Seasonal variation of coliform and total heterotrophic bacteria and antibiotic resistance of selected isolated strains from Biyyam backwaters

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ABSTRACT: The present study was carried out to investigate the seasonal variation of coliform load and THB, and to examine the antibiotic resistance of selected strains of bacteria. Highest coliform load and THB in water (coliform load (95-1400+/100ml), THB ($0.2\text{-}2\times10^6$cfu/ml)) and sediment (coliform load (20-1400+/100g), THB ($0.1\text{-}1.5\times10^6$cfu/g)) was observed during the monsoon season. However there was no significant correlation between the microbial load and physicochemical parameters in the present study. From the total 224 isolates obtained from the water and sediment samples, maximum number of isolates were present in the monsoon season (97 isolates), followed by post-monsoon (84 isolates) and pre-monsoon (43 isolates). Enterobacteriacea were the dominant forms in the monsoon (48%) and post-monsoon (74%), and *Bacillus* sp. (56%) for pre-monsoon. In this study most of the isolated bacteria were resistant to commonly used antibiotics. Of the 10 antibiotics used, 5 of them were resistant to Enterobacteriacea. The MAR index for Enterobacteriacea was 0.5 and was 0.2, 0.7 and 0.8 for *Staphylococcus* sp., *Bacillus* sp., and *Micrococcus* sp. respectively.

Keywords: Coliform load, THB, antibiotic-sensitivity, MAR index and Biyyam backwaters.

1. INTRODUCTION:

Bacteria are one of the important components of any food chain occurring in an ecosystem and hence its enumeration as an index of sanitary quality of water is important (Tortorello, 2003). Bottom sediments are also known to be reservoirs of enteric bacteria and therefore are implicated as an important component of overall water quality (Pachepsky and Shelton, 2011) and hence it is important to evaluate them as they can pose a potential risk to human health. A large proportion of the pathogenic organisms present in water may also become associated with the sediment which can be subjected to resuspension (Davies et al.,1995). In recent years there has been a considerable interest in studying the water borne pathogens in water and the associated environment (Pandey et.al, 2014). The quality of water and the extent of pollution may be described and indicated according to its physico-chemical and microbiological characteristics (Bhandari & Kapil,2008).

The type and number of bacteria may change with physico chemical parameters and seasons. Hence, it is important to investigate the microflora and physical parameters to assess the water quality. This in turn has a great effect on the quality of fish caught from the backwater.

Coliforms are a group of bacteria present in the digestive tract of animals, humans, and found in their faeces. It is used as an indicator organisms and gives a reasonable indication of presence of other pathogenic bacteria such as *Salmonella* spp., *Shigella* spp. and *Vibrio* spp., (Chandran et.al 2008). Faecal coliforms are known indicators of potential health hazards linked with faecal pollution (McLellan and Eren, 2014). Coliform bacteria are used as a criterion to assess the public health safety of discharges while faecal coliforms are used for modelling bacterial removal (Jagals and Lues, 1996).

The Malappuram district of Kerala has a large number of backwaters and among them Biyyam backwaters is the biggest with an area of 15 sq.km. Biyyam backwater popularly known as Biyyam kayal (lat- $10^0$45'-

$10^0$48' N and long- $75^0$58'E) , is a famous tourist attraction situated proximal to Ponnani township and the total basin area includes parts of Malappuram , Palakkad and Thrissur districts. This water body lies almost parallel to the Arabian sea and makes the southern boundary of Ponnani municipality.

The back waters of Kerala has been subjected to acute pressure of developmental activities, agricultural use, housing, aquaculture and has drastically affected the areas of flora and fauna and also its water quality. Water quality is among the most important factor affecting the health and safety of its users and the suitability for its utilization in various aspects (Lenart – Boron et.al, 2017). And this has resulted in the degradation of backwaters, this is the most serious problem faced by the back waters of Kerala and the Biyyam backwaters is no exception to this.

2. Materials and methods

2.1 Sampling: Water and sediment samples were collected in sterile vials seasonally (one month in each season) from 7 stations for bacteriological analysis (Fig.1).

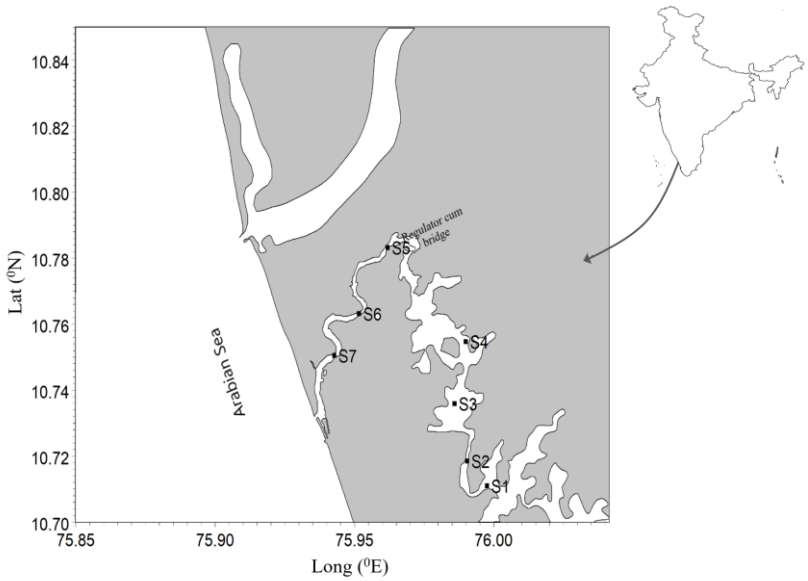

Fig. 1: Location of the study area and sampling stations

2.2 Physicochemical analysis: Salinity and pH was determined using a refractometer (ATAGO, S/Mill-E) and pH meter (Eutech Eco Tester pH1) respectively. Dissolved oxygen (DO) and nutrients (NO_3, NO_2, PO_4) were analysed following the standard methods (Grasshoff, 1983).

2.3 Bacteriological analysis: Water and sediment samples were analysed for coliform load and were enumerated by three tube Most Probable Number (MPN) technique using MacConkey broth (Speck, 1976). Samples were analysesd for heterotrophic bacterial load by pour plate method using Nutrient agar (APHA, 1998).

2.4 Isolation and identification: The colonies were isolated and identified upto genus level by following the standard scheme of Buchanan and Gibbons (1974).

2.5 Antibiotic sensitivity studies: antibiotic resistance of bacteria was determined for sediment samples (post-monsoon) following the disc diffusion method (Bauer et.al., 1966). The isolates were tested with 10 different antibiotics each for gram negative and gram positive bacteria. The isolates were scored as resistant, intermediate and sensitive according to the inhibition zone around the disc.

2.6 Multiple antibiotic resistance index : The MAR index was calculated as the ratio of the number of antibiotics to which the isolate displayed resistance to the number of antibiotics to which the isolate had been evaluated for susceptibility (Krumperman, 1983).

2.7 Statistical analysis: Pearson's correlation coefficient was used to find out the significant relationship of different environmental parameters.

3. RESULTS AND DISCUSSION:

In the present study highest coliform load and total heterotrophic bacteria (THB) was observed during the monsoon season in most of the stations followed by post-monsoon and pre-monsoon both in the water and sediments (Table 1 and 2). Higher counts during monsoon months may be due to low salinity, less exposure to light and temperature since these organisms cannot tolerate high salinity (Nallathambi et.al., 2002). Also, a heavy rain in the monsoon is known to increase the coliform load in both the water and the sediment (Goyal et.al 1977). Salinity showed a seasonal variation during the present study (Fig. 2). This is mainly due to the presence of the regulator with 24 shutters (between station 4 and 5) to avoid the saline water intrusion into the kole lands (proximity of station 1 to 4). These shutters are opened during the monsoons to avoid flooding of kole lands. Hence, stations 5 to 7 are observed to be freshwater during monsoon season, which is otherwise saline (once the regulator is closed after monsoons).

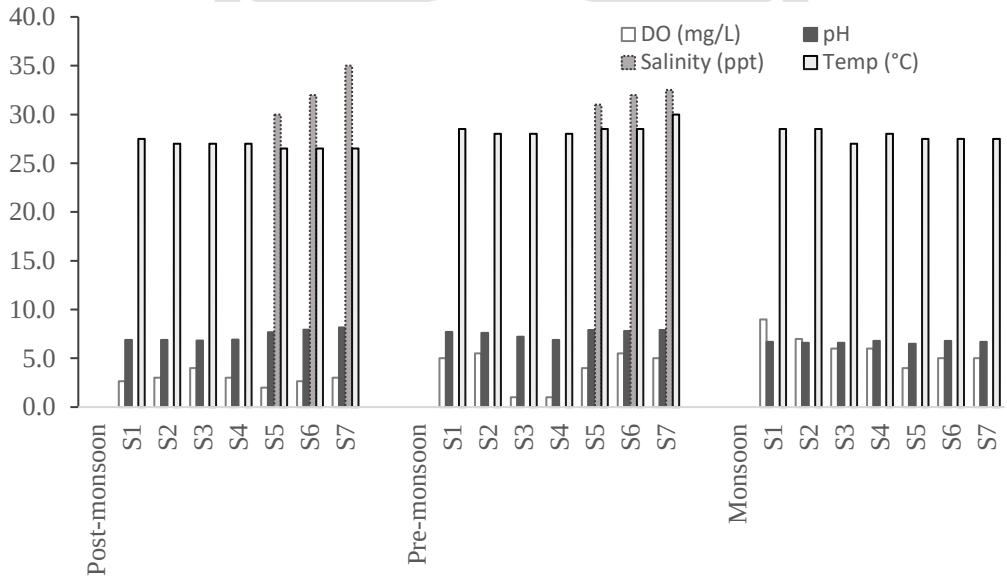

Fig.2: Seasonal variation of DO, Temp, Salinity and pH in the Biyyam backwaters

Table: 1 Seasonal variation of MPN index and THB of water from Biyyam backwaters

Stations	Post-monsoon		Pre-monsoon		Monsoon	
	MPN/100ml	THB Cfu/ml	MPN/100ml	THB Cfu/ml	MPN/100ml	THB Cfu/ml
S1	20	0.1×10^6	150	0.4×10^6	1400+	2×10^6
S2	150	0.4×10^6	20	0.1×10^6	1400+	1.6×10^6
S3	1100	1.1×10^6	75	0.2×10^6	450	0.6×10^6
S4	1100	1×10^6	1100	0.9×10^6	250	0.4×10^6
S5	40	0.2×10^6	11	0.1×10^6	1100	0.7×10^6
S6	450	0.6×10^6	9	0.1×10^6	1400+	1.3×10^6
S7	75	0.3×10^6	1400+	1.2×10^6	95	0.2×10^6

Large variation in coliform load and THB was observed between stations, especially during the pre-monsoon and post-monsoon (Table 1 and 2). It is not uncommon to find difference of 2-5 orders of magnitude between maximum and minimum concentrations observed at the same site or in the same watershed (Pachepsky and

Shelton, 2011). In a laboratory study higher variability between replicate sediment samples were observed compared to the water samples (Anderson et.al 2005).

Table: 2 Seasonal variation of MPN index and THB of sediment from Biyyam backwaters

Stations	Post monsoon		Pre monsoon		Monsoon	
	MPN/ 100g	THB Cfu/g	MPN/100g	THB Cfu/g	MP/100g	THB Cfu/g
S1	15	0.1×10^6	150	0.2×10^6	20	0.1×10^6
S2	30	0.3×10^6	20	0.1×10^6	115	0.3×10^6
S3	1100	2.1×10^6	95	0.2×10^6	40	0.2×10^6
S4	1100	1×10^6	20	0.2×10^6	200	0.4×10^6
S5	45	0.4×10^6	450	0.2×10^6	1100	0.9×10^6
S6	20	0.2×10^6	450	0.2×10^6	1400+	1.3×10^6
S7	150	0.6×10^6	160	0.2×10^6	1400+	1.5×10^6

Among physicochemical parameters, pH value did not show much variation between stations and seasons, the highest pH value was recorded as 8.1 (post-monsoon) and the lowest as 6.5 (monsoon season). Water temperature ranged between 26.5^0C to 30^0C and dissolved oxygen content varied between 1 to 5.5 mg/L in the present study (Fig. 2). Though higher nutrients were detected during pre-monsoon and post-monsoon compared to monsoon season (Fig. 3), the bacterial count in the present study did not show any significant correlation with the physicochemical parameters. Hence, the variation between stations in the present study could be due to other contributing factors, the patchy distribution of organisms in sediments and difficulty in dissociating bacteria from sediment particles (Pachepsky and Shelton, 2011) could also be a key factor.

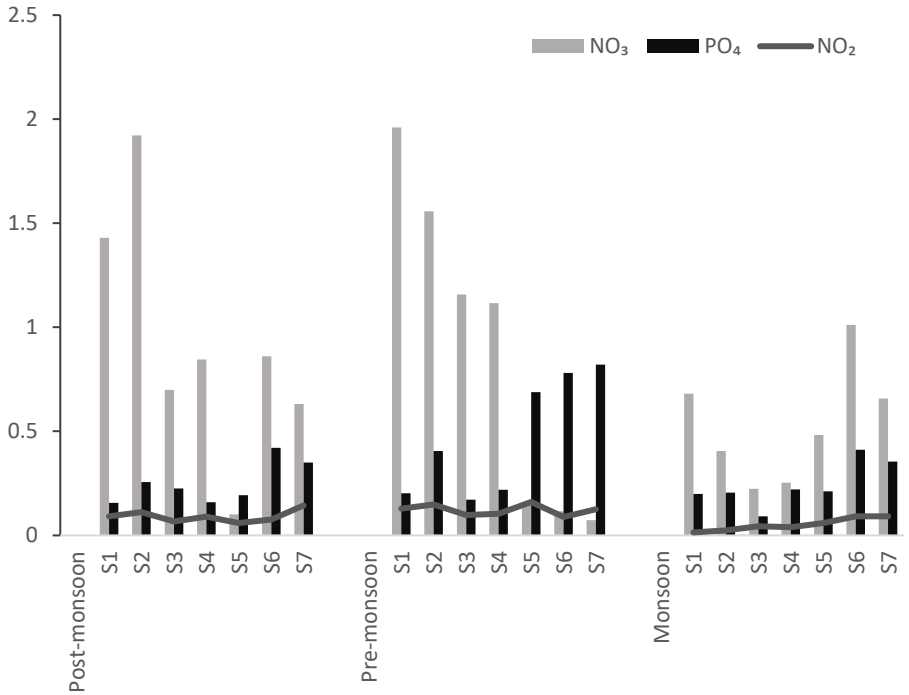

Fig. 3: Seasonal variation of Nutrients (µm/L) in the Biyyam backwaters

Water and sediment samples from Biyyam backwaters showed the presence of different genus of bacteria. The isolated bacteria from the present study were further characterized and identified upto genus level (Table 3). A total of 224 isolates from water and sediment samples were obtained in the present study, from which 84 isolates were in post monsoon, 43 in pre-mosoon and 97 in monsoon. Out of the 84 isolates (sediment and water) in post-monsoon 62 (74%) were Enterobacteriacae and 7 (8%) were *Bacillus* sp. And the remaining isolates of 12 (14%) *Micrococcus* sp. and 3 (4%) *Staphylococcus* sp. were present only in the sediment. In the pre-monsoon, *Bacillus* sp. were dominant with 24 (56%) isolates followed by Enterobacteriacae 6 (14%)

isolates, *Micrococcus* sp. 4 (9%) isolates.and *Aeromonas* sp. 3 (7%) isolates in the water and sediment. However, 1 (2%) isolate of *Vibrio* sp. and 5 (12%) isolates of *Staphylococcus* sp. were present only in sediment and water respectively. Highest number of isolates 97 (43%) were observed in monsoon season. Of the 97 isolates 47 (48%) isolates were Enterobacteriacae followed by *Bacillus* sp .25 (26%), *Vibrio* sp. 5 (5%) , *Listeria* sp. 11 (11%), and *Aeromonas* sp.9 (9%) .

Table: 3 Seasonal variation of number of isolates from Biyyam backwaters

Isolates	Post monsoon		Pre monsoon		Monsoon	
	Water	Sediment	Water	Sediment	Water	Sediment
Enterobacteriacae	32	30	5	1	22	25
Bacillus sp.	5	2	15	9	15	10
Micrococcus sp.	--	12	3	1	--	--
Aeromonas sp.	--	--	2	1	4	5
Vibrio sp.	--	--	--	1	3	2
Listeria sp.	--	--	--	--	6	5
Staphylococcus sp.	--	3	5	--	--	--

The antibiotic sensitivity for both gram negative and gram positive isolates revealed antibiotic resistant bacteria in the backwater of Biyyam. Among the 10 antibiotics applied, 5 of them viz., cefuroxime, cefoxitin, nitrofurantoin, nalidixic acid and tetracycline were resistant to gram negative Enterobacteriacae. It was also found to be a multidrug resistant. The findings of antibiotic resistance in Enterobacteriacae are extremely important as they taper towards the prevalence of antibiotic resistant microorganisms in drinking water sources. It has lately been discovered that such multiple drug resistant bacteria 'superbugs' are the suit of the worry nation's worldwide (Walsh et al., 2011).

The antibiotic sensitivity study for gram positive isolates are given in the table 4. Gram positive isolates (*Staphylococcus* sp., *Bacillus* sp., and *Micrococcus* sp.) showed 100% resistance to ampicillin and penicillin and also were resistant to gentamicin (33.33%), erythromycin (66.66%), norfloxacin (66.66%), linezolid (66.66%), ciprofloxacin (33.33%), vancomycin (66.66%), and tigecycline (33.33%). Most of the bacteria isolated were resistant to commonly used antibiotics, ampicillin (100%), penicillin (100%), cotrimoxazole (57.1%), cefuroxime (92.9%), erythromycin (92.9%) and therefore represent a public health concern (Khan and Malik, 2001). Koesak et al., (2012) have also previously detected bacterial resistance against ampicillin, gentamicin, erythromycin, tetracycline and ciprofloxacin at different times.

Table: 4 Antibiotic sensitivity study of selected gram positive isolates (*Bacillus* sp., *Staphylococcus* sp., *Micrococcus* sp.) from Biyyam backwater

Name of antibiotic	% of sensitive strains	% of moderately sensitive strains	% of resistant strains
Gentamicin	66.66	Nil	33.33
Erythromycin	Nil	33.33	66.66
Levofloxacin	100	Nil	Nil
Norfloxacin	Nil	33.33	66.66
Ampicillin	Nil	Nil	100
Penicillin	Nil	Nil	100
Linezolid	33.33	Nil	66.66
Ciprofloxacin	33.33	33.33	33.33
Vancomycin	33.33	Nil	66.66
Tigecycline	Nil	66.66	33.33

The MAR index is a good tool for health risk assessment which identifies if isolates are from a region of high or low antibiotic use. A MAR index >0.2 indicates a 'high-risk' source of contamination (Davis and Brown 2016). In the present study all the isolates showed Multiple Antibiotic Resistance, which ranged between 0.2 to 0.8 (Table.5). This may be due to their long term exposure to the pollutants in the backwater. The high incidence of multiple antibiotic resistance has been reported in the aquatic environment (Hatha et al.,2005).

Resistance to multiple antibiotics can lead to occurrence of newly emerging resistant bacteria which may be transferred to consumers causing infections that are hard to handle. The incidence of antibiotic resistance in this study corresponds with the findings of Rakic-Martinez et al., 2011, who reported the prevalence of MAR bacteria in waste water. According to Harakeh et al.,(2006), the emergence of antimicrobial bacteria increase in environments where antimicrobials are indiscriminately used by the public. Antibiotic resistant organisms are tough to be treated with, so proper monitoring should be carried out for better health of the backwater and as per report of Das et al., (2013) there is a need of effluent/run off treatment to avoid spread of antibiotic resistant bacteria in the aquatic environment.

Table: 5 MAR index of selected isolates from Biyyam backwaters

Isolates	Multiple antibiotic resistance	MAR index	No. of isolates
Enterobacteriacae	CXM,CX,NIT,NX.TE	0.5	30
Bacillus sp.	E,NX,AMP,P,LZ,CIP,VA	0.7	2
Micrococcus sp.	GEN,E,NX,AMP,P,LZ,TGC,VA	0.8	12
Staphylococcus sp.	AMP,P	0.2	3

Gentamicin (GEN), Erythromycin (E), Levofloxacin (LE), Norfloxacin (NX), Ampicillin (AMP), Penicillin (P), Linezolid (LZ), Ciprofloxacin (CIP),Vancomycin (V), Tigecycline (TGC).

4. Conclusion:

The high coliform load and THB during monsoons is due to the land runoff resulting from heavy rains. However, patchy distribution of organisms in sediments and difficulty in dissociating bacteria from sediment particles seem to be the key factor for the bacterial variation between stations rather than the influence of physicochemical parameters on them. Also, the excessive use of antibiotics in different sectors especially agriculture have led to their presence in the aquatic ecosystem too, leading to most of the selected isolates showing an antibiotic resistance in the present study. Hence, the overall results points to the deteriorating water quality of the Biyyam backwater and therefore represent a public health concern and hence preventive measures need to be applied.

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