Chapter 2
Sources and Distribution of Fecal Coliforms in the Coastal Environment: A Case Study from Chilika Lagoon, Odisha, India

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Abstract Worldwide, the contamination of coastal waters by fecal coliforms (FC) is an ongoing public health problem, and the Chilika Lagoon is no exception to it. Chilika, a brackish water coastal lagoon located in the Odisha state of India, is a biodiversity hotspot supporting commercial fisheries, water birds, and wildlife. Fisherman villages densely surround the lagoon, and dumping of solid waste and domestic sewage into the lagoon has become a common practice. We examined the long-term spatiotemporal distribution of FC in a 3-year period from 2017 to 2019, in the Chilika Lagoon and its drainage rivers. FC loads were represented as the most probable number (MPN) which varied seasonally and sectorally ranging from 0 to 2400 MPN/100 ml. The highest average FC load (17 MPN/100 ml) was recorded during monsoon and the lowest (7 MPN/100 ml) during summer. When FC loads of the lagoon were compared with Central Pollution Control Board (CPCB) guidelines for Class SW-II waters, >100 MPN/100 ml values were obtained from 5 (2017), 8 (2018), and 14 (2019) water samples. Kantabania (142 MPN/100 ml) and Kusumi (189 MPN/100 ml) rivers recorded much higher FC loads. Samples collected from Odialpur, a shoreline village, showed an average FC load of 279 MPN/100 ml, indicating a point source of fecal pollution. The runoff from rivers, sewage disposal from villages, birds, and livestock could be the possible sources of FC loads into the lagoon. Overall, FC loads in the lagoon were mostly within safe limits as prescribed for water used for bathing, contact water sports, and commercial fishing. The low FC load in the lagoon could be due to the quick inactivation and rapid mortality of fecal bacteria by the high salinity of the water. Salinity showed a statistically significant negative relationship ($r = -0.06$, p-value < 0.05) with MPN counts. Phylogenetic analysis of 16S rRNA gene sequences amplified from FC isolates revealed that they belonged to *Shigella flexneri* (seven isolates), *Klebsiella pneumoniae* (three

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isolates), and *Escherichia fergusonii* (one isolate). Antibiotic resistance profiles showed that all isolates were resistant to one or more antibiotics. The large data set on FC would be useful for wetland management authorities and decision-makers towards pollution control monitoring schemes in Chilika Lagoon.

Keywords Fecal coliforms · Salinity · Antibiotic susceptibility · MAR index · Lagoon · Bird guano

1 Introduction

Coastal lagoons are among the most productive and transitional ecosystems between land, freshwater, and marine waters (Pérez-Ruzafa et al. 2019). These coastal ecosystems are threatened by increasing anthropogenic activities such as land reclamation for agriculture, urbanization, tourism, and aquaculture (Pérez-Ruzafa et al. 2019). These activities have led to the dumping and discharge of solid waste and sewage, leading to microbial pollution of the coastal environments (Kataržytė et al. 2018). The microbial pollutants lead to the deterioration of the sanitary quality of water, creating severe health hazards for wildlife and the public. Therefore, continuous monitoring of microbial pollutants using indicator bacteria can elucidate the sanitary status of water which can ultimately serve as a risk assessment tool for recreational and other human activities (Sugumar et al. 2008).

1.1 Microbial Indicators of Bacteriological Quality of Water

The ideal microbial indicator should be (1) non-pathogenic, (2) present in densities that correlate with pathogens, (3) absent in non-contaminated samples, and (4) abundant and easy to detect (Cabral 2010; Motlagh and Yang 2019). Coliform bacteria fulfill most of these criteria and are considered as indicator species for the bacteriological quality of water. The most routinely used microbial indicators of water quality are total coliforms (TC). The FC, also known as thermotolerant bacteria, are a subset of TC that ferment lactose at 44 °C (Cabral 2010). FC are specifically present in the intestines of humans and other warm-blooded animals (Boyd 2015; Motlagh and Yang 2019). They have a relatively short lifespan in comparison to other coliform bacteria and are used as indicator bacteria for monitoring the sewage contamination in natural water bodies (Motlagh and Yang 2019). The presence of large FC loads in a water body suggests a high probability that other pathogenic bacteria, viruses, and protozoa may be present.

Coliforms are rod-shaped, gram-negative, non-spore-forming, β-galactoside permease-positive, β-galactosidase-positive, and aerobic or facultative anaerobic

bacteria (Clesceri et al. 1998; Campbell et al. 2011; Sengupta and Saha 2013). Coliforms of the *Enterobacteriaceae* family belong to the genera *Citrobacter*, *Enterobacter*, *Escherichia*, and *Klebsiella* and can ferment lactose with gas production at 35–37 °C (Cabral 2010). Some coliform bacteria, e.g., *Escherichia coli*, are also common occupants in the bird and mammalian intestinal tracts; others such as *Enterobacter* and *Klebsiella* present on the plant surfaces and in soils are not directly involved with fecal contamination. Therefore, the coliform group comprises both intestinal bacteria and other free-living bacteria that are non-fecal in origin. *E. coli* is constantly found in the feces of human, pets, and farm animals with a much higher abundance (i.e., approximately 10^9 bacteria/g feces) than other coliforms (Sengupta and Saha 2013). Thus, *E. coli* was considered as the only coliform that was directly associated with a fecal source (WHO 2012). A strong positive correlation has been shown between FC and *E. coli* abundances (Town 2001).

The increasing levels of multidrug-resistant bacteria in aquatic environments have been recognized as an emerging global issue. Multiple antibiotic resistances (MAR) have been used to distinguish the fecal pollution sources through antibiotic resistance profiling (Cimenti et al. 2007). The MAR characteristics of FC are useful to understand whether isolates are derived from the high- or low-risk sources of contamination where antibiotics are used frequently or rarely (Krumperman 1983). MAR index $\geq$0.20 (threshold value) denotes a high-risk source of contamination (Riaz et al. 2011).

1.2 Monitoring and Assessment of FC

FC loads have been used as indicators of fecal contamination and pathogen in natural freshwater sources. For instance, Davis et al. (2005) investigated the spatial and temporal distribution of FC from a drinking water reservoir in California, Canyon Lake. The study found a seasonal variation in the concentration of FC. The study also revealed that the correct interpretation of the fecal contamination was largely influenced by the choice of indicator bacteria and the sampling depth. Mitch et al. (2010) examined the accumulation of FC within the Quinnipiac River during winter when there was no disinfection treatment of wastewater effluents practiced. The study suggested a year-round disinfection process and also control of FC from the non-point sources such as river discharge and upstream or downstream of a wastewater outfall.

Various marine environments have also been assessed for the presence of FC bacteria. For example, Chigbu et al. (2004) examined Mississippi Sound, a coastal water body, for inter-annual variations in FC levels and their relationship with various water quality parameters. The study found a negative correlation between FC loads, salinity, and water temperature, whereas a positive correlation with rainfall suggested that freshwater input was a source of FC bacteria. Wiegner et al. (2017) determined the spatiotemporal variation of fecal indicator bacteria from Hilo Bay,

Hawaii, and demonstrated that fecal indicator bacteria increased alarmingly during the high flow period.

Numerous studies have been conducted on the distribution of FC bacteria from coastal lagoons and estuarine ecosystems. For instance, Konan et al. (2009) studied the spatial and temporal variations of FC from a eutrophic coastal lagoon: Grand-Lahou (south coast of West Africa). FC loadings were higher during the monsoon and lower during the dry season. The FC density was greater in the continental influence zone with anthropogenic inputs than in the oceanic influence zone of the lagoon. Yetis and Selek (2014) analyzed FC levels and their relationship with physicochemical parameters in Akyatan Lagoon (Mediterranean coast of Turkey) and revealed higher FC loads in the drainage channels than inside the lagoon. Cooksey et al. (2019) investigated fecal indicator bacteria, coliphages, and human adenovirus from estuarine recreation sites of a brackish Lake Pontchartrain located in southeast Louisiana, USA. The study found no correlation between fecal indicator bacteria/coliphage and human viral pathogens and suggested direct detection of pathogens using alternative microbial pollution monitoring tool. The spatiotemporal distribution of FC bacteria was assessed from Sontecomapan coastal lagoon (Gulf of Mexico) which revealed that FC exceeded USA-EPA maximum permissible values for services involving direct human contact such as harvesting or extracting shellfish (Soto-Castor and Esquivel-Herrera 2020). Furthermore, the study showed that human settlements and anthropogenic activities (cattle and poultry husbandry) as well as droppings from wildlife such as waterfowl and mammals were the sources for fecal contamination in the lagoon. Blackwater Estuary, UK, has also been examined for FC loads in the overlying water and shellfish (oysters) (Florini et al. 2020). The study found low FC levels in high saline water compared to the freshwater zone which was attributed to the increased bacterial cell inactivation under elevated salt concentrations.

The studies on FC from Indian coastal waters have been conducted mostly at small spatial and temporal scales. Mohandass and Bharathi (2003) studied the FC levels from the coastal water and sediments of Nagore situated on the east coast of India and recorded higher coliform counts in the sediments than the water column. In another study from Mumbai, the west coast of India, samples were collected from coastal areas, creeks, and effluent of wastewater treatment facilities, drains, and ocean outfalls (Vijay et al. 2010). FC levels exceeded the prescribed limits for SW-II class of water. Jayakumar et al. (2013) monitored two estuaries and two coastal lagoons along the southeast coast of India (Chennai) and found a considerable FC count, which evidenced the impact of anthropogenic activities. Latha and Mohan (2013) surveyed the FC levels from Kengeri Lake, Bangalore, India, and concluded that the lake's water was unfit for domestic and agricultural uses. In a study from coastal aquifer of Chennai, groundwater contamination was linked to an on-site sanitation system (Jangam and Pujari 2019).

Chilika, Asia's largest brackish water lagoon, is located in the Odisha state of India. The lagoon's shoreline, especially in the periphery of the central, northern, and southern sectors, is densely populated. The lagoon covers three districts of Odisha which are Puri, Khurdha, and Ganjam. There are 424 villages located within

a 2 km range of the lagoon (Kumar and Pattnaik 2012). These villages lack adequate sanitary facilities, and disposal of domestic sewage and open defecation along the shoreline are common. The industries are not well developed in the vicinity of Chilika Lagoon, and fecal pollution is primarily due to the lack of treatment for disposal of domestic sewage and solid waste. This has led to the disposal of considerable quantities of untreated wastes into the lagoon from peripheral villages. Daya River, a major freshwater source to the Chilika Lagoon, transports and disposes approximately 550 million l/day of untreated domestic sewage from the nearby city, Bhubaneswar, India (Ghosh et al. 2006; Joshi and Mishra 2017). Furthermore, wildlife such as birds and buffalo that use Nalabana Bird Sanctuary as a foraging ground may also contribute to FC in Chilika Lagoon.

Mukherjee (2016) used the Modelo Hidrodinâmico (MOHID), a three-dimensional water modeling system, to analyze the intrusion of FC from the Daya River into the Chilika Lagoon. The model predicted that the freshwater influx was mostly responsible for distributing FC in the lagoon, but their propagation was limited due to rapid inactivation. The FC are influenced by several physicochemical factors such as salinity, temperature, sediment texture, and organic matter in estuarine systems (Hassard et al. 2017; Karbasdehi et al. 2017). Parida et al. (2012) have isolated pathogenic bacteria such as *Shigella dysenteriae*, *Bacillus cereus*, *Klebsiella pneumonia*, and *Streptococcus lactis* from the Chilika Lagoon. So far, systematic monitoring of the spatiotemporal distribution of FC has not been carried out; therefore, no baseline data is available from Chilika Lagoon. Considering this knowledge caveat, a long-term monitoring was conducted for the (1) determination of the spatiotemporal distribution of FC in the lagoon, (2) determination of FC loads in major rivers that drain their freshwater into the lagoon, (3) molecular identification and phylogenetic analysis of FC isolates using 16S rRNA gene sequences, and (4) determination of antibiotic resistance profiles of FC isolates.

2 Materials and Methods

2.1 Study Area

Chilika (19° 28′–19° 54′ N and 85° 06′–85° 35′ E) is a coastal brackish water lagoon situated on the east coast of India (Fig. 2.1). The wetland was designated as the first Indian Ramsar site (no. 229) in 1981 due to its rich biodiversity (Srichandan et al. 2015; Behera et al. 2018a). The lagoon is highly productive due to shallow depth and supports an enormous diversity of flora and fauna (Pattnaik et al. 2019, 2020). The lagoon is a well-known wintering ground for thousands of migratory birds and is also home to the Irrawaddy dolphins. The average catchment area of the lagoon is approximately 4146 km^2. The freshwater inflow is mostly brought by Daya, Bhargavi, Luna, and Makara (Srichandan et al. 2015). The lagoon has been divided into four sectors: central, northern, and southern sector, and outer channel

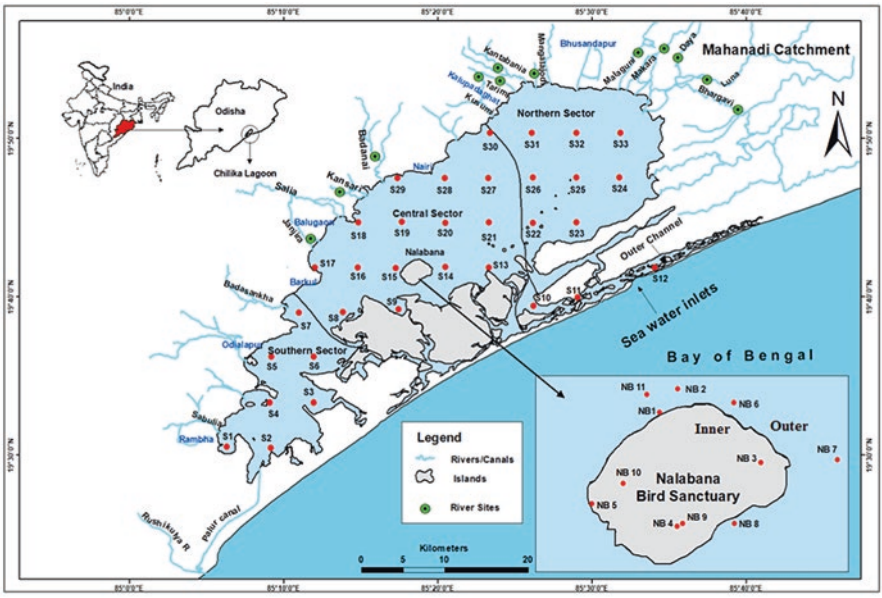

Fig. 2.1 Geographical location of the Chilika Lagoon. Water samples from the lagoon were collected from the 33 GPS fixed stations (shown with red closed circles) located in the four sectors. The sampling sites from the 12 major rivers are shown with green circled dot. The panel shows the detailed location of inner (NB3, NB4, NB5, NB9, and NB10) and outer (NB1, NB2, NB6, NB7, NB8, and NB11) stations in the Nalabana Bird Sanctuary. Shorelines of Barkul, Chandraput, and Odialpur villages were targeted for the FC survey

(Srichandan et al. 2015; Behera et al. 2017). The northern sector is the freshwater zone (salinity 0.5–5) with most of the river influx from Mahanadi River distributaries (Muduli and Pattnaik 2020). The southern sector experiences higher salinity than the central sector due to its connection with the Bay of Bengal (BoB) through the Palur Canal. The central sector is a brackish zone due to the mixing of freshwater and seawater. Both the southern and central sectors experience salinity ranging from 5 to 18. The outer channel is a marine zone with salinity ranging between 18 and 30 due to the direct connectivity to the BoB (Muduli and Pattnaik 2020). The Nalabana Bird Sanctuary covers an area of about 16 km^2 and is situated in the central sector of the lagoon. The sanctuary hosts congregation of millions of migratory and resident birds during winter and act as a nursery and breeding ground.

2.2 Water Sampling

Surface water samples (n = 1188) were collected from the 33 GPS (Global Positioning System) fixed stations. Samples were collected monthly for 3 consecutive years, from January 2017 to December 2019 (Fig. 2.1). A total of 84 water

samples were collected from 12 major rivers during peak monsoon during the 3 years (Fig. 2.1). Shorelines of Barkul, Chandraput, and Odialpur villages were also targeted for the FC survey with water samples ($n = 30$) collected in June 2017. Water samples ($n = 264$) from 11 GPS fixed positions were collected monthly from outside and inside the Nalabana Bird Sanctuary from January 2018 to December 2019 (Fig. 2.1). Water samples were transported on ice and processed on the same day for MPN assessment. Salinity was measured in situ using a Thermo Scientific™ Orion™ Star A212 Conductivity Benchtop Meter.

2.3 Detection of FC Bacteria

Analysis of FC bacteria was conducted using the multiple tube fermentation method and recorded as the MPN of organisms present in 100 ml of the water sample (WHO 1985). Tubes containing double- and single-strength lactose broth with inverted Durham tubes were sterilized. Ten milliliters of the sample was inoculated in three tubes of double-strength media. 3-3 tubes each for single-strength media were inoculated with 1 and 0.1 ml of sample. For each sample, nine tubes were inoculated and incubated at 44 °C for 48 h. The gas-producing tubes and color change of media from purple to yellow were considered positive, and the MPN index was determined by comparing the presumptive test results with the standard table prescribed by Dubey and Maheshwari (2012) (Table 2.1).

Water samples with the positive presumptive results in the multiple tube fermentation tests were further selected for confirmatory analysis on eosin-methylene-blue (EMB) agar plates. EMB is a differential media that can differentiate between lactose-fermenting and non-lactose-fermenting bacteria. In general, the lactose-fermenting bacteria can be differentiated with purple colonies with dark centers. Further, *E. coli* colonies can be differentiated from other lactose-fermenting colonies due to the distinct metallic green sheen (Fig. 2.2a).

The CPCB, New Delhi, has implemented various regulatory guidelines on water quality for different applications to obtain water quality standards (CPCB 1993). According to the guidelines for primary water quality criteria for Class SW-II (waters for bathing, contact water sports, and commercial fishing), the permissible level of FC load is 100 MPN/100 ml of the sample (CPCB 1993). All samples were compared with the CPCB guidelines for FC load risk assessment.

2.4 Molecular Identification and Phylogenetic Analysis

Lactose-fermenting isolates ($n = 11$) cultured from water samples collected during January to March 2019 were selected from the EMB agar plates, and pure colonies were obtained after streaking on Luria-Bertani (LB) agar plates (Table 2.2). The genomic DNA was extracted from pure cultures using FastDNA SPIN Kit, MP

Table 2.1 MPN index for various combinations of positive presumptive test results of FC when three tubes are inoculated with 10 ml, 1 ml, and 0.1 ml of water samples (Modified from Dubey and Maheshwari, 2012)

Sl no.	Combination of positives	MPN index/100 ml
1	0–0–1	3
2	0–1–0	3
3	1–0–0	4
4	1–0–1	7
5	1–1–0	7
6	1–1–1	1
7	1–2–0	1
8	2–0–0	9
9	2–0–1	14
10	2–1–0	15
11	2–1–1	20
12	2–2–0	21
13	2–2–1	28
14	3–0–0	23
15	3–0–1	39
16	3–0–2	64
17	3–1–0	43
18	3–1–1	75
19	3–1–2	120
20	3–2–0	93
21	3–2–1	150
22	3–2–2	210
23	3–3–0	240
24	3–3–1	460
25	3–3–2	1100
26	3–3–3	2400

Biomedicals (Behera et al. 2018b). The concentration of DNA and its purity were assessed spectrophotometrically using an Epoch™ Microplate Spectrophotometer (BioTek, Mumbai, India) and visualized by gel electrophoresis. PCR amplification of 16S rRNA genes was carried out using S1000 Thermal Cycler (Bio-Rad) with primer sets 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) as described earlier by Behera et al. (2018b). PCR conditions include initial denaturation at 95 °C for 1 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min 30 s, and final extension at 72 °C for 10 min. PCR products were confirmed and visualized using 1% agarose gel electrophoresis with ethidium bromide staining. Purification of PCR products was carried out with a HiPurA™ PCR product purification kit (HiMedia) and sequenced using ABI PRISM® 3700 DNA Analyzer (Applied Biosystems).

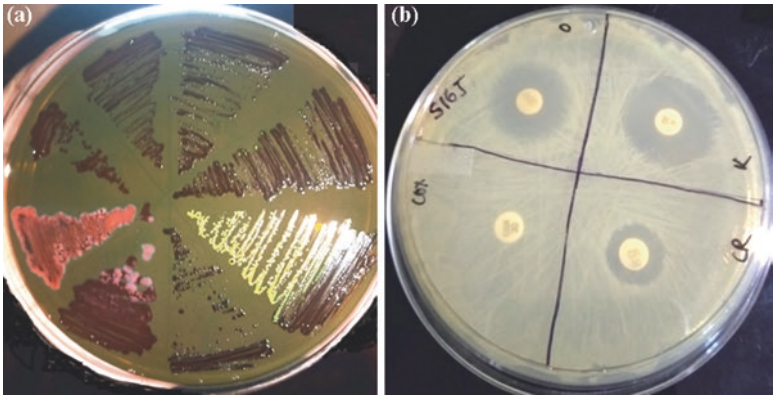

Fig. 2.2 FC isolates including high metallic green sheen producing colonies of *E. coli* on EMB agar plate (**a**) and antibiotic susceptibility testing of isolates showing resistance to cloxacillin and susceptibility to oxytetracycline, kanamycin, and cefaloridine (**b**)

The gene sequences were compared with GenBank sequences using BLASTN tool (http://www.ncbi.nlm.nih.gov/BLAST). Sequence alignments were executed using the ClustalW program (http://www.ebi.ac.uk/clustalw). The editing of aligned sequences and phylogenetic analysis were performed using software MEGA v 6.0 (Tamura et al. 2013). The neighbor-joining method was used for phylogenetic tree construction. Bootstrap test was performed based on 1000 replicates using a Kimura-2 nucleotide evolution model.

2.5 Antibiotic Susceptibility Profiling and MAR Index

The selected FC isolates were analyzed for antibiotic susceptibility profiling against 24 different antibiotics (HiMedia Laboratories, India) using the following discs (μg/disc): ofloxacin (5), trimethoprim (5), sulfadiazine (300), tobramycin (30), cefalexin (30), erythromycin (10), norfloxacin (10), oxytetracycline (30), nalidixic acid (10), nitrofurantoin (300), sulfamethizole (300), bacitracin (10), amoxicillin (30), kanamycin (30), furazolidone (50), amikacin (10), cefadroxil (30), cloxacillin (30), chlortetracycline (30), cefaloridine (30), novobiocin (30), carbenicillin (100), ciprofloxacin (30), and co-trimoxazole (25). The standard disc diffusion method was used to assess the antibiotic resistance pattern on LB agar plates (Bauer 1966). For this, bacterial suspension was inoculated using spread plate technique on solidified LB agar plate, and antibiotic discs were placed individually on the surface. After 24 h of incubation at 30 °C, the isolates were scored either susceptible or resistant for a particular antibiotic based on the appearance of zone around a disc. The experiments were conducted in triplicate, and mean values were considered for antibiotic resistance or susceptibility profiles. Data interpretation was carried out as per the performance standards for antimicrobial disc sensitivity testing recommended by

Table 2.2 Molecular identification, antibiotic susceptibility patterns, and multiple antibiotic resistance (MAR) index of FC isolates. The numbers in the parentheses represent BLAST percent identity scores of 16S rRNA gene sequences

Isolates	Closest neighbour	Antibiotic susceptibility profile and MAR index																							MAR index
		OF	TR	SZ	TOB	CN	E	NX	O	NA	NIT	SM	B	AMX	K	FR	AK	CFR	COX	CT	CR	NV	CB	CIP	COT	
S5J1	*S. flexneri* (NR026331) (99.24%)	S	S	S	S	S	S	S	**R**	S	S	**R**	**R**	S	S	S	S	S	**R**	S	S	S	S	S	S	0.17
S6J2	*E. fergusonii* (NR074902) (99.70%)	S	S	S	S	S	S	S	S	S	S	S	**R**	S	S	S	S	S	**R**	S	S	S	S	S	S	0.08
S8J3	*S. flexneri* (NR026331) (99.85%)	S	S	S	S	S	S	S	S	S	S	**R**	**R**	**R**	S	S	S	S	**R**	S	S	S	S	S	S	0.17
S13J4	*S. flexneri* (NR026331) (99.77%)	S	S	S	S	S	S	S	S	S	S	S	**R**	**R**	S	S	S	S	**R**	S	S	S	S	S	S	0.13
S16J5	*S. flexneri* (NR026331) (99.29%)	S	S	S	S	S	S	S	S	S	S	S	**R**	S	S	S	S	S	**R**	S	S	S	S	S	S	0.08
S25J6	*K. pneumoniae* (NR114715) (99.58%)	S	S	S	S	S	S	S	S	S	S	S	**R**	**R**	S	S	S	S	**R**	S	S	S	S	S	S	0.13
S26J7	*S. flexneri* (NR026331) (99.02%)	S	S	S	S	S	S	S	**R**	S	S	**R**	**R**	**R**	S	S	S	S	**R**	S	S	S	S	S	S	0.21
S28J8	*K. pneumoniae* (NR114715) (99.48%)	S	S	S	S	S	S	S	S	S	S	S	**R**	**R**	S	S	S	S	**R**	S	S	S	S	S	S	0.13

S33J9	*K. pneumoniae* (NR114715) (99.47%)	S	S	S	S	S	S	S	S	S	S	S	**R**	**R**	S	S	S	S	**R**	S	S	S	S	S	S	0.13
S7F1	*S. flexneri* (NR026331) (99.85%)	S	S	S	S	S	S	S	S	S	S	S	**R**	S	S	S	S	S	**R**	S	S	S	S	S	S	0.08
S16M1	*S. flexneri* (NR026331) (99.63%)	S	S	S	S	S	S	S	S	S	S	**R**	**R**	S	S	S	S	S	**R**	S	S	S	S	S	S	0.13

S: susceptible, *R:* resistant, *S. flexneri: Shigella flexneri*, *E. fergusonii: Escherichia fergusonii*, *K. pneumoniae: Klebsiella pneumoniae*, *OF:* ofloxacin, *TR:* trimethoprim, *SZ:* sulfadiazine, *TOB:* tobramycin, *CN:* cefalexin, *E:* erythromycin, *NX:* norfloxacin, *O:* oxytetracycline, *NA:* nalidixic acid, *NIT:* nitrofurantoin, *SM:* sulfamethizole, *B:* bacitracin, *AMX:* amoxicillin, *K:* kanamycin, *FR:* furazolidone, *AK:* amikacin, *CFR:* cefadroxil, *COX:* cloxacillin, *CT:* chlortetracycline, *CR:* cefaloridine, *NV:* novobiocin, *CB:* carbenicillin, *CIP:* ciprofloxacin, *COT:* co-trimoxazole

CLSI (Clinical and Laboratory Standards Institute) (CLSI 2006). The MAR index for a FC isolate was calculated and interpreted using formula: *a*/*b*, where "*a*" refers to the number of antibiotics for which the isolate was resistant and "*b*" refers to the total number of antibiotics used in antimicrobial sensitivity testing (Krumperman 1983).

2.6 Statistical Analysis

The water samples were grouped based on their locations, seasons, and years and were compared for FC load using one-way Welch's ANOVA, followed by the non-parametric post-hoc Games-Howell test at p-value < 0.05. Pearson's correlation coefficient (r) was computed to determine the relationship between FC and salinity.

2.7 Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequences of FC isolates have been submitted to GenBank database at NCBI under the accession numbers MW527410–MW527420.

3 Results and Discussion

3.1 Distribution of FC Bacteria

The FC distribution showed that the Chilika Lagoon was polluted with a varying degree of fecal contamination; however, FC loads were mostly within the safe limits established by CPCB for class SW-II water. In total 5, 8, and 14 water samples exceeded the threshold value of 100 MPN/100 ml of sample in 2017, 2018, and 2019, respectively (Fig. 2.3). The high FC loads were recorded from station S2 (Palur Canal), S3 (Malud-Talatala), S4 (Honeymoon Island), S5 (Gopakuda), S6 (Budhibaranasi), S9 (Veteswara), S14 (Nuapada), S15, S18 (Kalijugeswar), S22 (Tuagambhari), S23 (Tatebandha), S26 (Teeni Muhani Nali), S28 (Baulabandha), S30 (Sorana), and S31 (Kalupadaghat) which could be due to their proximity to shoreline villages or island with human settlements (Fig. 2.1). The mean FC load was 33 and 13 MPN/100 ml in water samples collected from inside and outside of the Nalabana Bird Sanctuary, respectively.

The average FC load from 33 stations of Chilika Lagoon over the study period was 14 MPN/100 ml. The highest FC load, i.e., 2400 MPN/100 ml, was found in S28 (~4 km from Baulabandha village; November 2019), followed by 1100 MPN/100 ml from both S15 (~1 km from Nalabana Bird Sanctuary; October 2019)

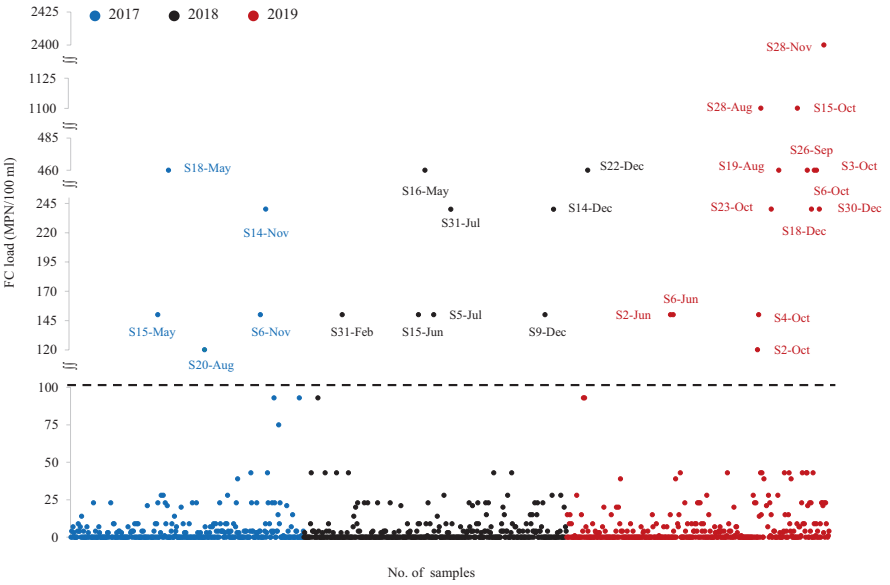

Fig. 2.3 Variation in the FC load in water samples collected during 3-year sampling period from Chilika Lagoon. Each dot represents a sample. Black dashed line denotes the threshold value (100 MPN/100 ml) prescribed as safe limit for FC load according to CPCB guidelines for Class SW-II waters

and S28 (August 2019) (Fig. 2.3). The occurrence of high FC load in S28 could be due to sewage discharge from the Baulabandha village which has a population of ~6660 individuals as per the 2011 census (Fig. 2.1). The monitoring from the peripheral villages showed the highest FC load in samples from Odialpur (279 MPN/100 ml), followed by Chandraput (37 MPN/100 ml) and Barkul (35 MPN/100 ml) (Fig. 2.4a).

The FC load from the rivers was analyzed during the monsoon season when freshwater discharge into the lagoon was the highest. River Kusumi showed the highest FC load (189 MPN/100 ml) followed by Kantabania (142 MPN/100 ml) and Badanai (71 MPN/100 ml) rivers, suggesting that drainage also contributed as a non-point source of FC load into the lagoon (Fig. 2.4b).

3.2 Spatiotemporal Distribution of FC Bacteria

Spatially, the mean FC loads varied between 4 (outer channel) and 19 (central sector) MPN/100 ml in the lagoon (Fig. 2.5a). A spatial pattern in FC distribution among monitoring stations reflected that salinity gradient and spatial factors could be an important factor in controlling the distribution. Consistently, the FC loads showed a statistically significant negative correlation ($r = -0.06$, p-value < 0.05)

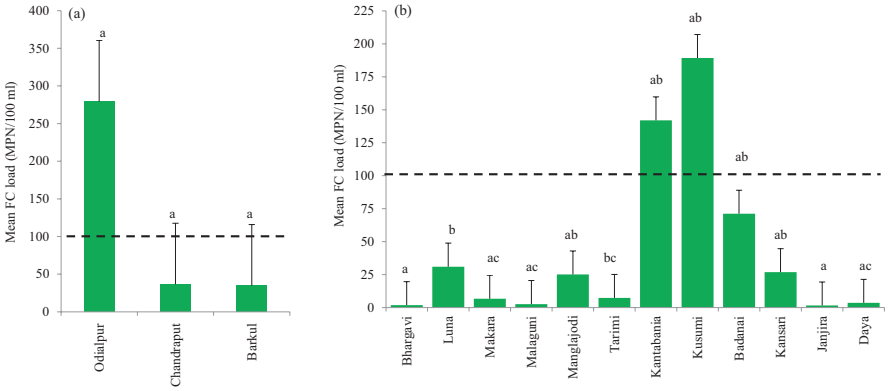

Fig. 2.4 Variation in mean FC load in water samples collected from 3 villages (**a**) and 12 major rivers (**b**) surrounding the Chilika Lagoon. Error bars represent Standard Error. The mean differences were tested by one-way ANOVA. Means with same alphabets are not significantly different (p-value ≥ 0.05). Black dashed line indicates the threshold value (100 MPN/100 ml) prescribed by CPCB guidelines as safe limit for FC load

with the salinity. The higher salinity in outer channel stations and periodic tidal flushing from BoB would lower the FC loads in this sector. The decrease in FC levels with increasing distances from the discharge points in northern sectors could be due to dilution, sedimentation, predation, and inactivation by high salinity and pH (Burkhardt et al. 2000). An earlier study from estuary found that enteric bacteria were typically abundant in head stations which were connected to the river and pollution sources than seawater inlets stations where salinity was high (Mallin et al. 2000). This could further explain the higher FC loads in the central sector where highly populated villages such as Barkul, Baulabandha, and Balugaon are located on the shoreline (Fig. 2.1). The decrease in FC load with increasing salinity was in agreement with studies from the coastal waters of Mississippi Sound (Chigbu et al. 2004), lagoon waters of Grand-Lahou (Konan et al. 2009), Persian Gulf in Bushehr coastal areas (Karbasdehi et al. 2017), and Hilo Bay (Wiegner et al. 2017). Earlier studies also concluded that with an increase in salinity, there is a decrease in the survival rate of FC (Evison 1988; Šolić and Krstulović 1992). This could be due to the increased bacterial cell inactivation because of high salt concentration in water (Hughes 2003; Florini et al. 2020).

Temporal patterns in FC could be influenced by climatic and seasonal factors that affect land runoff, river discharge, and water temperature. The mean FC level ranged between 17 (monsoon and winter) and 7 (summer) MPN/100 ml (Fig. 2.5a). The higher FC load in the monsoon could be attributed to increased land runoff during this season, which may have brought fecal inputs into the lagoon from various sources (Konan et al. 2009; Soto-Castor and Esquivel-Herrera 2020). During winter, the high FC loads in Chilika Lagoon could be due to the resting migratory birds. The lowest FC loads during the summer could be due to an increase in salinity as there is no freshwater flow from rivers into the lagoon. Furthermore, the higher solar

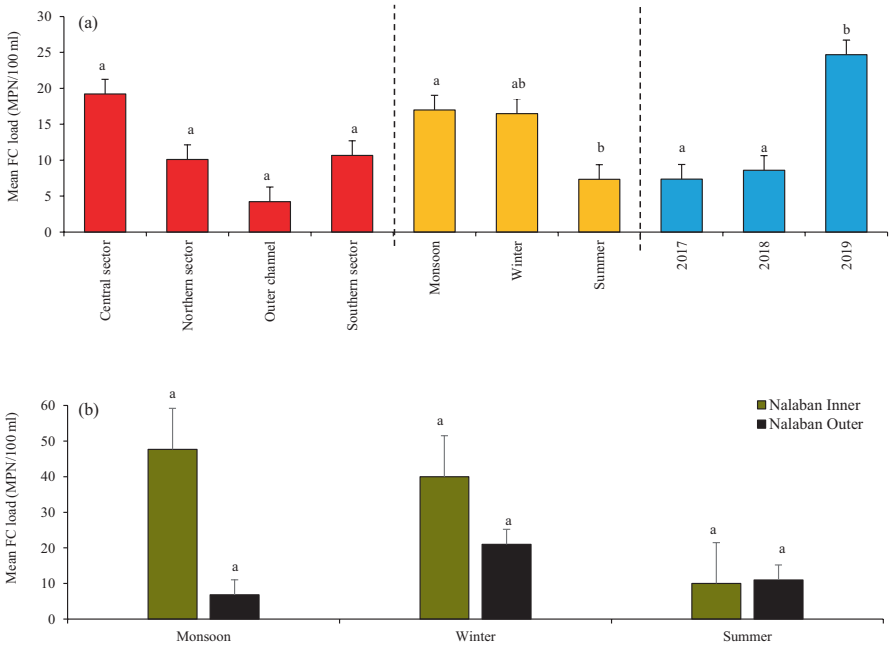

Fig. 2.5 Spatiotemporal variation in the FC load from Chilika Lagoon (**a**) and temporal variation in the FC load from Nalabana Bird Sanctuary (**b**). The value represents mean and error bars represent Standard Error. The mean differences were tested by one-way ANOVA. Means with same alphabets are not significantly different (p-value ≥ 0.05). Nalabana outer includes $n = 132$ samples collected from NB1, NB2, NB6, NB7, NB8, and NB11 stations. Nalabana inner included $n = 120$ samples collected from NB3, NB4, NB5, NB9, and NB10 stations

radiation during summer could also decrease FC viability (Hughes 2003). Seasonal variations in FC levels recorded from Chilika Lagoon were in agreement with earlier studies from marine and estuarine environments (Šolić and Krstulović 1992; Hughes 2003; Florini et al. 2020).

The average FC loads in Nalabana Bird Sanctuary also varied temporally (Fig. 2.5b). The FC levels in samples collected from inner stations of the sanctuary were higher during monsoon (48 MPN/100 ml) followed by winter (40 MPN/100 ml) and summer seasons (10 MPN/100 ml) (Fig. 2.5b). The average FC load varied spatially when compared between the samples collected from outside and inside of the sanctuary. FC loads in samples collected from outside stations (13 MPN/100 ml) of the sanctuary were much lower than inner stations (33 MPN/100 ml). A recent annual bird census carried out during January 2019 estimated a total of 1,047,968 birds from the entire Chilika Lagoon, of which 391,764 were sighted from the sanctuary. The higher FC load during winter could be due to thousands of birds that congregate in the sanctuary during winters for feeding and reproduction. Over 600 families in the vicinity of Chilika rely on the domestic and livestock animals (e.g., Chilika buffalo, cattle) for their livelihood. The total Chilika buffalo population has

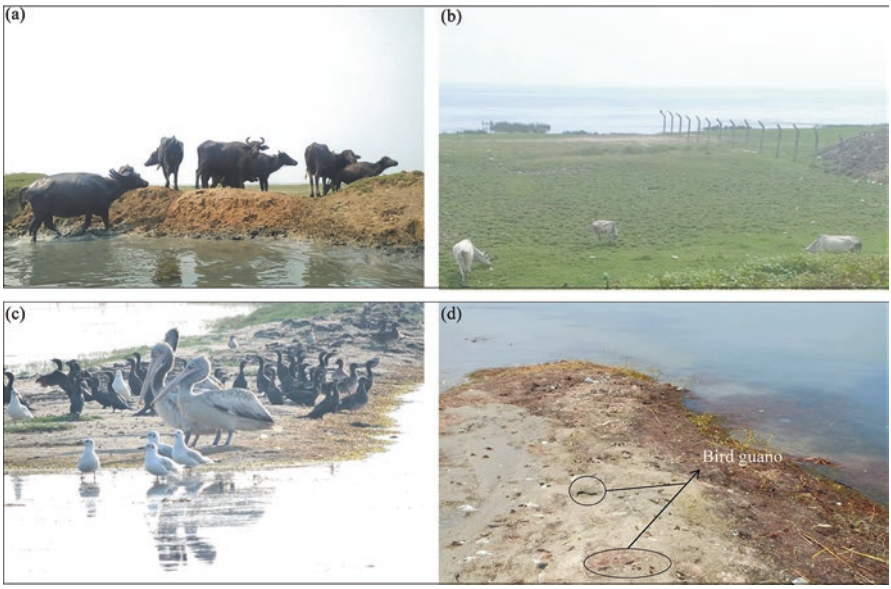

Fig. 2.6 Potential sources of fecal pollution in Chilika Lagoon. Livestock grazing inside Nalabana Bird Sanctuary during summer (**a**) and on the shoreline of Barkul village (**b**), bird flocks resting in the sanctuary (**c**), and their fecal guanos on the mudflats (**d**)

been estimated to be approximately 30,000 (Singh et al. 2017). These buffaloes stand out for their distinct habitat such as consumption of saline water and vegetations and their ability to cope well with high temperature (38–40 °C) during summer. These buffaloes are abundantly present in Bhusandpur, Satapada, Krushnaprasad, Rambha, Parikud, Malud, and Palur area and enter into the lagoon during dry season (Singh et al. 2017). Studies have shown the existence of FC in a wide variety of warm-blooded animals that congregate in coastal wetlands (Chigbu et al. 2004; Siewicki et al. 2007; Yetis and Selek 2014; Soto-Castor and Esquivel-Herrera 2020). Thus, guano and dung inputs from wildlife such as birds and buffaloes could also be one of the potential sources of FC (Fig. 2.6).

3.3 Inter-annual Variation in FC Bacteria

Since FC were monitored over 3 consecutive years from Chilika Lagoon, inter-annual variation in their abundances was also examined. The mean FC load (MPN/100 ml) of the lagoon was the highest (25) in 2019 and the lowest (7) in 2017 (Fig. 2.5a). Inter-annual variability was also observed in FC load from the water samples collected from inside stations of Nalabana Bird Sanctuary. The mean FC load was higher in 2019 (45 MPN/100 ml) compared to 2018 (20 MPN/100 ml). River Kusumi recorded the highest FC loads (534 MPN/100 ml), followed by River

Kantabania (377 MPN/100 ml) and River Badanai (153 MPN/100 ml) during 2019. The higher FC load during 2019 could be attributed to an extremely severe cyclonic storm *Fani* (a Category 4 cyclone) that made landfall on May 3, 2019. The cyclone *Fani* was accompanied by a high precipitation and runoff which could have brought sewage from a variety of sources that resulted in an increase in the FC load of the lagoon. The impact of cyclone *Fani* was consistent with other studies that demonstrated higher FC levels after cyclonic events (Mohandass and Bharathi 2003; Mosley et al. 2004; Wiegner et al. 2017). The mean annual salinity of the Chilika Lagoon was the lowest during 2019 (8.70), whereas the highest annual salinity was recorded during 2017 (13.05). This could further account for the recorded maximum FC load during 2019 when the salinity was the lowest.

3.4 Molecular Identification and Phylogenetic Analysis

Nearly complete 16S rRNA gene sequences (~1300 bp) were obtained from 11 FC isolates and used to generate a phylogenetic tree (Fig. 2.7). All FC isolates were affiliated to family *Enterobacteriaceae* that comprises enteric bacteria and are frequently isolated from water bodies with high fecal contamination (Singh et al. 2020). Isolates S25J6, S28J8, and S33J9 exhibited > 99% sequence similarity with *K. pneumoniae* (NR114715) (Table 2.2 and Fig. 2.7). Isolates S16M1, S7F1, S5J1, S26J7, S16J5, S13J4, and S8J3 displayed > 99% sequence similarity with *S. flexneri* (NR026331). S6J2 isolate showed maximum homology (99.70%) with *E. fergusonii* (NR074902) (Table 2.2, Fig. 2.7). *S. flexneri* can cause shigellosis that leads to death and morbidity in infants and immunosuppressed adults (Ranganathan et al. 2019). *K. pneumoniae* are the most common nosocomial pathogens that can cause various diseases such as pneumonia and urinary tract infections (Cabral 2010). *E. fergusonii* are seldom emerging pathogens related to intestinal and extra-intestinal infections in humans and animals (Wragg et al. 2009).

3.5 Antibiotic Susceptibility Profile and MAR Index

All FC isolates were susceptible to ofloxacin, trimethoprim, sulfadiazine, tobramycin, cefalexin, erythromycin, norfloxacin, nalidixic acid, nitrofurantoin, kanamycin, furazolidone, amikacin, cefadroxil, chlortetracycline, cefaloridine, novobiocin, carbenicillin, ciprofloxacin, and co-trimoxazole (Table 2.2). All FC isolates were resistant to bacitracin and cloxacillin. Six isolates were resistant to amoxicillin, four to sulfamethizole, and two to oxytetracycline. Multidrug resistance is defined as resistance to at least three classes of antibiotics and was recorded in S5J1, S8J3, S26J7, and S16M1 isolates (Table 2.2). Majority of the isolates showed MAR scores < 0.20 indicating that they were derived from low-risk contamination sources where antibiotics are rarely used (Krumperman 1983). However, isolate S26J7 had MAR

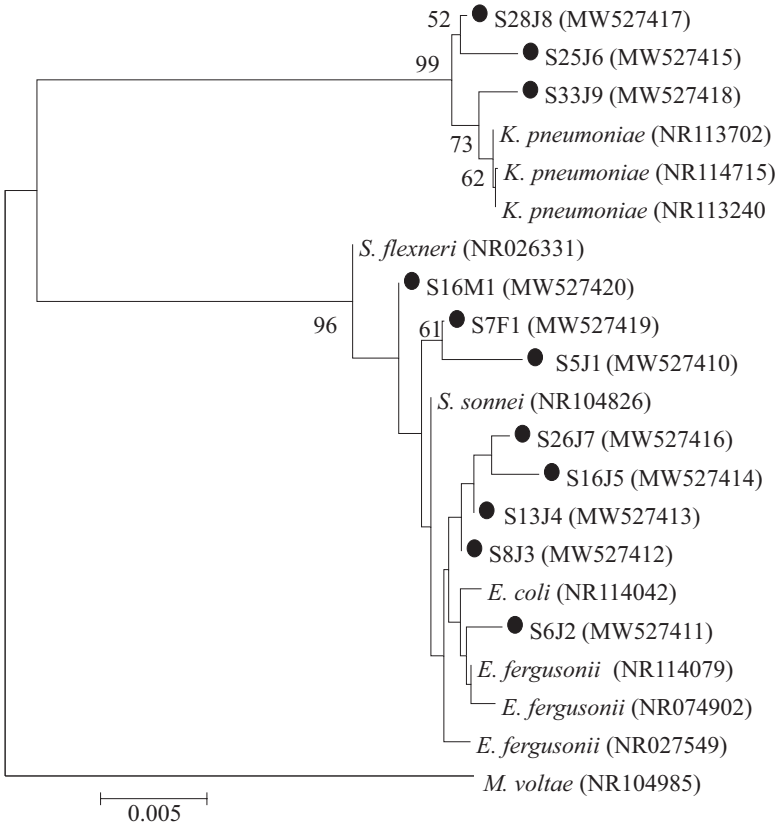

Fig. 2.7 Neighbor-joining phylogenetic tree derived from 16S rRNA gene sequences showing the positions of FC isolates and related organisms. The tree is based on a 1300 bp alignment of 16S rRNA gene sequences. *M. voltae* was used as an out-group to root the tree. GenBank accession numbers are given in parentheses. Numbers at the node points are bootstrap values (%) based on 1000 resamplings. Bootstrap values < 50% are not shown. Bar, 0.005 substitutions per nucleotide position. Black closed circles indicate fecal coliforms isolated in this study. *K. pneumoniae*: *Klebsiella pneumoniae*; *S. flexneri*: *Shigella flexneri*; *S. sonnei*: *Shigella sonnei*; *E. coli*: *Escherichia coli*; *E. fergusonii*: *Escherichia fergusonii*; *M. voltae*: *Methanococcus voltae*

index value of 0.21 indicating that it could have originated from a high-risk contamination sources (e.g., human wastes, commercial poultry farms, aquaculture farms, and dairy cattle and swine farms) where antibiotics are often used. Antibiotic-resistant FC bacteria can enter into the lagoon through sewage, land runoff, river discharge, and open defecation. Studies have also shown that migratory birds and livestock can also be a source of antibiotic resistance genes (ARGs) and bacteria (ARBs) in aquatic habitats (Huang et al. 2019; Cao et al. 2020). The ARGs may be transmitted to other microorganisms through horizontal gene transfer (Mishra et al. 2018). Furthermore, the ARB can enter into humans through contact with water

during fishing and recreational activities causing a potential threat to human health. The high prevalence of antibiotic resistance in the FC bacteria raises concerns about their continued use as safe indicator species.

4 Conclusion

The present study, for the first time, investigated the distribution of fecal bacteria in the Chilika Lagoon, major drainage rivers, and shoreline villages. The results indicated that the FC responded to salinity regimes (marine versus freshwater), locations (sectors), and seasons. Direct discharge from rivers (specifically Kusumi, Kantabania, and Badanai), wildlife, and untreated sewage and open defecation from shoreline villages were among the primary sources of fecal contamination in the Chilika Lagoon. A total of 27 water samples from the lagoon exceeded the MPN index when compared with the CPCB, New Delhi guidelines for Class SW-II waters. A total of 13 water samples from Nalabana Bird Sanctuary and 4-4 water samples each from the river and village sites exceeded the CPCB threshold value. Overall, the number of samples from the lagoon that exceeded the prescribed CPCB guidelines was not high, but continuous monitoring of FC should be practiced. Furthermore, the study revealed an inverse correlation between the FC loads and salinity which could be one of the reasons for the low abundances of FC in the lagoon. The high salinity of the lagoon combined with dynamic changes in physicochemical factors and climatic factors (e.g., solar radiation) could be the reason for the rapid inactivation of FC in the lagoon. The antibiotic-resistant profiles should be monitored on a continuous basis to foresee the emergence and widespread of MAR. Effective measures must be taken to prevent the development and spread of new resistance from various point and non-point sources of pollution. Further research should include FC assessment from sediments which could also act as reservoirs of FC.

Chilika Lagoon is a major resource for the state's commercial fisheries, recreation, tourism, biodiversity, and aesthetics; therefore, disease interception scheme must be implemented in order to protect the public. Thus, achieving and ensuring good water quality is a prime concern for wetland managers and policymakers. The microbial pollution from fecal bacteria is an emerging issue considering the lagoon's large size and lack of sanitary facilities in the shoreline villages. The present study provided a baseline data on spatiotemporal distributions, molecular identification, and MAR indexing of FC, which would be useful in formulating conservation plans and monitoring schemes in Chilika Lagoon.

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