SEASONAL VARIATION IN HUMAN PATHOGENIC BACTERIA OF TEREKHOL ESTUARY

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Submitted in partial fulfilment of Master

of Sciences in Marine Microbiology

by

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Under the Supervision of

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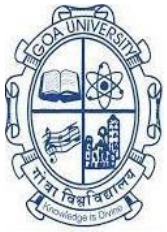

Goa University

Date: April 2023

Examined by: Seal of the School

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, “Seasonal variation in Human Pathogenic Bacteria of Terekhol Estuary” is based on the results of investigations carried out by me in the M.Sc. Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University under the Supervision/Mentorship of Dr. Varada S. Damare and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation. I hereby authorize the University authorities to upload this dissertation on the dissertation repository or anywhere else as the UGC regulations demand and make it available to any one as needed.

Aditi A. Palyekar
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COMPLETION CERTIFICATE

This is to certify that the dissertation report "Seasonal Variation in Human Pathogenic Bacteria of Terekhol Estuary" is a bonafide work carried out by Ms. Aditi Abhay Palyekar under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of Master of Science in the Discipline Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University.

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TABLE OF CONTENTS

INTRODUCTION 1.1 Introduction to Marine Microbiology

Marine Microbiology studies make up a very important part to study the earth's environment as a large part of the earth constitutes of the oceanic ecosystem. About 70 % of the earth's surface is covered by water which is even greater than the land mass. Microbes play a very important role in the oceanic ecosystem as they assist in various chemical as well as physical processes (Woods et al. YEAR). Human activity impacts marine ecosystems at a worldwide scale. Despite their importance as key players in important processes, the strain caused to microorganisms have been greatly neglected. Microbial communities respond to nutrients and chemical pollution by increasing cell numbers. There are important changes in community composition, increase in diversity and high temporal variability. These changes prove that the modification of the environmental conditions cause phylogeny stress. Altered microbial communities in human impacted marine environments will successively have harmful effects on human health (Nogales et al. 2011).

1.1.2 What is an estuary?

Estuaries measure among the foremost biologically productive ecosystems on the planet critical to the life cycles of fishes, different aquatic animals and also the creatures that go after them. The term 'ESTUARY ` comes from the Latin word easts which means heat, boiling, or tide. Specifically, *aethalium means* tidal. Thus, the Oxford

Dictionary defines estuary as "the tidal mouth of a great river, where the tide meets the current.". *Webster's Dictionary* is more specific with the definitions "(a) a passage, as the mouth of a river or lake where the tide meets the river current; more commonly, an arm of the sea at the lower end of a river; a firth. (b) In physical geography, a drowned river mouth, caused by the sinking of land near the coast." Perhaps the most widely quoted definition of an estuary in the scientific literature is given by Pritchard (1967):

"An estuary is a semi enclosed coastal body of water which has a free connection with the open sea and within which sea water is measurably diluted with fresh water derived from land drainage."

This definition still had a few drawbacks to address those, Fair bridge (1980) gave a more accurate definition for the term estuary;" An estuary is an inlet of the sea reaching into a river valley as far as the upper tidal rise."

According to Fair bridge an estuary can be divided into three different parts

1. Marine or lower estuary, this part has a free connection to the open sea.
2. A middle estuary, which is subjected to strong salt and freshwater mixing.
3. An upper or fluvial estuary, this part is known for the presence of fresh water which is subjected to daily tidal activity.

1.1.3 Estuarine ecosystem

Physical settings like climate, geomorphology, presence or absence of water salts etc. are the primary determinant of the kind of processes which will develop in an exceedingly specific location. The spirited discussion of what makes an estuary an extremely productive ecosystem is therefore a very exciting reason to study the ecology of an estuarine ecosystem. Four major reasons for this high productivity are listed

I. Varieties of primary production units that guarantee most use of the abundant sunlight (marsh grasses, benthic algae, phytoplankton)
II. Ebb and flow of water movements ensuing from recurrent event actions
III. Overabundance of essential nutrients
IV. Speedy regeneration and conservation of nutrients because of the activity of microorganisms and filter feeders. (Schalke et.al.2016).

1.1.4 Impactof humans on the Marine Ecosystem

Humans have lived in and around estuaries for tens of thousands of years. Early peoples harvested the wealthy primary and secondary productivity of estuaries. It is thought that the made resources of the coastal margin provided a very important energy grant that allowed the amendment from village based agricultural societies to the complicated social structure of civilization. Throughout the Holocene epoch, humans congregated close to the coast and lots of the world's current massive cities developed close to the estuaries. These areas were a vital supply of food, and rivers provided vital routes for navigation. Lower stream valleys supported wealthy agricultural areas. Humans have made changes to estuarial areas since the start of civilization.But huge changes in estuaries largely occurred within the twentieth century once human populations grew dramatically within the coastal zone. The act has physically modified coastal systems by debilitating and filling areas and by dredging channels for navigation, drainage, and access to minerals like oil. Industrial, agricultural, and concrete growth have introduced several toxic materials like serious metals and pesticides that poison estuarine organisms and nutrients and organic matter that junction rectifiers to eutrophication. As human impact grew, thus did the study of those impacts and efforts to scale back or mitigate them.

1.1.4.1 Changes in Temperature Salinity and pH of estuarine waters

a) Temperature:It is very important to know the temperature of the sea water as it affects the distribution of life in the ocean, the exchange of gases, and the survival of organisms. It also controls the rate at which organisms metabolize or break down food into usable nutrients. The ocean surface temperatures range from 0–30-degree C. The water temperature varies with the depth, geographical

location, season, elevation and climatic conditions and is influenced by stream flow, stream side vegetation, groundwater inputs, industrial effluents, etc.

b) Salinity:It is the term used to define the total amount of dissolved inorganic salts in the ocean. (Definition: - The salinity of seawater is defined as the total amount by weight of dissolved salts in one kilogram of seawater.) The average salinity of seawater is typically about 35%. Salinity varies globally across the surface and with the change in the depth. Evaporation, formation of sea ice increases the salinity of seawater while rainfall, run off, melting of sea ice decrease the salinity

c) pH: pH is often described as the 'MASTER VARIABLE' in sea water and other aquatic systems since many properties, processes and reactions are pH dependent. However, the sea water pH is usually considered as a part of the carbon dioxide system which provides the major pH buffer in seawater. Sea water is normally slightly basic with a surface water pH of 8.1-8.2, but as the ocean continues to absorb more CO2, the pH decreases and the ocean becomes more towards the acidic side.

1.1.4.2 Dissolved oxygen (DO)

It is the measure of how much oxygen is dissolved in the water. The amount dissolved in seawater can tell us a lot about its water quality. The concentration of dissolved oxygen in ocean water is typically between 7 to 8 milligrams per litre (mg/L). Water temperature and biological processes are the major factors affecting the dissolved oxygen concentration of seawater. Photosynthesis, stream flow, and aeration increase the DO of seawater, whereas respiration, decomposition decrease the DO of the sea.

The DO of seawater is estimated by Winkler's Method. This method is a type of iodometric titration which oxidizes iodine ion to iodine using manganese as a transfer

medium. This iodine is then titrated against sodium thiosulphate, the end point of redox titration is indicated with starch as it forms a complex compound with iodine resulting in a blue colour. Change from blue to colourless marks the end point of the titration.

1.1.4.3 Chlorophyll estimation

The concentration of chlorophyll is an indicator for the number of photosynthetic plankton or phytoplankton present in the ocean. Individual samples of chlorophyll are measured by filtering a known amount of sample through a glass fibre filter. The filter paper itself is used for the analysis. The chlorophyll is extracted using 90% acetone and then a spectrophotometric analysis is carried out to measure the absorbance at 665 and 750 nm wavelengths.The first spectrophotometric methodology for Protoctista pigments in brine was delineated by a semiotician with Thompson (J. Mar. Res. 11: 156, 1952), some enhancements to the present methodology were urged by Parsons and designer (J. Mar. Res., 21: 155, 1963) and new spectrophotometric equations by Jeffrey and Humphrey (Beachem. Physiol. Franzen. 167: 191, 1975).

1.1.4.4 Dissolved nutrients

Probably the foremost vital property of sea water in term of its result on the life forms within the oceans is that of the concentrations of dissolved nutrients. Nitrogen, phosphorus and silica as they play a crucial role in enhancing the primary productions of the marine organismsNutrients within the ocean are cycled by a method called biological pumping, whereby living being extract the nutrients out of the surface water and mix them in their organic matrix. Then once the plants die, sink and decay, the nutrients are turned back to their dissolved state at deeper levels of the ocean. The abundance of nutrients determines how fertile the oceans are. A measure of this fertility is the primary production, that is, the rate of fixation of carbon per unit of water per unit time.

a) NITROGEN: Dissolved nitrogen gas is hardly used by all marine organisms and is therefore converted to nitrite or nitrate by nitrogen cycle. The total nitrogen concentration in the ocean is 0.5 p.m. a very small fraction is in nitrite or nitrate derived from decomposition of organic matter on land and supplied to the oceans by rivers, rainfall by lightning discharge and as fallout from industrial pollution. Also, acid rain is a significant source of nitrite in oceans •Nitrile is produced due to excretion of nitrile due to incomplete assimilatory reduction of nitrate by phytoplankton and chemoautotrophic ammonium oxidation.

b) PHOSPHORUS: Phosphorus is present in the ocean water in the form of dissolved inorganic phosphates, it affects the water quality by causing excessive algal growth. Phosphorus is normally present in surface water at a rate of only 0.02 ppm. Phosphate in seawatercomes from a range of sources, naturally by erosion of rocks and also due to fertilizers that wash away from the crops in the rain. Many other human activities cause phosphate pollution in water like the industries catering to chemical fertilizers, pulp and paper industry etc.

c) SILICA: The solid crust of earth contains 80-90 % silicates or silicates plus other compounds of silica or silicon dioxide. Water moving over and through natural deposits will dissolve a small amount of various silicate minerals making it the most common contaminant of most ocean waters. Silica is present in the lowest concentration among the other mentioned nutrients. Silica is an essential nutrient for many marine organisms that produce their skeleton with the help of silica, for example phytoplankton's.

1.1.4.5 Human pathogenic bacteria

Invisible to the optic, there's an abundant world of microbes living within the ocean with a complexness and variety that rivals all alternative life on Earth. They include bacteria, viruses, archaea, protists fungi etc. 90% of the weight would be of microbes if all marine organisms are weighed. Just because these microbes can't be seen doesn't mean they're unimportant.Microorganisms are normally the motors of environments that in any case wouldn't approach the food and supplements they have. a few are the guardians of sound environments, improvement the expanse of waste and inconsistently protective against illness as opposed to spreading it. (Hall.et.al 2019)

Coastal square areas are wedged through pollution inputs because of changes in land use and geophysical science, with large amounts of our wastes coming into on a dayto-day basis. Ocean and estuarine ecosystems will so impact the extent to which human exposure to microorganism pathogens embrace each marine-indigenous pathogens and outwardly introduced microorganism contaminants.

Human pathogenic organism risks are greater in locations with high population densities or wide business enterprise, and therefore the volume of fresh water and coastal discharges is important to be analysed. Unsafe microorganism levels in coastal waters may end up in shellfish harvest home limits, fish kills, and, if unmarked or disregarded, health problems in individuals and different creatures. (Corned et.al 2019)

MacConkey agar may be a selective and differential media used for the isolation and differentiation of non-fastidious gram-negative rods, particularly members of the bacteria family and therefore the genus *Pseudomonas etc*. The inclusion of antimycotic agent and digestive juice salts within the media stops the expansion of gram-positive microorganism and fastidious gram-negative microorganism, like *Neisseria* and Pasteurella. The tolerance of gram-negative eubacterium to digestive juice is part a

result of the comparatively bile-resistant outer membrane that hides the bile-sensitive cytoplasmic membrane (Mikado, 1996). different species-specific bile-resistance. Thiosulfate-citrate- bile salts -sucrose agar or TCBS agar is a selective media used to isolate *Vibrio* species. Sodium thiosulphate and citrate are present in high concentrations which inhibit the growth of *Enterobacteriaceae. Thymol* blue and bromothymol blue are present to detect pH changes.

Salmonella Shigella (SS) Agar is moderately selective and differential medium for the isolation, cultivation and differentiation of *Salmonella* spp. and some strains of *Shigella* spp. SS Agar is a modification of the Deoxycholate Citrate Agar. It is recommended for testing clinical specimens and food testing for the presence of Salmonella spp. and some *Shigella* spp.

Zobel marine Agar is used for the isolation and enumeration of marine bacteria as it has the composition that closely mimics the composition of the sea water which allows the bacteria to grow abundantly outside its natural habitat.

1.2. AIMS AND OBJECTIVE

AIM: To study the variation in the prevalence of human pathogenic bacteria with respect to time and study of various physicochemical parameters of the Terekhol estuary.

OBJECTIVES:

- To examine the existence of human pathogenic bacteria.
- To enumerate human pathogenic bacteria in the estuarine waters
- To determine variation in their population with time
- To correlate the abundance of these bacteria with physicochemical parameters of the estuarine waters.

MATERIALS AND METHODS

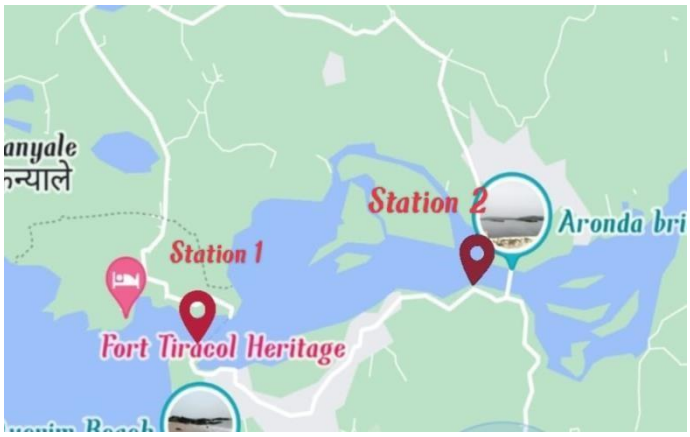
2.1 Site study and sampling

Two locations along the Terekhol river estuary were selected for sampling of water.

Location 1:15.7190802” N, 73.6928307” E situated near the mouth of the estuary.

Location 2:15.7241878” N, 73.7194422” E situated further along the river.

Fig.1. Location for sampling.



Sampling: Surface water was first collected in a bucket.The clean plastic sampling bottles were first rinsed with the water from the sampling location and then filled with the same water. Bottles were brought to the lab within 2 hours of sampling for further analysis. Water was also collected in glass stoppered bottles carefully without any mixing or formation of bubbles and fixed immediately with Wrinkler’s A and B reagents for DO analysis. Temperature was also measured soon after collection.

Table 1: Sample numbers with the sampling dates

SAMPLE NUMBER	SAMPLING DATE
1	22-September 2022
2	13-October 2022
3	18-October 2022
4	09-November 2022
5	22-November 2022
6	08-December 2022
7	14-December 2022
8	05-January 2023
9	21-January 2023
10	03-February 2023
11	15-February 2023
12	09-March 2023
13	20-March 2023

2.2 Detection of Temperature, Salinity and pH

2.2.1 Temperature

Temperature of each water sample was checked on site using a mercury thermometer.

2.2.2 Salinity

Several drops of the seawater sample were placed on the angled prism of the refractometer. Clear plate was sealed on top of it (ensuring no air bubbles were trapped). The refractometer was pointed towards a direct light source, and observed through eyepiece, and reading was noted down.

2.2.3 pH pH of each seawater sample collected was measured using pH meter (EUTECH

INSTRUMENTS, pH 700 pH/mV/ºC/ºF meter) in the laboratory.

2.3 Estimation of chlorophyll

Five hundred ml seawater was filtered through GF/F filter paper. A drop of $MgCO_3$ was added while filtering. The filter paper was then placed in an amber-coloured glass vial. Ten ml of 90% acetone was poured inside a vial containing the filter paper. Filter paper was crushed gently and kept undisturbed for 24 hours in the refrigerator. Next day samples were analysed spectrophotometrically at 665 nm, then 2 drops of HCL acid was added. Absorbance was measured at 750 nm. Readings were recorded and calculations were done.

2.4 Estimation of Dissolved oxygen

DETERMINATION OF REAGENT BLANK: 50 ml of distilled water taken in conical flask to that 1 ml of 50% H2SO4 + 1ml alkaline iodide (Winkler's B) + 1ml manganous chloride reagent (Winkler's A) was added. mixed thoroughly to avoid precipitation. 1 ml of starch was added (if blue colour was developed then titration needed to be carried out further, there was no blue colour.)

STANDARDISATION OF THIOSULPHATE SOLUTION: Solution was prepared same as prepared for blank. 10 ml of 0.01N potassium iodate solution was added. Solution was mixed and kept in dark for 3min to liberate iodine. Liberated iodine was titrated against thiosulphate till the solution turned pale yellow. 1ml starch was added and titration was counted. till the colour changed from blue to colourless (should remain colourless for 30 sec approx..). This was carried out in triplicates to obtain mean burette reading.

2.5Estimation of nutrients

2.5.1 Nitrate

Different concentrations of nitrate standard solution (0.069 g of anhydrous sodium nitrite ($NaNO_3$) are dissolved in 100 ml distilled water) i.e., 0,2,7.7,10 µm was prepared. All the samples from the locations were taken in test tubes. 25 ml of the standard concentration and 25 ml of sample were added to test tubes. 1 ml of Sulphanilamide was added to each tube and mixed and 1 ml of N (1-naphthyl)ethylenediamine dihydrochloride was added. The tubes were shaken and the azo dye was allowed to form for 15 min. The absorbance was measured at 540 nm and standard graph was plotted and calculation was done based on the equation of the line obtained.

2.5.2 Phosphate

Standard solution of known concentration of phosphate (136.09 mg potassium dihydrogen phosphate 75 ml distilled water +0.2 ml sulphuric acid and diluted up to 100 ml) i.e., 0,1,2.5,4,6 µm was prepared. To the prepared concentrations 1 ml of ascorbic acid + 1 ml of mixed reagent was added. (12.5g of ammonium pentamolybdate tetrahydrate + 125ml distilled water. To this 350ml of reagent 1 was added. 2) 0.5g of potassium antimony tartrate + 20 ml distilled water). Absorbance was measured at 880 nm and standard graph was plotted; calculation was done based on the equation of the line obtained.

2.5.3 Silicate

Different concentrations of standard silicate solution (188.06mg of silicomolybdic acid in100 ml distilled water) as 0, 10, 25, 50, 100µm were prepared**.** 25ml of the standard concentrations and samples were added to test tubes. 1 ml of acid molybdate reagent was added to all tubes, mixed and kept for 10-20 min.1ml of oxalic acid followed immediately by 0.5 ml ascorbic acid was added.Absorbance was measured at 640 nm and a standard graph was plotted.

2.6 Bacterial analysis

2.6.1 Enumeration of bacteria by viable count and examination of human pathogenic bacteria on differential media.

Dilutions up to 10^{-2} were prepared under sterile conditions using sterile sea water.

Dilutions were spread plated on Zobel Marine agar, TCBS agar, MacConkey's agar and SS agar plates.ZMA plates were incubated for 24 hours at RT and colonies were counted for viable count. TCBS, SS and MacConkey agar plates were incubated for 48 hours for the isolation of human pathogenic bacteria.

2.6.2 Identification of human pathogenic bacteria

Gram staining was done for the bacteria isolated

Biochemicals tests according to Berge's manual were carried out to confirm bacterial isolates.

1. Motility test (*Salmonella, Shigella*): Nutrient agar butts were prepared, 24 hours old bacterial culture was stabbed inside the agar with the help of a sterile pointed nichrome loop. Incubated for 24 hours and checked for diffusion of the culture in the agar.

2. Glucose fermentation (*Vibrio cholera, Vibrio parahaemolyticus*): Bacteria was grown on different agars like SS. TCBS, MacConkey's and morphological characteristics were noted down.
3. Citrate test (*E. coli*): Slants were prepared of Simmons citrate agar and bacteria was streaked on it. If the colourof the media changes from green to blue it will indicate a positive result.
4. Urease test (*Salmonella, Shigella*): Slants were prepared of Urease agar. Bacteriawere streaked on it. Colour change to pink after incubation of 24 h indicated positive result.
5. Indole test (*Salmonella, Shigella, E. coli*): 1% L-Tryptophan was added to Nutrient Broth and was autoclaved at 121°C. bacteria were inoculated in broth and was incubated for 24 hours at room temperature. Next day 2-3 drops of Kovac's reagent were added. Pink ring formation indicated positive results.

2.3 Statistical analysis

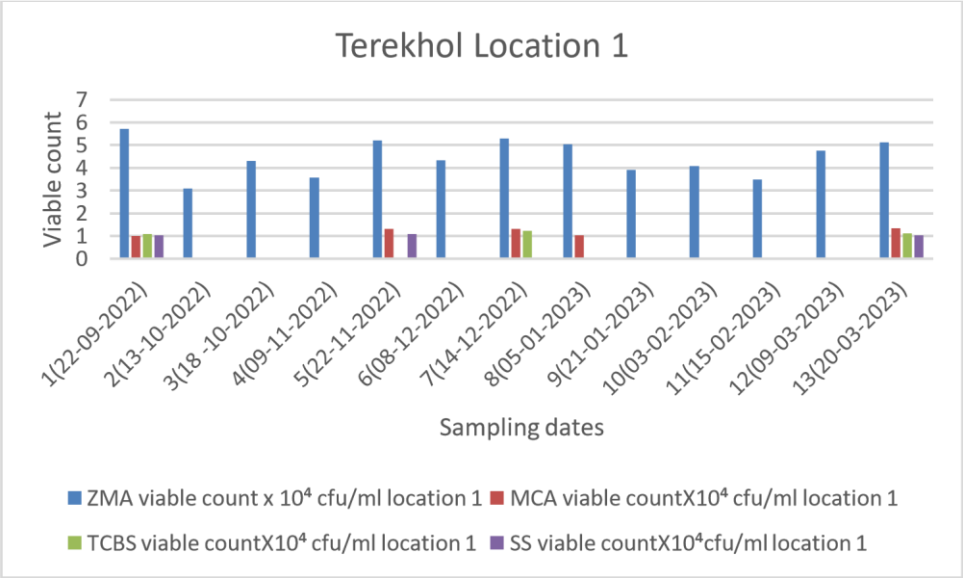
Two-way ANOVA and correlation analysis for the entire data set of location 1 and location 2 were carried out separately using Microsoft Excel 2021.

RESULTS

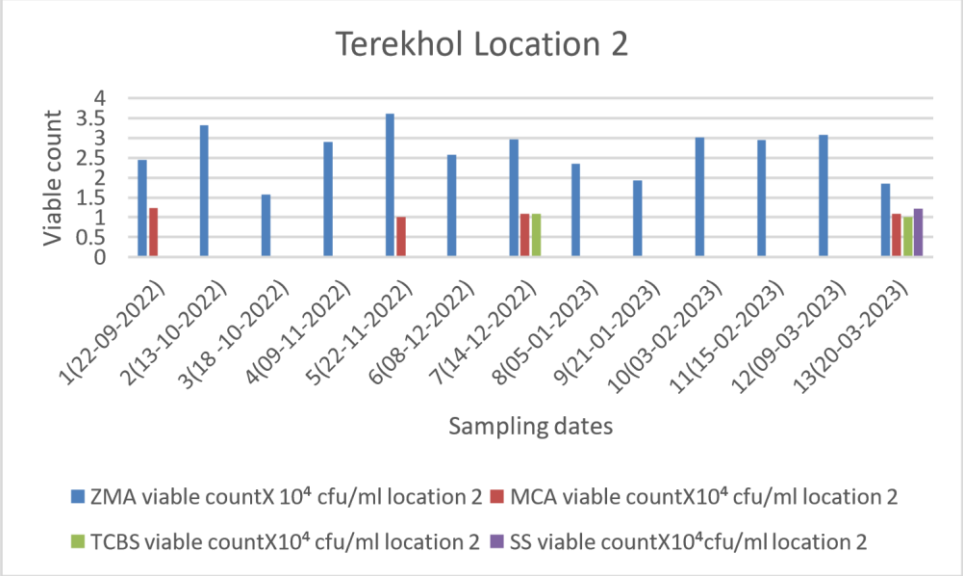
Among the 13 sampling times, the presence of human pathogenic bacteria on TCBS, MCC, SS Agar was observed only during 5 sampling times (Fig. 2 a, b).

Fig. 2 Abundance of human pathogenic bacteria in Terekhol water sample at the two sampling locations

(a)Location 1



(b)Location 2



Sampling 1 on 22th September 2022 showed growth on all the three selective media for location 1. Highest growth was seen on TCBS agar. Location 2 showed growth only on MCC agar. Sample 5 on 22nd November 2022 showed growth on MCC at both the locations. Location 1 also showed growth on SS agar. Highest growth was seen on

MCC at location 2. Sample 7 on 14th December 2022 showed growth on MCC and TCBS at both the locations with higher growth at location 2. Growth was seen at only location 1 on MCC agar on 8th January 2023. The final sampling on 20th March 2023 showed growth on all the media with highest growth seen in location 2.

For all the samples the temperature range was from 24°C to 30º C. The highest temperature was observed on 9th March 2023 at location 1 and the lowest was on 21st January 2023 also at location 1(Fig.3 a). Salinity on the other hand showed a wide range for all the samples with lowest being 18 on 18th October 2022 at location 2 and highest being 29 on three occasions i.e., 13th October 2022 at location 1, 3rd February 2023 at location 2 and 9th march 2023 at location 1(Fig.3 b). The pH range varied from 6 to 8 for all the samples. Lowest pH observed at location 2 on 9th November 2022 and highest at location 1 on 22nd November 2022 (Fig.3. c).

Chlorophyll concentrations were between the range of 1 mg/m^3 to 3 mg/m^3. The highest concentration was seen at location 2 on 22nd September 2022 and lowest was on 13 October 2022 at location 1(Fig.4 a). Dissolved oxygen was highest at location 1 on 21 January 2023 and lowest at 22nd September 2022 at location 2 (Fig .4 b).

Fig. 3 Temperature, salinity and pH of the Terekhol estuary water at the two sampling locations during the months of study.

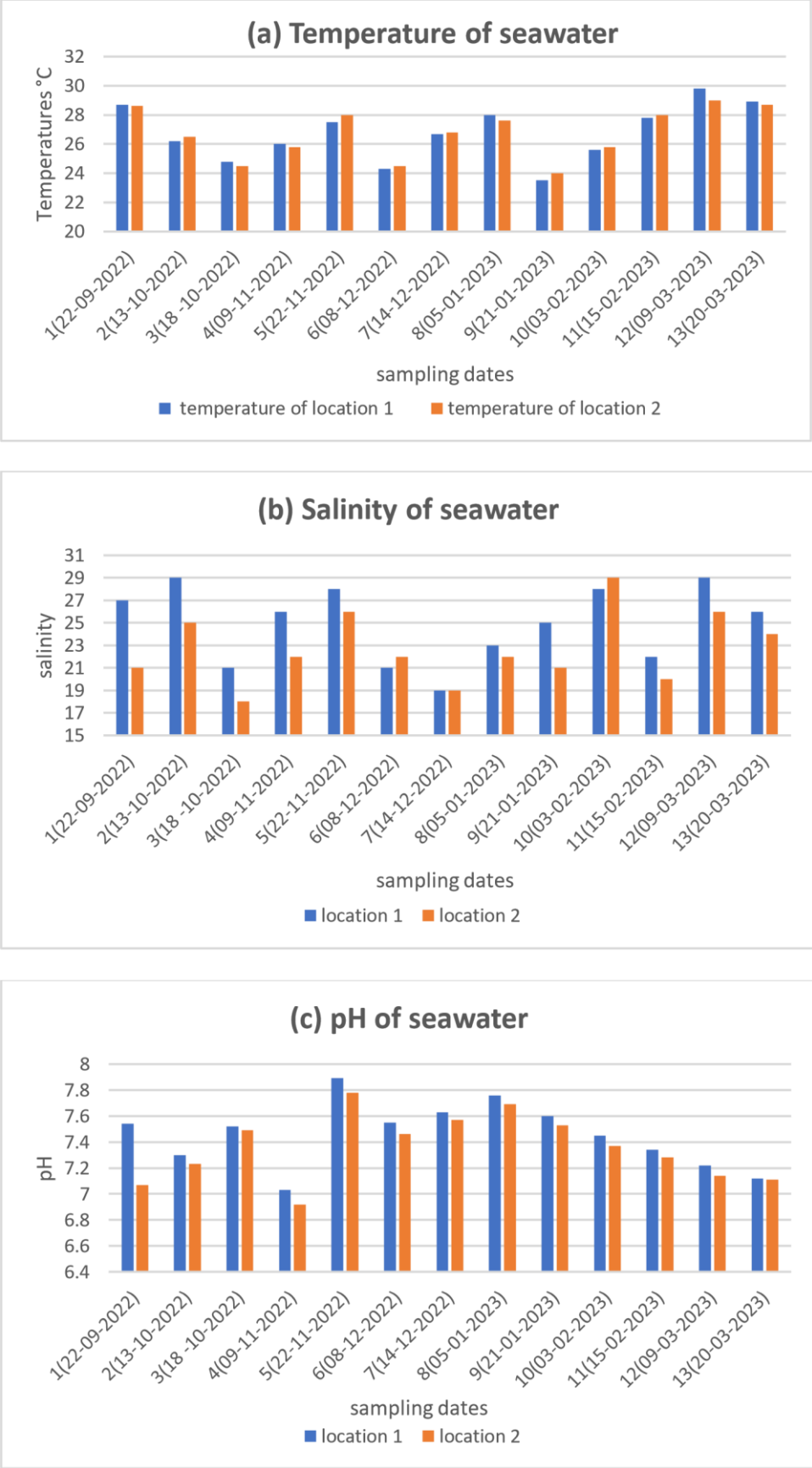
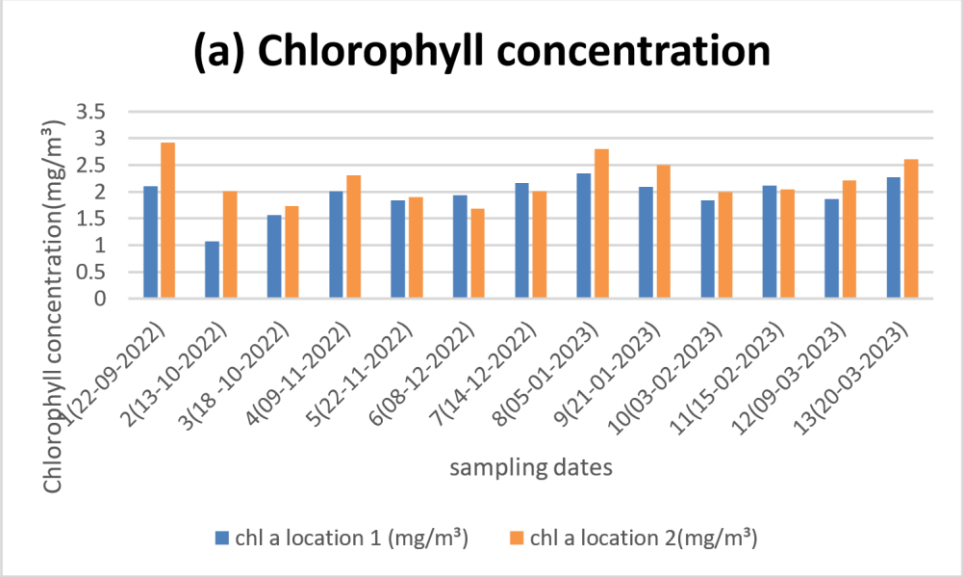


Fig. 4 Concentration of chlorophyll and dissolved oxygen in the Terekhol estuary water at the two sampling locations during the months of study.



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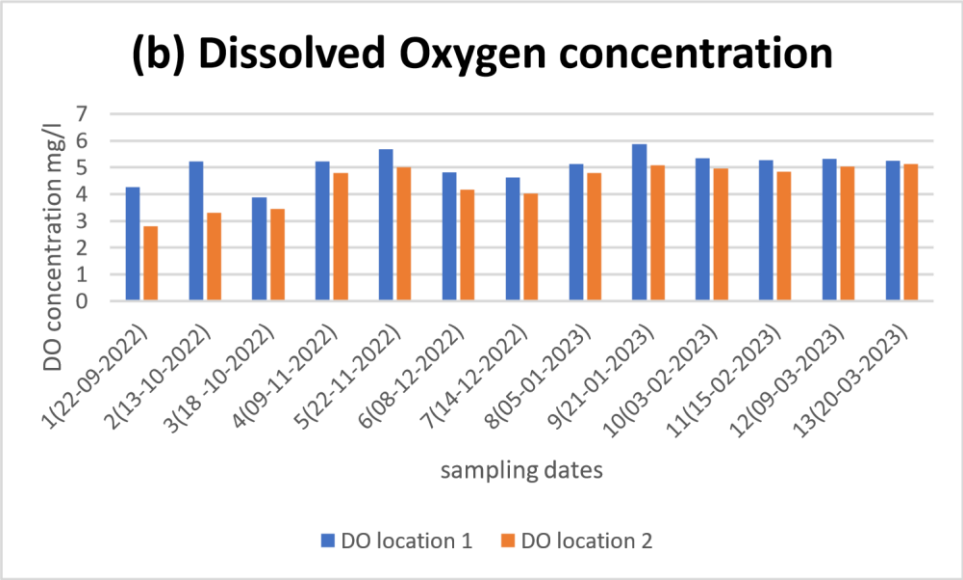
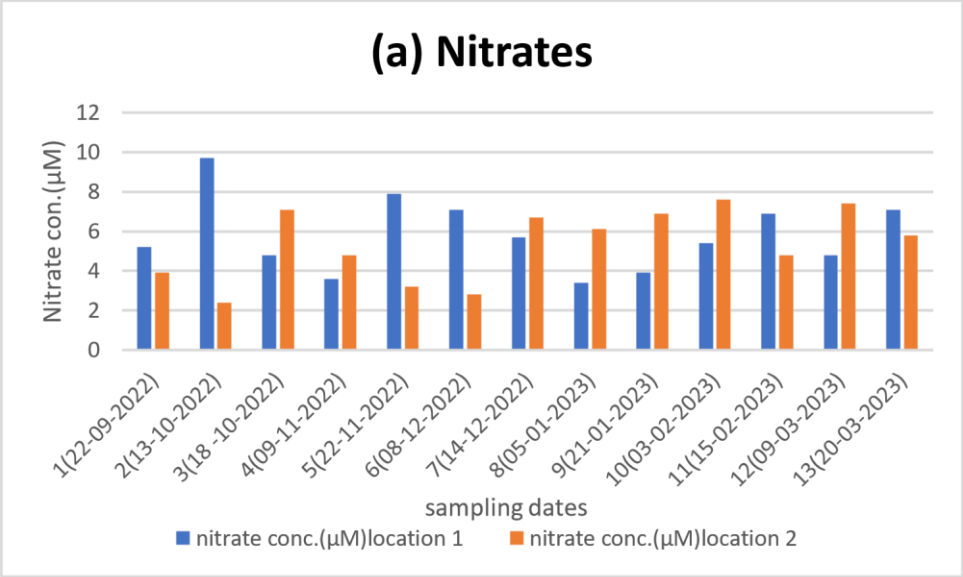
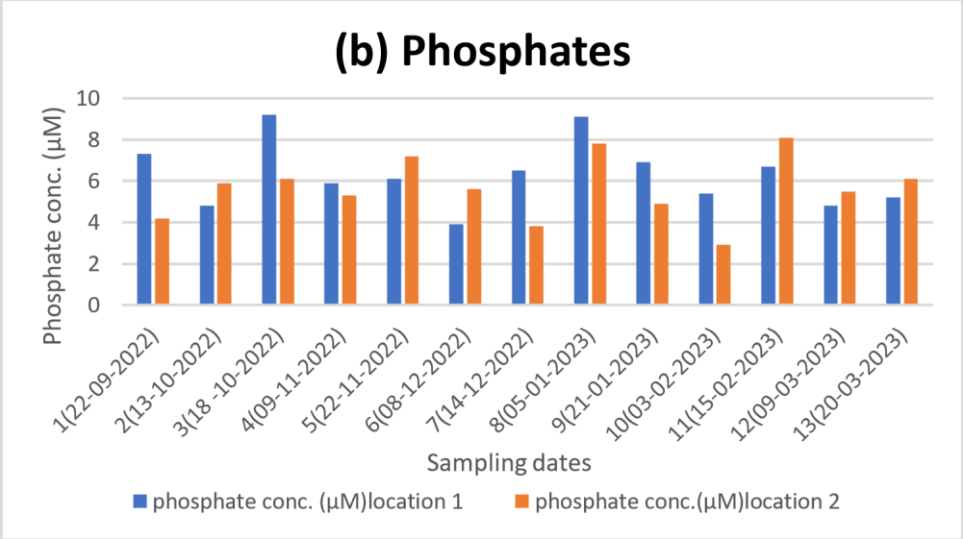
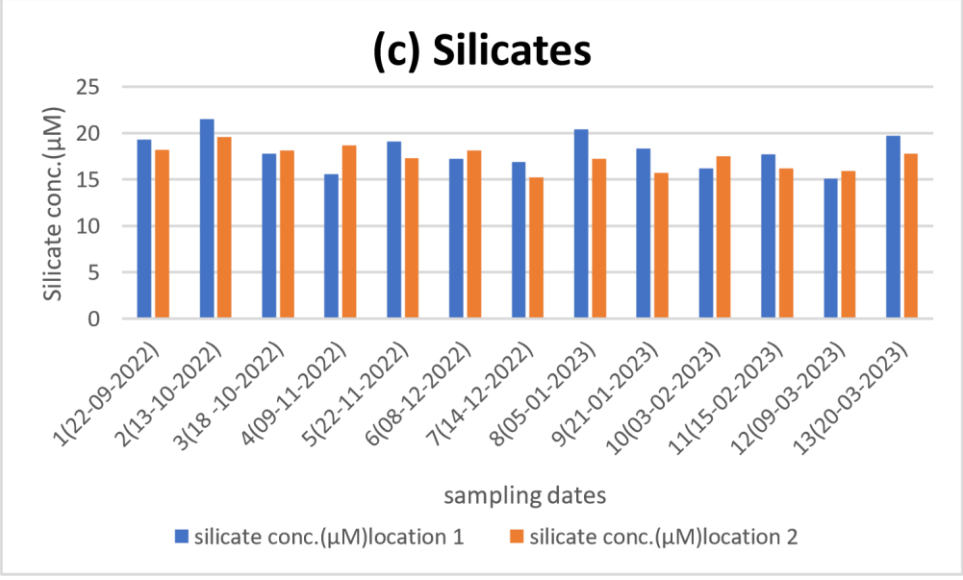


Nutrient analysis showed the presence of silicate in the highest concentrations within a range of 15 to 22 µM, nitrate between 2-9 µM and phosphate between 2-10 µM (Figs.5 a, b, c).

Fig.5 Concentration of nutrients in the Terekhol estuary water at the two sampling locations during the months of study.


(a) Nitrates
Nitrate con.(µM)
12
10
8
6
4
2
0
1(22-09-2022)
2(13-10-2022)
3(18 -10-2022)
4(09-11-2022)
5(22-11-2022)
6(08-12-2022)
7(14-12-2022)
8(05-01-2023)
9(21-01-2023)
10(03-02-2023)
11(15-02-2023)
12(09-03-2023)
13(20-03-2023)
sampling dates
nitrate conc.(µM)location 1
nitrate conc.(µM)location 2


(b) Phosphates
Phosphate conc. (µM)
10
8
6
4
2
0
1(22-09-2022)
2(13-10-2022)
3(18 -10-2022)
4(09-11-2022)
5(22-11-2022)
6(08-12-2022)
7(14-12-2022)
8(05-01-2023)
9(21-01-2023)
10(03-02-2023)
11(15-02-2023)
12(09-03-2023)
13(20-03-2023)
Sampling dates
phosphate conc. (µM)location 1
phosphate conc.(µM)location 2


(c) Silicates
Silicate conc.(µM)
25
20
15
10
5
0
1(22-09-2022)
2(13-10-2022)
3(18 -10-2022)
4(09-11-2022)
5(22-11-2022)
6(08-12-2022)
7(14-12-2022)
8(05-01-2023)
9(21-01-2023)
10(03-02-2023)
11(15-02-2023)
12(09-03-2023)
13(20-03-2023)
sampling dates
silicate conc.(µM)location 1
silicate conc.(µM)location 2

Among all the bacterial colonies that were isolated from the seawater 8 different isolates were identified with the help of biochemical tests (Table 2.). Among the 8 isolates, isolate 1, 4 and 8 showed positive results for *E. coli.* Isolate 2 and 7 showed positive results for *V. cholerae.* Isolate 3 was positive for *Salmonella, Shigella* for Isolate 5 and Isolate 6 was *V. parahemolyticus.*

Table 2. Biochemical tests

Biochemical tests	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Motility	-	-	+	-
Indole Test	+	-	-	+
Citrate Test	-	-	-	-
Urease Test	-	-	-	-
Glucose Fermentation	+	+	+	+
Gram Character	Gram negative	Gram negative	Gram negative	Gram negative
Organism	*E. coli*	*V. cholerae*	*Salmonella*	*E. coli*

Biochemical tests	Isolate 5	Isolate 6	Isolate 7	Isolate 8
Motility	-	-	-	-
Indole Test	-	-	-	+
Citrate Test	-	-	-	-
Urease Test	-	-	-	-
Glucose Fermentation	-	+	+	+
Gram Character	Gram negative	Gram negative	Gram negative	Gram negative
Organism	*Shigella*	*V. parahemolyticus*	*V. cholerae*	*E. coli*

Two-way ANOVA for both the locations showed no significant values, thus accepting the null hypothesis that the variance between the samples as well as the parameters is

same (Tables 3 and 4). Correlation analysis showed no significant correlation between any physicochemical parameter and abundance of bacteria (Tables 1.6 and 1.7).

Table 3. ANOVA analysis for location 1.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	29.27210549	11	2.661100499	1.252823471	0.2616247175	1.876731984
Columns	7309.65813	10	730.965813	344.1324849	0	1.91782714
Error	233.6490827	110	2.12408257			
Total	7572.579318	131				

Table 4. ANOVA analysis for location 2.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	16.55486409	11	1.504987645	0.8386378953	0.6020469656	1.876731984
Columns	6500.758375	10	650.0758375	362.2476464	0	1.91782714
Error	197.4018129	110	1.794561936			
Total	6714.715052	131				

Table 5. Correlation analysis for location 1. (r values in red are close to or above 7)

Location 1												
	Temp	Sal	pH	DO	chl a (r	nitrate	phospl	silicat	ZMA	MCC	TCBS	SS
Temp	1											
Sal	0.3	1										
pH	-0.24	-0.3	1									
DO	0.17	0.64	-0.01	1								
chl a (mg/	0.25	-0.4	0.11	0.19	1							
nitrate (µl	0.11	0.23	-0.01	0.07	-0.54	1						
phosphate	-0.09	-0.44	0.42	-0.37	0.22	-0.53	1					
silicate (µ	0.04	0.08	0.29	0.1	-0.22	0.48	0.21	1				
ZMA	0.43	-0.21	0.45	-0.12	0.53	-0.16	0.16	-0.01	1			
MCC	0.46	-0.14	0.39	0.05	0.46	0.12	0.17	0.38	0.81	1		
TCBS	0.29	-0.32	-0.12	-0.19	0.39	0.12	-0.09	0.07	0.55	0.68	1	
SS	0.39	0.31	0.12	0.3	0.16	0.4	-0.16	0.34	0.51	0.68	0.36	1

Table 6. Correlation analysis for location 2. . (r values in red are close to or above 7)

Location 2												
	Temp	Sal	pH	DO	chl a (	nitrat	phos	silicat	ZMA	MCC	TCBS	SS
Temp	1											
Sal	0.35	1										
pH	-0.16	-0.15	1									
DO	0.4	0.39	-0.08	1								
chl a (mg/	0.34	0.05	-0.13	0.54	1							
nitrate (µl	-0.01	-0.08	-0.01	0.3	0.31	1						
phosphate	0.41	-0.25	0.16	0.06	0.19	-0.42	1					
silicate (µ	-0.21	0.23	-0.33	-0.44	-0.18	-0.57	0.1	1				
ZMA	0.39	0.52	-0.01	0.06	-0.31	-0.44	-0	0.02	1			
MCC	0.43	0.02	0.23	0.15	0.06	-0.06	-0	-0.24	0.11	1		
TCBS	0.3	-0.21	-0.06	0	0.2	0.2	-0.3	-0.29	-0.2	0.8	1	
SS	0.39	0.11	-0.34	0.28	0.42	0.06	0.1	0.13	-0.4	0.54	0.64	1

DISCUSSION

Occasional examining of the Mandovi and Zuari estuary in 2002-2003 demonstrated higher coliform counts during monsoon which may be attributed to increased surface overflow into these estuaries conveying the feces from the land (Rodrigues et al.2011). In addition, La Rosa et al. (2001) suggested that high pollution during the monsoon may be caused by excessive surface runoff that introduces pathogenic microorganisms into the water column (Rodrigues et al.2011). Another study showed that November had the abundance of the examined human pathogenic bacteria (Ramaiah et al.2004).

In the present study bacterial growth didn't follow a particular trend but the highest growth was observed during the month of September for location 1 and November for location 2. September falls within the southwest monsoon period. The increase in the numbers of bacteria in November could be due to the sand mining activities that were taking place in that area around the time of sampling.Wastewater discharges containing fecal coliforms, nutrients, and BOD are the sole factors that are accountable for the disparity in water quality between different locations (Fulke et al.2019). Studies also showed that change in temperature and salinity showed variation in bacterial population, low temperatures showed growth in *E. coli* and *Vibrio* species (Hassard et al.2017). During the present study, the lowest temperature was observed in October but there was no growth of the bacteria. Comparing all the physicochemical parameters at both the locations, location 1 showed higher range of temperature, pH, salinity and DO than location 2. Chlorophyll concentrations on the other hand were higher at location 2, this could be due to the mixing of the water at this location during sand mining. Nutrient concentrations on both the locations did not follow any specific trend.

Relationship between viable count and different parameters was likewise inspected in another study. The relationship between overall bacterial abundance and

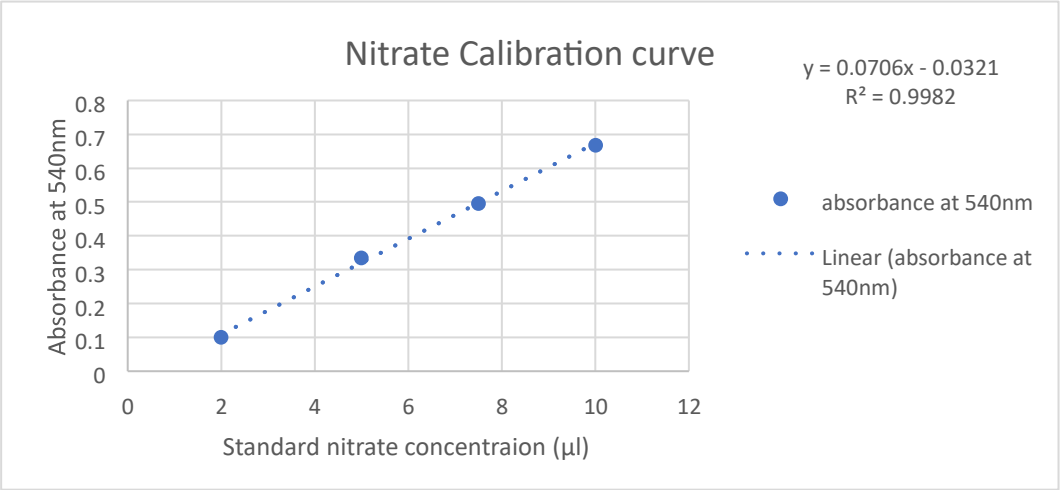
physicochemical parameters changes with location but does not significantly change with seasons, according to a two-way ANOVA of the entire data set (Damare et al. 2020). Similar analysis was used for the present study using Two Way ANOVA. No significant correlations between the physicochemical parameters and the counts obtained for bacterial growth was observed during correlation analysis. There was no significant change with the changes in season (Table 3 and 4).

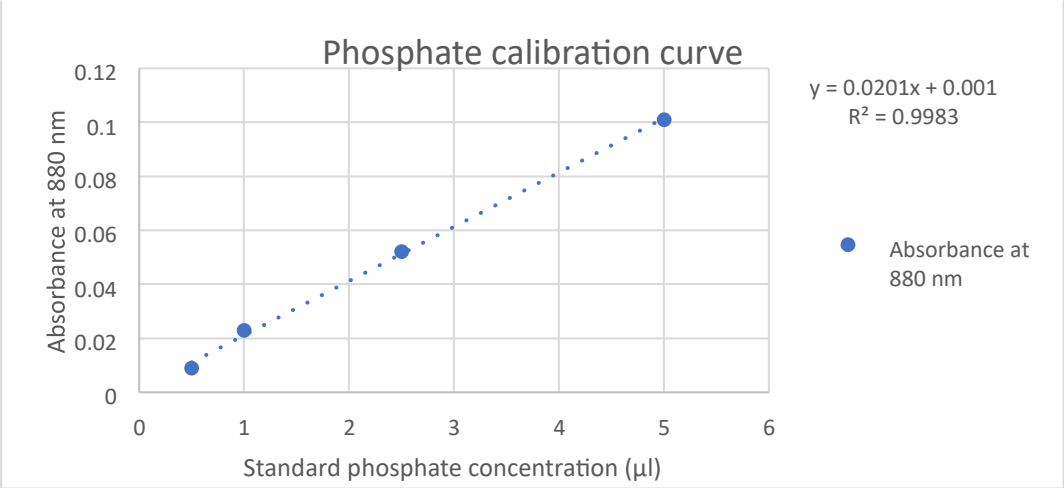
CONCLUSION

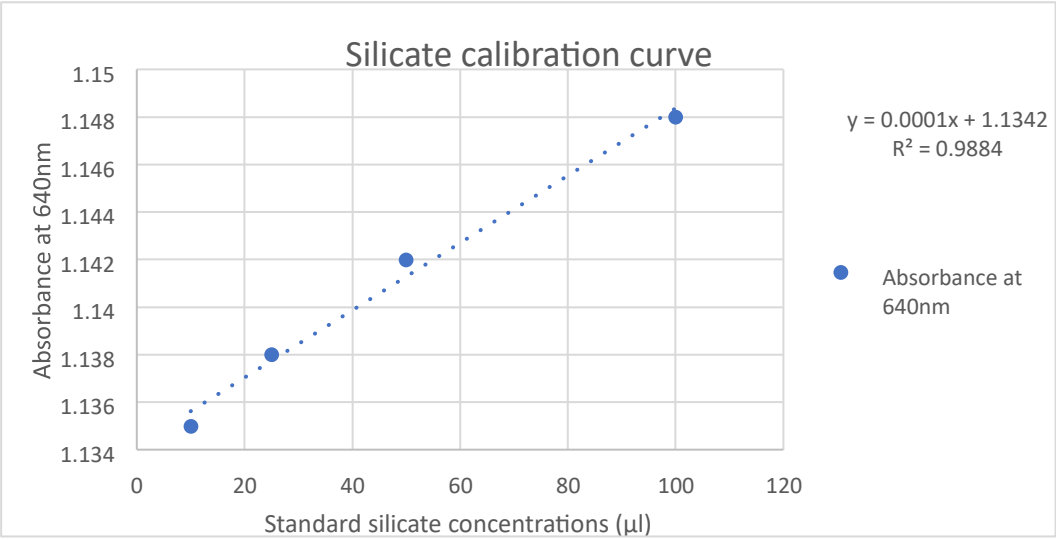
The study carried out in the Terekhol estuary showed the presence of human pathogenic bacteria at both the study locations. As no significant correlation was observed between the bacterial growth and the physicochemical parameters it can be said that the variations in the bacterial growth was not due to the change in the seasons. So, the presence and variation in the bacterial count of human pathogenic bacteria can be attributed to the anthropogenic activities that take place in and around the Terekhol river estuary like waste disposal from the surrounding restaurants or human habitations, sand mining from the river, etc. At the same time the variations could also be have been due to the tides or surface runoffs.

APPENDIX

Calibration curve graphs







MEDIA PREPARATION

1. Zobel Marine Agar: 5.525g of ZMA in 100ml of Distilled water. Autoclaved and plates were poured.

2. MacConkey's Agar: 5.507 g of MCC agar in 100 ml of Distilled water. Autoclaved and plates were poured.

3.Thiosulfate- Citrate- Bile salt -Sucrose Agar: 6.235 g of TCBS agar in 80 ml Distilled water. The media was heated at 100 °C in a water bath for 10 min .20 ml of separately autoclaved agar was added to the media and plates were poured.

4.Salmonella Shigella Agar: 5.735 of SS agar component 1 and 0.322 ml of component 2 in 80 ml of distilled water. The media was heated at 100 °C in a water bath for 10 min .20 ml of separately autoclaved agar was added to the media and plates were poured.

Agar for TCBS and SS: 2 g of agar powder in 20 ml of distilled water. Autoclaved and added to the media before pouring plates.

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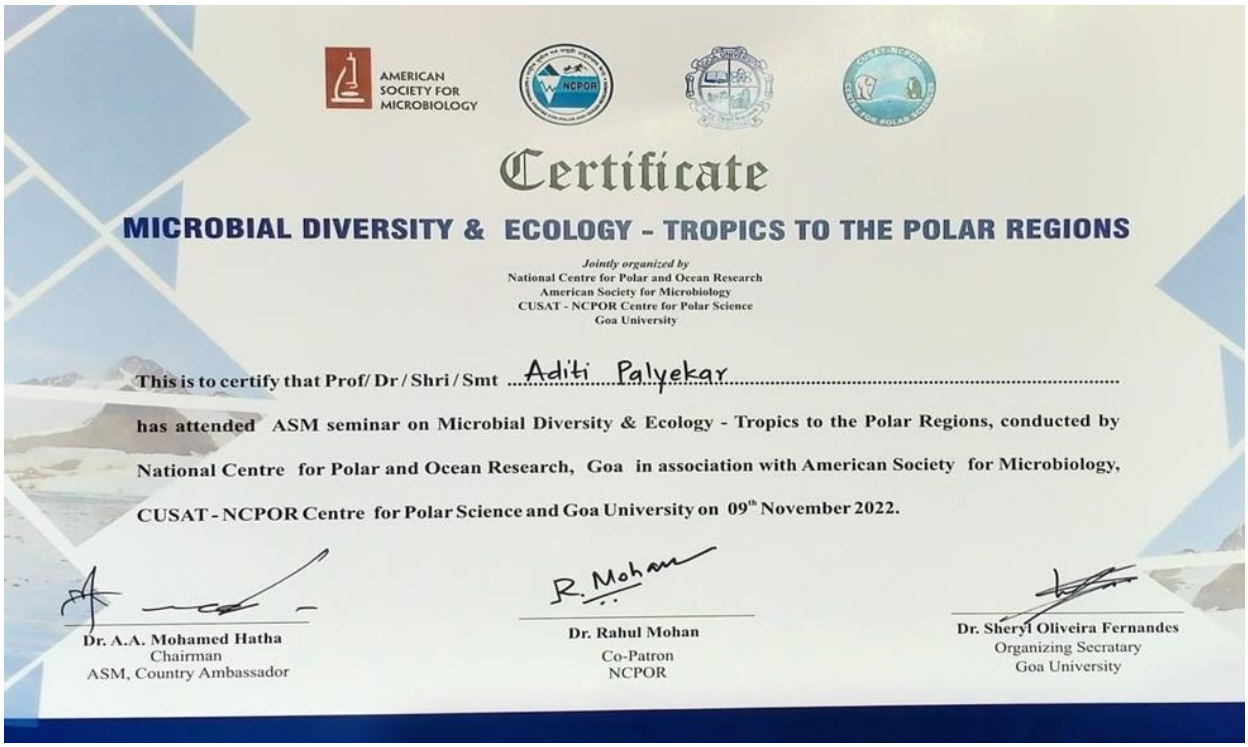

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DISTRIBUTION OF BACTERIA AND FUNGI IN THE COASTAL WATERS OF GOA

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Abstract: Goa being a tourist destination, the health of its water bodies and beaches is important for recreational activities. The presence of bacteria that indicate sewage contamination such as faecal coliforms have been reported at a few instances in the monsoonal estuaries of Goa. The present study is carried out to examine if the presence of different bacteria would fall in support of the previous studies or not. Different locations in the North Goa district were chosen for this study. Seawater samples were plated on various nutrient media such as Zobell Marine Agar, Thiosulphate Citrate Bile salts Sucrose Agar (TCBS), Salmonella-Shigella Agar and Mac Conkey's Agar to check for the growth of bacteria, and on Malt Extract Agar and Potato Dextrose Agar for the growth of fungi. Vibrio-like colonies were found to grow after plating most of the samples. Coliforms were found only in a few samples and their numbers were within the permissible limits. Surface waters of the Mandovi and Zuari estuary comprised more number of human pathogenic bacteria than the beach seawater samples. Different types of fungi were obtained from the beach seawater than the estuarine seawater. The fungi could be responsible for restricting the human pathogenic bacterial load.

INTRODUCTION

- Goa: popularly known as 'Pearl of the orient' and tourist paradise.
- Rapid and uncontrolled tourism development in coastal areas has exposed their fragile ecosystem to an ever-increasing risk of environmental degradation.
- Negative impacts of tourism that lead to environmental degradation include: crowding, poor sewage disposal, boat/trawler-generated waste, over-fishing, etc.
- Waste disposal in waters shoot up the nutrient levels, and consequently the autochthonous microbial populations, and expose them to allochthonous microflora.
- Aquatic microbiota: an important factor in the sustainability of the natural water ecosystems.
- Hence it is significant to study the distribution of microorganisms in these waters.

METHODOLOGY

Collection of surface seawater & making dilutions up to 10^{-5} using sterile seawater (collected in advance from the same location) as diluent

↓

Spread plating 0.1 mL sample of 10^{-3}, 10^{-4} and 10^{-5} dilutions on Zobell Marine Agar (ZMA), 10^{0}, 10^{-1} and 10^{-2} on Thiosulphate Citrate Bile salts Sucrose Agar (TCBS), Salmonella Shigella Agar (SS), MacConkey's agar, Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA). MEA and PDA supplemented with antibiotics (ampicillin 5µg/ml and chloramphenicol 5µg/ml)

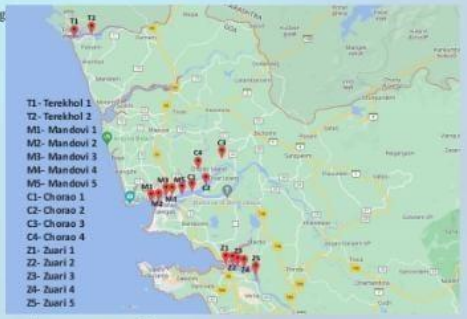
↓ Incubating at RT/24 h for bacteria and 3-5 days for fungi

Counting of colonies and subculturing for identification

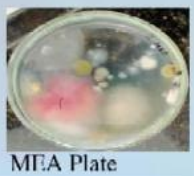
↓

To carry out antibacterial assays

SAMPLING LOCATIONS



Growth of bacteria and fungi on various media

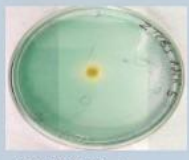


MEA Plate

MEA Plate

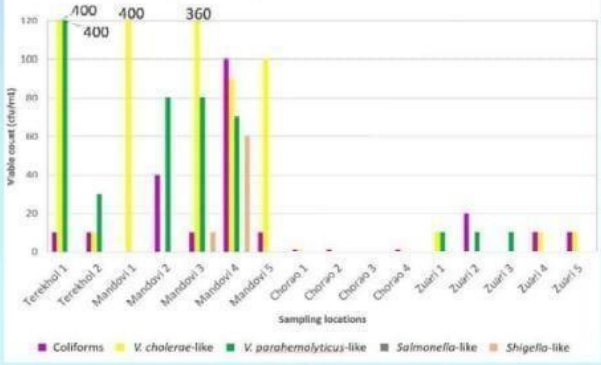
ZMA Plate

ZMA Plate



TCBS Plate

Human pathogenic bacterial counts in the estuarine waters



HARMFUL EFFECTS OF PATHOGENIC BACTERIA

Exposure to microbially polluted recreational waters

- Human health risk
- Recreational waterborne illness (RWI)
- Gastrointestinal illnesses, neurological infections, skin infections, earaches, eye infections, and respiratory infections

SIGNIFICANCE OF ANTIBACTERIAL ACTIVITY

- Search for new, effective bactericidal materials is significant for combatting drug resistance.
- Fungi naturally produce antibiotics to kill or inhibit the growth of bacteria.
- Fungi are well known for their production of substances with antimicrobial activities, several which have formed the basis for development of new clinically important antimicrobial agents.

ANTIMICROBIAL ACTIVITY

Antifungal activity of a few isolates.

- Antibacterial activity study is in progress.

SUMMARY

Sea water sampling from different locations in the estuaries of Goa showed the presence of human pathogenic bacteria and various fungi. Numbers of the former were within permissible limits during the sampling time studied. More work in these locations will shed more light on the prevalence of these bacteria in the waters. Different fungi obtained seem to be promising for antifungal activity, hence this activity and their antibacterial profile will be further tested.

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INTRODUCTION 1.1 Introduction to Marine Microbiology Marine Microbiology studies make up a very important part to study the earth's environment as a large part of the earth constitutes of the oceanic ecosystem. About 70 % of the earth's surface is covered by water which is even greater than the land mass. Microbes play a very important role in the oceanic ecosystem as they assist in various chemical as well as physical processes (Woods et al. YEAR). Human activity impacts marine ecosystems at a worldwide scale. Despite their importance as key players in important processes, the strain caused to microorganisms have been greatly neglected. Microbial communities respond to nutrients and chemical pollution by increasing cell numbers. There are important changes in community composition, increase in diversity and high temporal variability. These changes prove that the modification of the environmental conditions cause phylogeny stress. Altered microbial communities in human impacted marine environments will successively have harmful effects on human health (Nogales et al. 2011). 1.1.2 What is an estuary? Estuaries measure among the foremost biologically productive ecosystems on the planet critical to the life cycles of fishes, different aquatic animals and also the creatures that go after them. The term 'ESTUARY ` comes from the Latin word easts which means heat, boiling, or tide. Specifically, aethalium means tidal. Thus, the Oxford Dictionary defines estuary as "the tidal mouth of a great river, where the tide meets the current.". Webster's Dictionary is more specific with the definitions "(a) a passage, as the mouth of a river or lake where the tide meets the river current; more commonly, an arm of the sea at the lower end of a river; a firth. (b) In physical geography, a drowned river mouth, caused by the sinking of land near the coast." Perhaps the most widely quoted definition of an estuary in the scientific literature is given by Pritchard (1967): "An estuary is a semi enclosed coastal body of water which has a free connection with the open sea and within which sea water is measurably diluted with fresh water derived from land drainage." This definition still had a few drawbacks to address those, Fair bridge (1980) gave a more accurate definition for the term estuary;" An estuary is an inlet of the sea reaching into a river valley as far as the upper tidal rise." According to Fair bridge an estuary can be divided into three different parts 1. Marine or lower estuary, this part has a free connection to the open sea. 2. A middle estuary, which is subjected to strong salt and freshwater mixing. 3. An upper or fluvial estuary, this part is known for the presence of fresh water which is subjected to daily tidal activity. 1.1.3 Estuarine ecosystem Physical settings like climate, geomorphology, presence or absence of water salts etc. are the primary determinant of the kind of processes which will develop in an exceedingly specific location. The spirited discussion of what makes an estuary an extremely productive ecosystem is therefore a very exciting reason to study the ecology of an estuarine ecosystem. Four major reasons for this high productivity are listed I. Varieties of primary production units that guarantee most use of the abundant sunlight (marsh grasses, benthic algae, phytoplankton) II. Ebb and flow of water movements ensuing from recurrent event actions III. Overabundance of essential nutrients IV. Speedy regeneration and conservation of nutrients because of the activity of microorganisms and filter feeders. (Schalke et.al.2016).