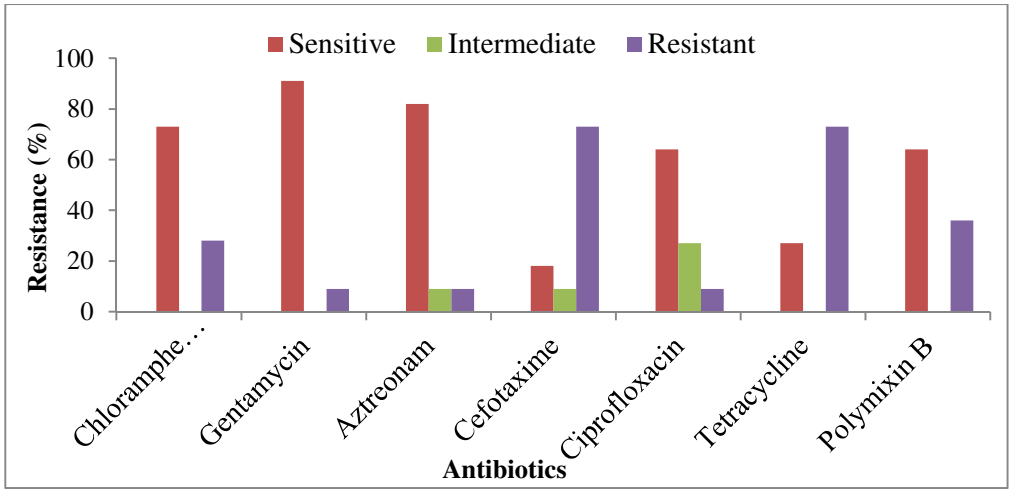


Figure 4.32: Antimicrobial resistance in *V. fischeri* isolated from Ponnani estuary

Antibiotic resistance patterns and multiple drug resistance (more than three antibiotics) observed in selected *V. fischeri* strains. Among ten strains, one displayed multidrug resistance (MDR) of more than three antibiotics and 3 strains displayed multidrug resistance of more than two antibiotics. Highest number of resistant antibiotics (CM-AZ-CX-TC) was observed to strain 7 (Table 4.28).

Table 4.28: Antibiotic resistance profiles for *V. fischeri*

No. of resistant antimicrobes	No. of strains	Antimicrobial resistance profiles	%	Resistance classification
2	1	CX-TC	10	NMDR
1	1	CX	10	NMDR
3	1	CX- CIP-TC	10	MDR
3	1	CM-CX-TC	10	MDR
1	1	TC	10	NMDR
4	1	CM-AZ-CX-TC	10	MDR
3	1	GM-CX-TC	10	MDR
1	1	CX	10	NMDR
2	1	CX-TC	10	NMDR
2	1	CM-TC	10	NMDR

*Cefotaxime-CX, Tetracycline-Tc, Gentamycin-Gm, Chloramphenicol-Cm, ciprofloxacin-Cip, Polymixin B-PmB,
*MDR - Multiple drug resistance, NMDR - Non multiple drug resistance.

4.10.2.1 Cluster analysis to show similar response of antibiotics to *V. fischeri*

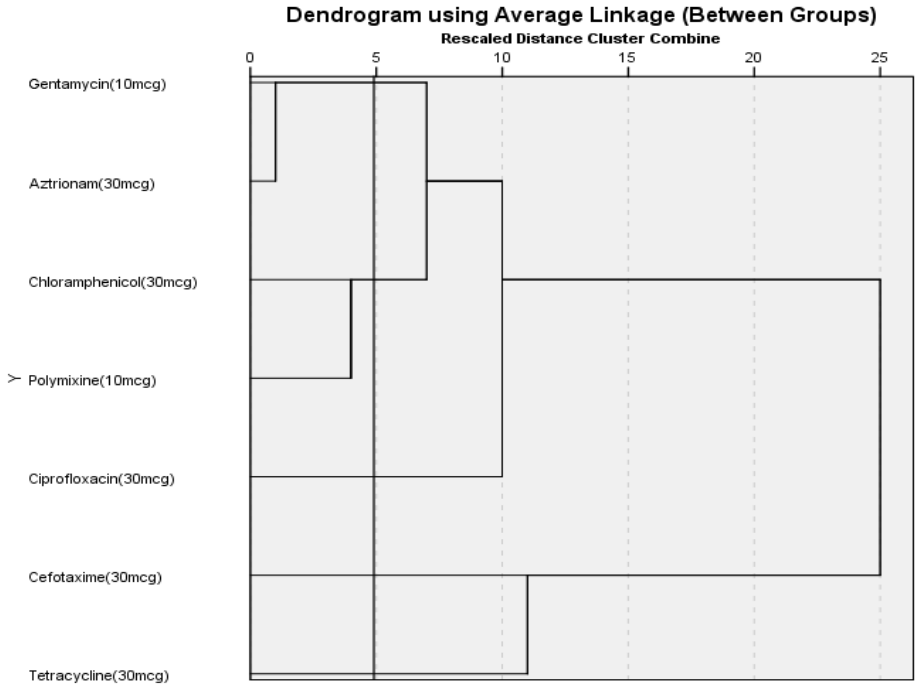


Figure 4.33: Antibiogram showing the antibiotic susceptibility pattern of *V. fischeri*

Table 4.29: Response of *V. fischeri* to antibiotics by different samples and cluster membership

Frequency	Resistant	Intermediate	Sensitive	Cluster No
Aztrionam(30mcg)	1 (9.1)	1 (9.1)	9 (81.8)	1
Gentamycin(10mcg)	1 (9.1)		10 (90.9)	
Chloramphenicol(30mcg)	3 (27.3)		8 (72.7)	2
Polymixin(10mcg)		4 (36.4)	7 (63.6)	
Ciprofloxacin(30mcg)	1 (9.1)	3 (27.3)	7 (63.6)	3
Cefotaxime(30mcg)	8 (72.7)	1 (9.1)	2 (18.2)	4
Tetracycline(30mcg)	8 (72.7)		3 (27.3)	5

Values in the brackets are percentages

Cluster analysis was done for drawing antibiogram which will help to find out which of the antibiotics are having similar response. By allowing 20 Percent variability, aztrionam and gentamycin are having almost similar response, more than 80% of the cases are sensitive to these antibiotic. Chloramphenicol and polymixin also had similar pattern. Responses of other antibiotics were not similar to one another. More cases (72.7%) were resistant by cefotaxime and tetracycline. Still their performance was not similar because the sample which was found to be resistant to cefotaxime may not be resistant to tetracycline.

4.11 Heavy metal susceptibility of bioluminescent bacteria (Alex, 2010)

Isolated strains of *V.fischeri* and *P.leiognathi* were tested for its heavy metal susceptibility by agar disc diffusion method. Susceptibility of *P.leiognathi* and *V. fischeri* to various concentrations of heavy metal salts (mercury chloride, copper sulfate, lead nitrate, cadmium acetate and cobalt nitrate) was evaluated.

Figure 4.34: Plates showing heavy metal susceptibility

4.11.1 Heavy metal susceptibility of *P. leiognathi*

Isolated strains of *P. leiognathi* was resistant to lead and sensitive to mercury, cobalt, cadmium and copper and sensitivity follows the order mercury>cobalt>cadmium>copper. Zone of inhibition in different metal concentrations (40 µl, 30 µl, 20 µl and 10 µl) showed maximum for Mercury where the zone diameter was 20mm followed by Cobalt (19mm), cadmium (15mm) and Copper (14mm). Effect of *P. leiognathi* strain UMTGB206 to different heavy metals were showed in the table 4.30

Table 4.30: Effect of different heavy metals on *P. leiognathi*

Heavy Metals	Zone of inhibition(mm)on different metal concentrations(µl)			
	40 µl	**30 µl**	**20 µl**	**10 µl**
Hg	S(20)	S(15)	S(10)	S(10)
Co	S(19)	S(14)	S(10)	S(9)
Cd	S(15)	S(14)	S(12)	S(9)
Cu	S(14)	S(13)	S(12)	S(8)
Pb	R(0)	R(0)	R(0)	R(0)

R-Resistant; S-Susceptible.

Values in paranthesis are zone of inhibition in mm.

4.11.2 Heavy metal susceptibility of *V. fischeri*

V. fischeri was resistant to mercury and lead and sensitive to cadmium, cobalt and copper and sensitivity follows the order cadmium>cobalt>copper. Zone of inhibition in different metal concentrations (40 µl, 30 µl, 20 µl and 10 µl) showed maximum for Cadmium where the zone diameter was 19mm followed by Cobalt (18mm) and Copper (15mm). Effects of *V. fischeri* to different heavy metals were showed in the table 4.31.

Table 4.31: Effect of different heavy metals on *V. fischeri*

Heavy Metals	Zone of inhibition(mm)on different metal concentrations(µl)			
	40 µl	**30 µl**	**20 µl**	**10 µl**
Hg	R(0)	R(0)	R(0)	R(0)
Co	S(18)	S(12)	S(9)	S(8)
Cd	S(19)	S(16)	S(15)	S(10)
Cu	S(15)	S(11)	S(10)	S(7)
Pb	R(0)	R(0)	R(0)	R(0)

R-Resistant; S-Susceptible.

Values in paranthesis are zone of inhibition in mm.

RAMINA.P.P. “DISTRIBUTION AND ABUNDANCE OF LUMINESCENT BACTERIA IN PONNANI ESTUARY.” THESIS. POST GRADUATE DEPARTMENT OF AQUACULTURE AND FISHERY MICROBIOLOGY, MES PONNANI COLLEGE, PONNANI, UNIVERSITY OF CALICUT, 2018.

CHAPTER 5

DISCUSSION

Estuaries are dynamic ecosystems, forming transition zone between river and ocean environments subjecting to marine influences such as tides, waves and the influx of saline water and other riverine influences. Bioluminescent bacteria were highly inhabited in marine environment especially in seawater, sediments, marine plants and animals. Microbial activities play a vital role in marine food webs, nutrient mineralization and recycling. Ecology of bioluminescent bacteria had paid attention chiefly on marine environment (Nealson and Hastings, 1979), Vellar estuary (Ramesh *et al.*, 1989), Skidaway river estuary (Fricsher *et al*., 2005), Campania coastal waters (Sardo *et al*., 2008), Florida estuary (Audrey Carson, 2011).

The present study was aimed to investigate the distribution and abundance of bioluminescent bacteria in relation with the physico-chemical parameters of Ponnani estuary and to assess the pollution of this area by BLR ratio.

5.1 Physico-chemical parameters

The primary productivity of an ecosystem depends on the favorable status of the physical characteristics and hydrodynamic conditions of the estuary (Webster *et al*., 2003).Different physico-chemical parameters like pH, salinity, temperature, dissolved oxygen (DO) and biological oxygen demand (BOD) were analyzed.

5.1.1pH

pH of the water concludes the stability of the balance between the different forms of carbonic acid linked to the system buffer developed by carbonates and bicarbonates. It is a determinant factor in control and fixation of the phosphorous by sediment. pH of the estuary was weekly alkaline (6.8-8.4) during the study period and it was highest during the pre-monsoon (8.2-8.4) and it gradually decreased in the post monsoon (6.8-6.3) and monsoon (7.1-

8.1).This result agrees with the findings of Ranjith et al. (2017) where pH ranged 6.2-8.1 and Thasneem *et al*. (2018)where pH ranged 7.07 to 7.47. Studies by Leena, 2003; Jyotsna *et al*. (2018) also supports our study. Two-way ANOVA showed a significant difference between stations ($F=12.2$, $P<0.01$) and season ($F=77.6$, $P<0.01$) for pH. The high pH values during pre monsoon might be due to sea water penetration and high biological activity (Srinivasan, 2014). Similarly low pH observed during monsoon season might be due to removal of CO_2 by photosynthesis and fresh water influx, reduction of salinity and temperature and organic matter decomposition (Rajasegar, 2003 and Srinivasan, 2014).

5.1.2 Salinity

In the present study, salinity ranged between 2-32ppt and it was highest during pre-monsoon (18-32ppt) and it gradually decreased in the post-monsoon (2-20ppt) and monsoon (4-16ppt) with three stations (Kalur, Chamravottom kadav and Ishwaramangalam) in monsoon being purely fresh water. Similar results were obtained by Thasneem *et al*. (2018) and Ranjith *et al*. (2017) from Cochin estuary and Vambanad estuary from Kerala where in their study showed a salinity range between (0.5-32ppt) and (0.6-34ppt) repectively. Similar results were also reported from southern coastal estuary of India (Uthukrishnan and Umayoru, 2014; Srinivasan, 2014). Two-way ANOVA showed a significant difference between stations ($F=45.2$, $P<0.01$) and seasons ($F=339.9$, $P<0.01$) for salinity.

Normal Seawater has a salinity of 35g/l of chlorides and fresh water contain up to 5g/l. The salinity factor controls the distribution of fauna, flora and minerals. The normal salinity water shelters stenohaline forms (Brachiopods, crustaceans and lamellibranches), where as brackish water and fresh water shelters the euryhaline organisms and freshwater organisms respectively. Lower salinity during monsoonis due to the dilution of

precipitation inputs and higher concentration of salts during pre-monsoon is because of high evaporation of water bodies.

5.1.3 Temperature

Natural water temperature acts as a vital part in the distribution of the species by its extreme levels and its diurnal or seasonal variations that act as a significant factor in the life of a river estuary. Water temperature in the present study varied between 28^0C -32.5^0C and it was highest during pre-monsoon (31-32.5^0C) and it gradually decreased in the post-monsoon (29-30^0C) and monsoon (28-30^0C). Two-way ANOVA showed a significant difference between stations ($F=3.6$, $P<0.01$) and seasons ($F=123$, $P<0.01$) for temperature. Govindasamy *et al*. (2000) reported that surface water temperature is changed by the intensity of solar radiation, evaporation, fresh water influx. Hencemonsoon season witness heavy rainfall that reduces surface water temperature and high temperatures were observed during summer due to high solar radiation (Kannan and Kannan, 1996; Kannapiran *et al.,* 2008; Srinivasan, 2014). Leena, (2007) and Ranjith *et al*. (2017) also recorded similar range in temperature from Veli and Vempanad estuaries in Kerala. Similarly,water in Thengapattinam estuary in Kanniyakumari districts showed uniformity in the temperature range between 25.1 and 34.8^0C (Uthukrishnan and Umayoru, 2014) and surface water temperature fluctuated from 22°C to 32.8°C in the assessment of physico-chemical characteristics and pollution status of Tapi estuary (Surana *et al*., 2007). The above studies are in agreement with the temperature values obtained in the present study

5.1.4 Dissolved oxygen (DO)

Dissolved oxygen in the water is an excellent indicator of water quality. Dissolved oxygen in surface water plays a major role in the self-purification and maintenance of aquatic life. In the present study, dissolved oxygen values obtained in Ponnani estuary were very low due to pollution and nearly constant

for the three seasons (3.55-3.75 mg/L)and it was highest during monsoon (2.28 -5.08mg/L) and it gradually decreased in the post-monsoon(2.26-4.6mg/L) and pre-monsoon(3.15-4.57mg/L). Two-way ANOVA showed a significant difference between stations only ($F=7.4$, $P<0.01$) and not for seasons in the case of DO. Similar results were obtained by Thasneem *et al*. (2018) in Cochin estuary (<5mg/L). Depletion of DO was due to the upwelled propagation of high saline water mass and its increased residence time in the lower estuary. Solubility of oxygen in water decreases with increased temperature and salinity.

5.1.5 Biological oxygen demand (BOD)

BOD measurements were performed to evaluate the quality of coastal waters, rivers and lakes (Selanno, 2009, Jin *et al*., 2009; Ginting, 2012 and Waroy, 2013). Besides water quality, BOD is also carried out to measure the environmental contamination of soil and water due to the waste in landfills. In the present study, BOD concentrations ranged between (0.61-2.6mg/L) in all the seasons. Two-way ANOVA showed a significant difference between stations ($F=5.6$, $P<0.01$) and seasons ($F=7.3$, $P<0.01$) for BOD. Similar result was obtained by Thasneem *et al*. (2018) in Cochin estuary for BOD values (2.22-2.91mg/L).

5.2 Distribution of bioluminescent bacteria from different stations of Ponnani estuary

The present study focused on the distribution and abundance of bioluminescent bacteria in water, sediment, fish and invertebrates from different stations of Ponnani estuary. The present work is first of its kind from Ponnani estuary. Water and sediment colony forming unit (cfu) for the bioluminescent bacterial population density varied from 1 to 8 cfu /ml during the study period. Similar results were obtained for bioluminescent bacteria in Vellar estuary withan average count of 2-9 cfu/ml(Ramesh *et al*., 1989), 1-8 cfu/ml in water of Palk Strait of South coast of India (Srinivasan , 2014) and 3

– 5 cfu/ml in seawater and sedimentin mangrove ecosystem of Roach Park of Tuticorin (Arulmoorthy, 2014). Moreover, higher concentration of bioluminescent bacteria was also observed in a study conducted by Kannahi and Sivasankari, (2014) in the waters of Nagapattanam Seashore (160-200 cfu/ml).Ramaiah and Chandramohan, (1992; 1993), reported that toxicants lowers the occurence of bioluminescent bacteria. In the present study, the reduction in bioluminescent load may be attributed to the toxicants of Ponnani estuary.

In the present study, a strong seasonal variation was observed for the bioluminescent bacterial load. Where in, maximum density was observed in the pre-monsoon (7cfu/ml) followed by post-monsoon (3cfu/ml). However, in monsoon season bioluminescent bacteria was present only at one station (Jangar-1cfu/ml).

Sea water salinity and colony forming unit of bioluminescent bacteria are known to show a strong correlation (Abraham *et al*., 2003). In the present study too a strong positive correlation was obtained between sea water salinity and bioluminescent bacteria($r=0.6$, $n=37$, $p<0.001$). Similar results were observed by Ramaiah and Chandramohan, (1993); Abraham *et al*. (2003); Srinivasan, (2014). Seasonal variation was observed where in highest density of bioluminescent bacteria were observed in premonsoon and lowest in monsoon season (Ruby and Nealson, 1978; Ramesh *etal*., 1989). Salinity-oriented seasonal distribution of luminous bacteria in the waters of Vellar estuary had been recorded earlier (Ramesh *et al*., 1989). In contrast to the current findings, viable luminescent bacteria were highest in monsoon compared to post-monsoon and pre-monsoon period in waters of the Mystic River estuary due to rich nutrient concentrations during spring blooms in estuaries (Kathleen *et al*., 1984).

Sea water temperature and colony forming unit of bioluminescent bacteria are known to show significant positive correlation (Srinivasan, 2014). The present study also showed strong positive correlation between water temperature and bioluminescent bacteria (r=0.7, n=37 and P-value=0.0001).

5.3 Identification of the bioluminescent bacteria based on phenotypic characteristics and 16S rRNA sequencing

Phenotypic characteristics were affected by biochemical activities of each bacterial strain, therefore morphological, biochemical, and molecular characterizations were executed to confirm the identification of luminous isolates. Among total bioluminescent organisms, luminous bacteria were the most copious organisms existing in nature. They exist omnipresent in marine but less in freshwater and terrestrial environments. There are two common genera of bioluminescent bacteria in estuarine ecosystem namely *Vibrio* species and *Photobacterium* species. In the present study too, *Vibrio* species and *Photobacterium* species were the only genus isolated from bioluminescent bacteria. Many scientists unanimously revealed the fact that within genera Vibrio, *V.fisheri* is found either as free living or symbiotic in the marine environments (Aisha and Nuzhat, 2011; Thompson *et al*., 2017; Nagamani *et al*., 2017).

Vibrio and *Photobacterium* species were isolated from Andaman waters (Bayyana *et al*., 2018), Australian waters (Ferreira *et al.,* 2013), Eastern coast (Balan *et al*., 2013) that strongly agrees with the present study. However, few studies show the presence of bioluminescent *Vibrio* species only from the marine environments (Jabalameli *et al*., 2015; Neha *et al.,* 2017). In the present study pure genomic DNA isolated from the bioluminescent strains were subjected to 16SrRNA gene identification. 16S region of isolated DNA were amplified with 16SF and 16SR primers, electrophoresis of amplicon were

conducted which was subjected to BLAST search in genbank. . BLAST result showed highest similarity to *Vibrio fisheri* and *Photobacterium leiognathi.*

Different luminescent bacterial strain of *Vibrio* sp. viz: *V. harveyi, V. chigasi* and *V.gigantis* were isolated from different ecological niches by various researchers (Rashmi and Arti, 2017; Uzma *et al*., 2012). Variuos researchers identified and characterized bioluminescent bacteria by 16s rRNA identification *as V.harveyi* (Gomez *et al.,* 2004; Chiang *et al.,* 2006; Rashmi and Arti, 2017; Nagamani *et al*., 2017; Aisha and Nuzhath, 2011). Similar to the present study, molecular identification by 16s rRNA sequencing identified bioluminescent bacteria as *V.fischeri* from marine ecosystems of Dapoli beach, Kokan, Maharashtra (Neha and Arti, 2014), from Chah Bahar Port of Iran (Jabalameli *et al*., 2015), from the marine environment of Puerto Rico (Malave *et al*., 2010) and from Sagami bay, Japan (Tsukamoto *et al*., 2006).

The *P. leiognathi* was isolated from Marina beach of Tamilnadu by Ramesh and Mohanraju, 2017. Similar to the present study, *P.leiognathi* were identified by 16s rRNA sequencing from squid of Malaysia (Noor *et al*., 2014), from Caspian Sea (Mohseni and Salehghamari, 2014), from marine environment of Puerto Rico (Malave *et al*., 2010) and from Philippine squid and octopus host of Philippine marine environmemt (Naguit *et al*., 2011) and from Sagami bay, Japan (Tsukamoto *et al*., 2006).

5.4 Effect of different parameters on the luminescence of bioluminescent bacteria

Factors affecting growth of bioluminescent bacteria include temperature, salinity, nutrient concentration and pH level. In the present study, maximum light emission for bioluminescent bacteria was observed at 3-6% of sodium chloride, for pH 7, for glycerol concentration of 0.3% and incubation temperature of 30ºC. Similarly study by Alex, (2010), obtained a maximum

peak for NaCl at 3-6% and at pH 7. However the peak obtained in their study for glycerol concentration was different compared to the present study. Also, Fang *et al*. (2008) obtained a maximum peak for pH 7. But the peak obtained in their study for temperature (35^{o}C) and NaCl (2%) were different compared to the present study. Neha *et al*. (2017) also observed a maximum bioluminescence at pH 7, 3-6%NaCl and 0.3% glycerol concentration similar to the present study. However the peak obtained in their study for temperature (25^{o}C) was different compared to the present study.

5.5 Pollution status of Ponnani estuary through BLR (Bioluminescent ratio)

BLR is an indicator of variety of anthropogenic contamination of estuarine ecosystems. Many studies have described bioluminescent bacteria as indicator of marine water quality (Frischer *et al*., 2005; Sardo *et al*., 2008; Girotti *et al*., 2008; Perego *et al*., 2002). Bioluminescent ratio inhibition was observed after short-term exposure to a contaminant suggesting a physiological rather than a population response of native microbial communities (Frischer *et al*., 2005). Considerable seasonal variability had been revealed from the results associated with the BLR (Frischer *et al*., 2005). Bioluminescent ratios were maximum during the summer 0.25 and lowest during spring (0.03). Similar results were observed in the present study, wherein BLR decreased with increasing total bacterial abundance.

However study by Ramaiah and Chandramohan, (2003) showed that abundance of luminescent colonies in clean and polluted waters in summer is more influenced by seasonal variations, reaching a peak in summer and no change due to organic pollution and are affected by toxic substances causing inhibition of light emission leading to a decrease of its values. Sardo *et al*. (2008) described percentage of luminescent bacteria that implies pollution

status showing reduced values in contaminated areas(less than 4%), and greater values in unaffected waters(8-38%).

The BLR ranged between 0.5-7.2% with lowest ratio at Pallikadav (0.5%), Jangar (0.7%) and Harbour (1%) during post-monsoon, monsoon and post-monsoon respectively. Hence the three stations indicate increased pollution in these areas. The main source of pollution in Pallikadav is its connection with Canoli canal and Tirur-Ponnani rivers, which were loaded with domestic sewages. However Jangar and Harbour were contaminated due to the intense fishing activities and dumping of fish waste in these areas. Kootai showed the highest BLR (7.2%), while for the remaining 5 stations the values ranged from 2-3.8%. This indicates that the Ponnani estuary is in the onset of pollution and measures to control it need to be taken.

5.6 Antibiotic susceptibility pattern

Over usage of antibiotics may results in gaining resistance against *Photobacterium* and *Vibrio* species. Resistant strains may be developed with the development of new antibiotic because of the tendency of developing antibiotic resistance genes only when antibiotics were used in medicine and fields industry (Zulkifli, 2009). Different usage patterns of antimicrobial agents are expected to cause some impact on the distribution of antimicrobial resistance phenotypes and resistant determinants (Aarestrup, 2000). Rational use of antimicrobial agents and surveillance on antibiotic administration may reduce the acquisition of resistance by microorganisms of aquatic ecosystems.

In the present study, majority of *V.fischeri* and *P.leiognathi* isolates were resistant to cefotaxime and tetracycline. *P.leiognathi* were also resistant to gentamycin (27%) and were sensitive to aztreonam (91%) followed by chloraphenicol and ciprofloxacin (73%). *V. fischeri* were resistant to polymixin B (36%) and chloramphenicol (28%) and were sensitive to gentamycin (91%), aztreonam (82%), ciprofloxacin (64%) and polymixin B (64%).

Olayinka and Anthony, (2018) reported that 43% of the*Vibrio* isolates from Eastern Cape of South Africa were found to be resistant to tetracycline and (17%) resistant to cefotaxime that supports the present result. Similarly Andrianne *et al*. (2018) examined the antibiotic resistant profile of bioluminescent *Vibrio* species obtained from Philippine aquaculture systems. The bioluminescent *Vibrio* species also showed resistance to tetracycline.

Antimicrobial susceptibility of *Vibrio* species isolated from brackish water shrimp culture environment showed sensitivity to chloramphenicol (100%) and ciprofloxacin (100%) (Hossain *et al*., 2012). Also bioluminescent *Vibrio* species isolated from sea water of Izmir Gulf (Turkey) were found to be sensitive to gentamycin and chloramphenicol (Esra and Atakan, 2009). Various isolated Vibrio species from seafood were sensitive to ciprofloxacin and gentamycin (Chigozie and Samuel, 2015; Nsofor *et al*., 2014).

Bioluminescent isolates from shrimp farm environments of west Bengal were sensitive to chloramphenicol (100%) and gentamycin (82%) (Sengupta *et al*., 2003) that is comparable with the present investigation wherein 27% and 73% of *Photobacterium* was sensitive to gentamycin and chloramphenicol respectively.

Similar to the present study Emmanuel and Etinosa, (2017) reported the incidence of multiple drug resistant V*ibrio* species from abattoir effluents in Nigeria. *Vibrio* species in their study showed resistance to ampicillin (60-67%), trimethoprim (80-100%) and tetracycline (60-83%), However in the present study *Vibrio* species showed resistance to chloramphenicol (28%), cefotaxime (73%) and tetracycline(36%). According to Vande and Stobberingh (2000), indiscriminate application of antibiotics results in high incidence of multidrug resistance that will replace drug sensitive bacteria from antibiotic saturated environment.

5.7 Heavy metal susceptibility

Bioluminescence is employed as one of the indicators for monitoring aquatic samples with toxicants like pesticides, PCBs, alkanes, alcohols and heavy metals as it is easily operatable, highly responding and due to its low cost of performance (Unitzur *et al*., 2002; Weitz *et al*., 2002). Heavy metals like copper, cadmium, zinc, lead and mercury exist in nature and accumulate in food chains. Heavy metal acts as contaminant in ecosystem due to anthropogenic activities (De silva et *al*., 2012).

In the present study isolated bioluminescent bacterial isolates were sensitive to cadmium, cobalt and copper and resistant to lead and mercury. In contrary, in the study carried out by Neha and Arti, 2015, bioluminescent bacterial isolates showed resistance to $CuSO_4$ and sensitivity to $FeCl_3$, $HgSO_4$ and Pb $(NO_3)_2$.

Amutha and Shamini, (2014) examined metal tolerance of bioluminescent bacteria isolated from marine organisms where metal toxicity test was conducted by using zinc sulphate, copper chloride and Lead nitrate. Isolated bioluminescent bacteria showed resistance to all the three metals concluding its resistance to heavy metals, unlike the present study, was resistant only for lead. In contrast to the present observation studies have shown bioluminescent bacterial resistance to other metals other than lead and mercury (Uzma *et al*., 2012; Alex, 2010).

Luminescent bacterial strain analyzed during present investigation is extremely reactive to high concentrations of cadmium, cobalt and copper except for Pb $(NO_3)_2$ and $HgSO_4$ can be employed in quantifying their bioavailability in polluted water samples. Latest studies have reported recombinant GFP based bacterial biosensor responded to Pb2+ in the range of 50–400 μM (Chakraborty *et al*., 2008). Collectively, earlier and present studies indicates the necessity for development of lead and mercury specific luminescence based recombinant

bacterial strain which could provide a wide range for lead metal sensing and mercury metal sensing in terms of luminescence changes.

5.8 Symbiotic association

Bioluminescent bacteria often exist in symbiotic relationship with fish and squid. Bioluminescent bacteria identified in the current studies include *V.fisheri* and *P.leiognathi*. *V.fischeri* was obtained from the alimentary canal of *M. dobsoni* (50%) and light organ of *U. duvaucelii* (28%) and *P. leiognathi* from the light organ of *S. ruconius* (46%), *U. duvaucelii* (17%) and alimentary canal of *M. dobsoni* (30%). However, *V. fischeri* and *P.leiognathi* show a pathogenic association with *M. dobsoni* (Karunasagar *et al*., 1994; Thyssen *et al*., 1998; Venkateswara, 2015).

P. leiognathi and *S. ruconius* exist in symbiotic relationship with each other. Similar symbiotic association is seen between *V. fischeri* and *L. duvaucelii* (McFall-Ngai, 1999; Dunlap and Kita-Tsukamoto, 2006). *U. duvaucelii* and *S. ruconius* utilize the bacterial bioluminescence for counter-illumination camouflage during its night-time activity and in return *U. duvaucelii* and *S. ruconius* assist the bacteria by offering a microhabitat rich in nutrients.

The present investigation was supported by Mcfall-Ngai, (1999) who reported the presence of at least three species of luminous bacteria in squid species existing in symbiotic associations. Similar to our study symbiotic association of lolignid squid with *P. leiognathi* was observed by Mcfall-Ngai, (1999). However *V. fischeri* existed in symbiotic relation with sepiolid squids in their study. In squids, the light organs where the symbiotic associations occur are bilobed organs found ventrally within the mantle cavity, adjacent to the ink sac (Dunlap and KitaTsukamoto 2006). Squids attain their symbiotic luminous bacteria by parallel transfer from the surrounding seawater.

Various studies have shown symbiotic relationship of fish and bacteria and fish maintain their bacteria in light organs (Dunlap *et al*., 2007; Sparks *et al.*, 2005). Four bacterial species, *V. fischeri*, *P. kishitanii*, *P. leiognathi*, and *P. mandapamensis*, are the mainly identified bioluminescent symbionts of fish (Urbanczyk *et al*., 2007; Kaeding *et al*., 2007; Dunlap*et al*., 2007)**.** *V. fischeri* and *P. phosphoreum* are symbiotic bacteria that live in high densities in the specific organs of marine bioluminescent fishes and squid (Balan *et al*., 2013).

In the present study *S. ruconius* existed in symbiotic relationship with *P. leiognathi***.** Fish acquire their luminescent symbionts through horizontal transfer from the surrounding seawater (Dunlap and Kita-Tsukamoto 2006). According to Dunlap *et al*.(2004), specificity of the *P. leiognathi* and Leiognathid fish symbiosis is maintained at the species level in contrast to the individual level.

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CHAPTER 6
SUMMARY AND CONCLUSION

Luminescent bacteria are the widely distributed light emitting organisms that exist mostly in sea water and in terrestrial and fresh water environment to a greater extent. Light emission of bioluminescent bacteria was catalysed by luciferase enzyme. The study area, Ponnani estuary is a transition zone wherein mixing up of fresh water and marine water occurs.

In the present study physico-chemical parameters - pH, salinity, temperature, alkalinity, dissolved oxygen and biological oxygen demand, for water samples from different stations of Ponnani estuary were evaluated over two years (February 2014-January 2016) using standard methods (APHA, 2012 and Grasshoff, 1999). Salinity and temperature plays a major role in the growth of bioluminescent bacteria. In the present study, salinity and temperature ranged between 2-32ppt and 28-32.5^{0}C repectively. Stations having high salinity (Jangar, Pallikadav, Purathoor, Kootai, Chandappadi and Harbour) harbour greater bioluminescent load when compared to Kalur, Ishwaramangalam and Chamravottom kadav with lower salinity. High temperature areas (Pallikkadav, Jangar, Purathur, Harbour and Chandappadi) are known to harbour more bioluminescent bacteria. The DO varied between 1.02-5.69mg/L and BOD (0.72-3.56mg/L) showed more or less constant values for the nine stations.

Furthermore, THB (Total heterotrophic bacteria), BLB (bioluminescent bacteria) and (TVLO) Total *Vibrio* like Organisms were isolated from nine different stations of Ponnani estuary for duration of two years (2014-2016) and enumerated for distribution. Since our focus of study was bioluminescent bacteria, they were selected for further analysis. Bioluminescent bacteria were also isolated from *S. ruconius*, *U. duvaucelii* and *M. dobsoni* with an intend to know its symbiotic relationship with bioluminescent bacteria. Sea water complex agar was used as the media for its isolation.

Bioluminescent bacterial count in different stations of Ponnani estuary will provide distribution pattern and the pollution status of the estuary.

Estuarine water and sediment colony forming unit (cfu) for the bioluminescent bacterial population density varied from 1 to 8 cfu/ml during the study period. Maximum bioluminescence was obtained in pre-monsoon period followed by post-monsoon and monsoon period. Highest count of bioluminescent bacteria in water was obtained from Kootai (8cfu/ml) during pre-monsoon period, however, highest BLB was obtained in sediment samples from Jangar (7 cfu/ml) during the same season. Kalur, Chamravottom Kadav and Ishwaramangalam were entirely devoid of bioluminescent bacteria. Bioluminescent bacterial distribution in *S. ruconius*, *U. duvaucelii* and *M. dobsoni* was more or less uniform for the two years.

The bioluminescent isolates were biochemically characterized and molecularly identified by 16s rRNA sequencing as *Photobacterium leiognathi* strain UMTGB206 and *Vibrio fischeri*ES114. Optimum conditions required for the growth of bioluminescent bacteria were established by varying the concentration of sodium chloride, pH, temperature and glycerol concentration. Maximum bioluminescence for the isolated bacteria was observed at pH 7, sodium chloride of 3-6%, temperature of 30^0C and glycerol concentration of 1.5%.

*Photobacterium leiognathi*strain UMTGB206 and *Vibrio fischeri*ES114 were distributed in different estuarine samples unevenly. *P. leiognathi* strain UMTGB206 was observed in *S. ruconius* (141 cfu/mg) where they exist in symbiotic relationship followed by *M. dobsoni* (94 cfu/mg). The number is greatly reduced in sediment (12 cfu/mg) and water (9 cfu/ml) samples. Likewise greater number of *V. fischeri* inhabited *U. duvaucelii* (72 cfu/mg) where they exist in symbiotic association, followed by their high occurrence in *M. dobsoni* (132 cfu/mg), water (30 cfu/ml) and sediment (26 cfu/mg). In *M. dobsoni* they are known to exist as pathogens.

In the present study, pollution status of estuarine ecosystem was evaluated by determining BLR Ratio. Bioluminescent ratio is a measure of anthropogenic impact of estuarine ecosystem. The lowest BLR indicates pollution and was obtained at Pallikadav (0.5%) signifying highly polluted area followed by Jangar (0.7%) and harbour (1%). The main source of pollution in Pallikadav is its connection with Canoli canal and Tirur-Ponnani rivers, which were loaded with domestic sewages. However Jangar and Harbour were contaminated due to the intense fishing activities and dumping of fish waste in these areas. Kootai showed the highest BLR (7.2%), while for the remaining 5 stations the values ranged from 2-3.8%. This indicates that the Ponnani estuary is in the onset of pollution and measures to control it need to be taken.

*P. leiognathi*strain UMTGB206 and *V. fischeri*ES114 were tested for their susceptibility towards heavy metals like mercury, lead, cadmium, cobalt and copper. *P. leiognathi*strain UMTGB206 was reactive towards mercury, cadmium, cobalt and copper, but was resistant to lead. For the case of *V. fischeri*ES114, lead and mercury resistance were observed and are reactive towards cadmium, cobalt and copper. From these observations, *P. leiognathi*strain UMTGB206 were utilized for the construction of biosensor for monitoring heavy metal contaminants than *V. fischeri*ES114 since heavy metal sensitivity were maximum for *P. leiognathi* strain UMTGB206

Over usage of antibiotics may results in gaining resistance against *Photobacterium* and *Vibrio* species. Isolated strains of bioluminescent bacteria were tested for its antibiotic susceptibility pattern following the procedures of Baur *et al*. (1966). Eleven strains of *V.fischeri* and *P.leiognathi* had been tested to perceive their antibiotic susceptibility pattern. Different antibiotics had been tested for its susceptibility towards the isolated bioluminescent strains of *P.leiognathi* and *V.fisheri*. In this study, the highest resistance (73%) among total *V. fischeri* strains were observed to cefotaxime and tetracycline. 91% of

V. fischeri isolates were sensitive to gentamycin followed by aztreonam (82%), ciprofloxacin and polymixine B (64%). Multiple drug resistance was observed in selected *V.fischeri* strains wherein strain 7 showed highest MDR (CM-AZ-CX-TC). *P leiognathi* strains were found to be resistant to cefotaxime (91%), followed by tetracycline (45%) and gentamycin (27%). Ninety one percentage of *P. leiognathi* strains were found to be sensitive to aztreonam (91%) followed by chloramphenicol and ciprofloxacin (73%). Multiple drug resistance was observed in selected *P. leiognathi* strains wherein strain 5 showed highest MDR (CM-CX-CIP-TC). The development of MDR in bacteria isolated from Ponnani estuary might be due to anthropogenic wastes from different water sources reaching estuary.

From the present study it can be concluded that

- The Ponnani estuary harbours very low bioluminescent load due to the salinity and temperature fluctuations of the estuary. Salinity and temperature showed a strong positive correlation with distribution of bioluminescent bacteria as evidenced by the absence of bioluminescent bacteria in the low saline and temperature conditions.
- *P. leiognathi* and *V. fischeri* were isolated from Ponnani estuary that forms symbiotic association with *S. ruconius* and *U. duvaucelii* respectively.
- Antibiotic and heavy metal resistance were observed for isolated strains of bioluminescent bacteria of Ponnani estuary. This might be due to the increased level of heavy metal and antibiotics in estuarine water from different sources often developing resistance to particular microbes by long-term exposure.

- Bioluminescent bacteria signify pollution status of Ponnani estuary through BLR ratio. Hence using BLR as a pollution indicator, it can be stated that the Ponnani estuary is on the verge of pollution.

Owing to the economic significance of the Ponnani estuary, measures to control the pollution due to anthropogenic activities should be carried out.

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LIST OF PUBLICATIONS

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ANTIBIOTIC SENSITIVITY AND ENZYME PRODUCTION OF BIOLUMINESCENT VIBRIO SPECIES ISOLATED FROM SQUID AND PENAEID SHRIMP OF PONNANI ESTUARY, KERALA

Ramina, P.P*, Sabira, K.M, and Razia Beevi, M.
Department of Aquaculture and Fishery Microbiology, MES Ponnani College, Malappuram
**Email: mail2remie@gmail.com*

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Abstract: Luminous bacteria are the most abundant and widely distributed of the light emitting organisms and are found on marine fresh water and terrestrial environments. The review of literature reveals that most of the microbiological work from Ponnani estuary emphasized on the coliform load in the area. However a detailed study on the bioluminescent bacteria, its abundance and distribution is lacking. During the present work occurrence of bioluminescent bacteria in squid (*Loligo duvauceli*) and Penaeid shrimp (*Metapenaeus dobsoni*) from Ponnani estuary were studied. Objectives of the present study include Isolation and Identification of bioluminescent bacteria, antibiotic sensitivity studies and its enzyme production. The bioluminescent bacteria were isolated by using Sea water complex agar. The isolated strains were subjected to antibiotic sensitivity test (Kirby Bauer disc diffusion method) against nine different antibiotics. Isolates obtained from the squid were resistant to all nine antibiotics used for the test. Strains isolated from shrimp were highly sensitive only to two antibiotics gentamycin and chloramphenicol and are resistant to Amoxicillin, vancomycin and cefotaxime. The enzyme lipase produced by the *Vibrio* spp. were also isolated and purified.

Key words: Bioluminescent bacteria, Sea water complex agar, Antibiotic sensitivity, Lipase

INTRODUCTION

Luminous bacteria are most abundant and widely distributed of the light emitting organisms and are found in marine, freshwater and terrestrial environments. The most common habitats are free living species in the ocean as saprophytes growing on dead fish or meat as gut symbionts in the digestive tracks of marine fish and as light organ symbionts in the squid (Hasting *et al*., 1986).These bacteria are all gram negative motile rods and can function as facultative anaerobes (Bauman *et al*., 1983). Almost all luminous bacteria have been classified into three genera, Vibrio, Photobacterium and Xenorhabdus with most of the species being marine in nature (Campbell *et al*., 1989). Literature survey reveals that antibiotic sensitivity studies of luminous *Vibrio* species from hatcheries all over the world were sufficient, but the information regarding antibiotic sensitivity studies of luminescent bacteria from estuarine environment is lacking. So the present study focus about the antibiotic sensitivity studies of *Vibrio* species isolated from squid (*Loligo duvauceli*) and shrimp (*Metapenaeus dobsoni*) of Ponnani estuary. Antibiotics have been used to control luminous vibriosis in grow out ponds with little success. The principle drawback of antibiotic application is the development and spread of antibiotic resistant organisms. There are no universally acceptable pharmaceutical agents that are approved by FDA for treating infections in shrimp aquaculture, although studies are underway to improve disease, control and treatment (Reed *et al*., 2004) .Hence there is need to develop suitable chemotherapy to control luminous vibriosis.

Marine microorganisms which are halotolerant produce many enzymes to meet our therapeutic requirements. Bioluminescent *Vibrio* species produces many enzymes including lipase, esterase, chitinase, and asparaginase. It is believed that sea water ,which is saline in nature and chemically closer to the human blood plasma could provide microbial products, in particular the enzymes, that could be safer having no or less toxicity or side effects when used for

therapeutic applications to humans (Sabu, 2003).The bioluminescent bacterium *Vibrio fischeri* produces lipase enzyme under specific substrate concentration. Lipases have a dual role in catalyzing hydrolysis and synthesis of esters formed from glycerol and long chain fatty acids. These enzymes exhibit broad substrate specificity and degrade tweens and phospholipids often with positional, stereo and chain length selectivity (Jaeger *et al*., 1994). Lipases have been recognized as very useful biocatalysts because of their wide ranging versatility in industrial applications such as food technology, detergent, chemical industry, biomedical sciences (Jaeger and Reetz, 1998; Jaeger *et al*., 1999; Ghanem *et al*., 2000; Gupta *et al*., 2004). Information of lipolytic enzymes produced by marine *Vibrio* species from estuarine environment is scanty. Therefore, this research article also focuses about the production of lipase enzyme of *Vibrio* species isolated from squid and shrimp.

MATERIALS AND METHODS

Samples of squid and shrimp were collected aseptically from Ponnani estuary and brought to the laboratory for analysis. Presence of bioluminescent bacteria was detected by streak plate technique. The plates were then incubated for 24 hours at 37°C and at every six hours the appearance of luminescent colonies were observed. The distinct isolated luminescent colonies were marked while observing for luminescence and were further purified by sub-culturing in Sea Water Complex Agar plates. Species identification done with the help of Bergey's manual of Determinative Bacteriology (Holt *et al*., 1994).

Vibrio species isolated from estuarine squid and shrimp were subjected to antibiotic sensitivity testing against commonly used antibiotics with the help of Agar disc diffusion method of Baur *et al*., (1966). Different antibiotic discs (Chloramphenicol, Ciprofloxacin, Gentamycin, Azithromycin,Tobramycin,Erythromycin, Amoxicillin, Cefotaxime, vancomycin) with effective concentrations were placed over the plates swabbed with isolated *Vibrio* species in Muller Hinton Agar medium and incubated at 37°C for 24 hours and the zone of inhibition were measured.

Isolation, Purification and enzyme assay

Purification and enzyme assay for lipase was done by following the procedures of Ranjitha *et al.*, (2009). All strains were pre cultivated on Sea Water Complex Agar medium. For detection of lipolytic activity Zobell 2216E modified media was used. After inoculation, incubated at 30°C for 10 days. The total diameter, minus the diameter of the colony was considered to be proportional to the lipolytic activity rate. After 1-10 day incubation the halos or clear zones were measured. Cell free extract was made by inoculating 150 µl of culture into 200ml of sea water medium with substrate and incubated at 30°C for 3 days. The portion was centrifuged at 4°C for 30 min. The supernatant was filtered and the obtained cell free filtrates were used as the crude enzyme in the purification experiments and were purified by ammonium sulphate fractionation.

RESULTS AND DISCUSSION

During the present study *Vibrio* species were isolated from estuarine squid and shrimp of Ponnani. Phenotypic studies of bacterial isolates revealed that all the strains isolated were Gram negative, motile, rod shaped catalase and oxidase positive and halophilic. Sugar fermentation tests including Mannitol,Glucose and Sucrose were tested for bacterial isolates and only positive result was obtained for mannitol. Srinivasan and Ramaswamy(2009) reported positive result for D-Glucose without gas production .Many studies on the taxonomy of Marine luminous bacteria both free living and symbiotic have been carried out (Hendrie *et al.*,1970; Reichelt and Baumann ,1973). Lee and Ruby, (1994) reported that squid receives a significant input of cells of symbiotic *V. fischeri* from the habitat.

Sensitivity pattern of luminous bacteria from squid and shrimp to selected antibiotics

Antibiogram pattern showing varying degrees of susceptibility were given in Table 1.

Sensitivity pattern for luminous bacteria from squid and shrimp

Isolates obtained from squid were resistant to all nine antibiotics which were used for the test.

Table 1. Antibiogram pattern showing varying degrees of susceptibility

Antibiotics	Squid		Shrimp			
			Vibrio spp 1		*Vibrio* spp 2	
	Zonedia	S,R or I	Zonedia	S,R or I	Zonedia	S,R or I
Chloramphenicol	12	R	22	S	22	S
Ciprofloxacin	15	R	23	S	21	S
Gentamycin	12	R	16	S	15	S
Azithromycin	15	R	23	S	23	S
Tobramycin	12	R	16	S	14	I
Erythromycin	13	R	19	I	16	I
Amoxicillin	12	R	12	R	12	R
Cefotaxime	14	R	14	R	14	R
Vancomycin	9	R	9	R	9	R

Although the antibiotic sensitivity studies of bioluminescent bacteria from shrimp and shrimp hatcheries were abundant, related studies on antibiotic resistant pattern of bioluminescent *Vibrio* spp isolated from squid was lacking.

The luminescent *Vibrio* species isolated from shrimp were highly sensitive to only two antibiotics namely gentamycin and chloramphenicol. Studies related to antibiotic resistance pattern obtained from bioluminescent *Vibrio* spp from estuarine shrimp is lacking. But studies from shrimp hatcheries were sufficient. Devika and Jayabalan (1996) revealed a similar result to that of the present observation. Ciprofloxacin and azithromycin are also sensitive in border lines for *Vibrio* species 1. Tobromycin and erythromycin show intermediate level for *Vibrio* species 1 while tobromycin were sensitive in border line for the *Vibrio* species 2. Similar result were shown by Srinivasan and Ramaswamy, (2009). Amoxicillin, Vanco-mycin and Cefotaxime are resistant to both the *Vibrio* species isolated from shrimp. The incidence of antibiotic resistance was higher in amoxicillin, vancomycin and cefataxamine than other antibiotics used in this study. Resistance of amoxicillin was also focused by Srinivasan and Ramaswamy (2009). Adeleye, *et al.*, (2008) and Jun *et al.*, (2003) while studying the antimicrobial susceptibility test showed that all the *Vibrio* isolates (100%) were resistant to amoxicillin, augmentin, chloramphenicol and nitroforantoin. Manjusha *et al.,* (2008) focused multiple resistance patterns of *Vibrio* species to gentamycin, nitrofurantoin, tetracycline, augmentin, chloramphenicol, amoxicillin, ofloxacin, cotrimozazole, ceftriazone, and ciprofloxacin.

Screening of Lipase Enzyme

In ZoBell modified media, the isolated *Vibrio* spp.Showed 27 mm, 29 mm, 25 mm of halos respectively for one strain isolated from squid and two from shrimp. These halos indicate little lipase activity. Similar study were done by Ranjitha *et al.*, (2009) with *V. fischeri* in ZoBell modified media and showed halos of 40mm indicating significant lipase activity than the present work. Bruni *et al.*, (1982) reported that most strains of *Pseudomonas* sp. NCMB 1082 split all tweens, tributyrin, but not triolein of the growth media. Lipase production at the end of log phase was reported (Sarkar *et al.,* 1998; Kiran *et al.*, 2008; Ramani *et al.*, 2010). Kouker and Jaeger (1987) detected a plate assay for bacterial lipase in a medium containing triacylglycerol and the fluorescent dye rhodamine B. The low lipase activity obtained in the present study may be due to difference in substrate ingredients.

Purification of lipase enzyme

After 3 day incubation at 30°C in sea water containing glycerol, highest yield of enzyme was obtained. The purification procedures were performed at 4°C in order to reduce the loss of enzyme activity. After dialysis, the specific activity of lipase containing dialysate was increased than that in culture supernatant fluid. Nawani and Kaur (2000) used phenyl sepharose chromatography as a single step process that yielded a high active and pure lipase. (Ranjitha *et al*., 2009 and Khunt *et al*., 2012) reported similar result for *Vibrio* species. In the present study the quantity of enzyme obtained by enzyme activity assay is too low. The minimum amount may be due to enzyme loss during purification procedures.

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ANTIBIOTIC RESISTANCE PATTERN AND ESTERASE PRODUCTION OF BIOLUMINESCENT *VIBRIO SPECIES* ISOLATED FROM *LEIOGNATHUS*

Ramina PP*, Shafeeque Mohammed CK and Razia Beevi M

Department of Aquaculture and Fishery Microbiology, MES Ponnani College, Malappuram

*Email: mail2remi@gmail.com

Abstract

The aim of this study is to evaluate antibiotic resistance pattern and esterase production of bioluminescent bacteria isolated from the gut of leiognathus.Isolated bioluminescent vibrio species were subjected to Kirby baur disc diffusion method to obtain the antibiotic resistance pattern to selected antibiotics (Chloramphenicol, Ciprofloxacin, Gentamycin, Azithromycin, Tobramycin,Erythromycin Amoxicillin, Cefotaxime and Vancomycin) and were resistant to the antibiotics vancomycin,cefotaxime and amoxicillin. Marine microbes especially bioluminescent vibrios synthesise various enzymes of therapeutic importance like esterase, lipase, aspariginase and chitinase.In this contest , the present study also focus on the isolation ,purification and enzymatic assay for esterase.The isolated strains were cultured in ZoBell 2216E Modified Media to indicate esterase production and enzyme was purified from the concentrated culture supernatant and enzymatic assay were done by using purified enzyme.

Key words: antibiotic resistance, esterase, bioluminescent vibrios.

Introduction

Luminous bacteria are most abundant and widely distributed of the light emitting organisms and are found in marine, freshwater and terrestrial environments. The most common habitats are free living species in the ocean as saprophytes growing on dead fish or meat as gut symbionts in the digestive tracks of marine fish and as light organ symbionts in the squid (Hasting *et al*., 1986).These bacteria are all gram negative motile rods and can function as facultative anaerobes (Bauman *et al*., 1983).Almost all luminous bacteria have been classified into three genera, *Vibrio, Photobacterium* and *Xenorhabdus* with most of the species being marine in nature (Campbell *et al*., 1989).especially bioluminescent bacteria occur as gut organ symbionts in leiognathus.These strains were isolated from the fish by using SWCA and used for further analysis.Antibiotic resistant pattern of isolated strains to

selected antibiotics were done as this vibrio species causes major infection and reduce Leiognathus production.

Marine microorganisms which are halo tolerant produce many enzymes to meet our therapeutic requirements. Bioluminescent *Vibrio* species produces many enzymes including lipase,esterase, chitinase, and asparaginase. It is believed that sea water, which is saline in nature and chemically closer to the human blood plasma could provide microbial products, in particular the enzymes, that could be safer having no or less toxicity or side effects when used for therapeutic applications to humans (Sabu, 2003). The bioluminescence bacterium *Vibrio fischeri* produces esterase enzyme when the medium contained specific substrate. The lipase and esterase were purified from the concentrated culture supernatant. Esterases are distinguished from lipases in that their action is generally restricted to short-chain fatty acids. Esterases catalysis of a large number of aliphatic and aromatic esters. Although the molecular and catalytic properties of this protein from mammalian sources have been well studied, only limited investigations have been made in to properties of isolated microbial esterases. Because of the potential food applications, the general economic attractiveness of extracellular enzyme is higher than the intracellular microbial industrial enzymes (Meghji *et al.,* 1990). Information of lipolytic enzymes produced by marine *Vibrio* spp is particularly limited. Therefore, the objective of this study also focuses on the esterase production of *V. fischeri* isolated from leiognathus and on the purification and partial characterization of its esterase.

Materials and Method

Leiognathus fish samples were collected aseptically from Ponnani coastal area and brought to the laboratory for analysis. Presence of bioluminescent bacteria were detected by plating on sea water complex agar plates. The plates were then incubated for 24 hours at 37^0C and at every six hours the appearance of luminescent colonies were observed. The distinct isolated luminescent colonies were marked while observing for luminescence and were further purified by subculturing in Sea Water Complex Agar plates.Species identification done with the help of Bergey's manual of Determinative Bacteriology(Holt *et al*., 1994).*Vibrio* species isolated from leiognathus were subjected to antibiotic sensitivity testing against commonly used antibiotics with the help of Agar disc diffusion method of Baur *et al*., (1966). Different antibiotic discs(Chloramphenicol, Ciprofloxacin, Gentamycin, Azithromycin, Tobramycin, Erythromycin, Amoxicillin, Cefotaxime, vancomycin) with effective concentrations were placed over the plates swabbed with isolated

Vibrio species in Muller Hinton Agar medium and incubated at 37^0C for 24 hours and the zone of inhibition were measured.

Isolation, Purification and enzyme assay

Purification and enzyme assay for esterase was done by following the procedures of Ranjitha *et al.*, (2009). All strains were pre cultivated on Sea Water Complex Agar medium. For detection of lipolytic activity Zobell 2216E modified media was used. After inoculation, incubated at 30^0C for 10 days. The total diameter, minus the diameter of the colony was considered to be proportional to the esterase activity rate. After 1-10 day incubation the halos or clear zones were measured. Cell free extract was made by inoculating 150 µl of culture into 200ml of seawater medium with substrate and incubated at 30^0C for 3 days. The portion was centrifuged at 4^0C for 30 min. The supernatant was filtered and the obtained cell free filtrates were used as the crude enzyme in the purification experiments and were purified by acid precipitation

Results and Discussion

During the present study *Vibrio* species were isolated from Leiognathus obtained from ponnani coastal area. Phenotypic studies of bacterial isolates revealed that the strains isolated were Gram negative, motile, rod shaped catalase and oxidase positive and halophilic. Sugar fermentation tests including Mannitol, Glucose and Sucrose were tested for bacterial isolates and only positive result was obtained for glucose. Srinivasan and Ramaswamy(2009) reported positive result for D-Glucose without gas production .Many studies on the taxonomy of Marine luminous bacteria both free living and symbiotic have been carried out (Hendrie *et al.*,1970; Reichelt and Baumann ,1973).Lee and Ruby(1994) reported that habitats of Leiognathus must receive significant input of cells of symbiotic *vibrio* species.

.Sensitivity pattern of luminous bacteria from Leiognathus to selected antibiotics

Antibiogram pattern showing varying degrees of susceptibility were given in Table 1.

Table 1. Antibiogram pattern showing varying degrees of susceptibility

Antibiotics	**Zone diameter**	**S,R and I**
Chloramphenicol	20	S
Ciprofloxacin	22	S
Gentamycin	19	S
Azithromycin	25	S
Tobramycin	13	I
Erythromycin	16	I
Amoxicillin	-	R
Cefotaxime	-	R
Vancomycin	-	R

Sensitivity pattern for luminous bacteria from Leiognathus

Isolates obtained from Leiognathus were resistant to Amoxicillin,Cefotaxime and Vancomycin. Although the antibiotic sensitivity studies of bioluminescent bacteria from shrimp and shrimp hatcheries were abundant, related studies on antibiotic resistant pattern of bioluminescent *Vibrio* spp isolated from Leiognathus was lacking.The luminescent *Vibrio* species isolated from Leiognathus were highly sensitive to antibiotics namely gentamycin ,Ciprofloxacin, chloramphenicol and Azithomycin . Devika and Jayabalan (1996) revealed a similar result to that of the present observation.Tobromycin and erythromycin show intermediate level for *Vibrio* species .Similar result were shown by Srinivasan and Ramaswamy,(2009).The incidence of antibiotic resistance was higher in amoxicillin,vancomycin and cefotaxime than other antibiotics used in this study. Resistance of amoxicillin was also focused by Srinivasan and Ramaswamy (2009). Adeleye*, et al*., (2008) and Jun *et al.*, (2003) while studying the antimicrobial susceptibility test showed that all the *Vibrio* isolates (100%) were resistant to amoxicillin, augmentin, chloramphenicol and nitroforantoin. Manjusha *et al.,* (2008) focused multiple resistance patterns of *Vibrio* species to gentamycin, nitrofurantoin, tetracycline, augmentin, chloramphenicol, amoxicillin, ofloxacin, cotrimozazole, ceftriazone, and ciprofloxacin.

Screening of esterase Enzyme

In ZoBell modified media, the isolated *Vibrio* spp.Showed 29 mm of halos after 10 days of incubation which indicates that the strain showed significant esterase activity. It actively splits tween 20 than tributyrin as good substrate. Similar study were done b y Ranjitha *et al*., (2009) with *V. fischeri* in ZoBell modified media and showed halos of 36mm indicating significant esterase activity than the present work. Bruni *et al*., (1982) reported that most strains of *Pseudomonas* sp. NCMB 1082 split all tweens, tributyrin, but not triolein of the growth media. Wood et al., (1995) found that esterolytic activity at moderate temperatures (eg. 20ºC - 30ºC) was high with structurally diverse estersubstrates including aliphatic, cyclic and sugar esters.3.3.2 pH .This test found that the best buffer was phosphate buffer at optimum pH for the production of esterase enzyme. In order to favour the secretion of extracellur lipolytic enzymes the effect of temperature and carbon source has been studied by Dominguez et al., (2007). The esterase had narrow substrate specificity; it exhibited a high activity only on compounds having both polyoxyethylene and fatty acyl moieties such as tweens (Tomioka, 1983)

Purification of esterase enzyme

The crude extract was precipitated by slowly adding 1N HCl at 4ºC with stirring until pH 4.3 was attained. The precipitate containing the esterase activity was collected by centrifugation 10,000 rpm for 30 min. The pellet was dissolved in 10mM phosphate buffer and brought buffer pH 7.5 by the addition of 1N NaOH and are subjected to enzyme assay.Ranjitha et al.,2009 reported similar result for *vibrio* species.They also discussed about further purification procedures including ion exchange chromatography and gel filtration chromatography. Separation on Sephacryl S-300 gave a symmetrical single peak (Kakariari et al., 2000). Smacchi et al., (2000) reported that the chromatography in both DEAE-cellulose and on Sepharose 6B resolved the esterase activity into one peak corresponding to the major protein peak

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