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Antibiotic and Heavy Metal Resistance in Bacteria from Organs of Sewage Fed Farm Fishes

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ABSTRACT

Bacterial populations from organs (viz., liver, spleen, kidney and gill) of *Clarias batrachus* of the sewage fed water areas near IISCo slag disposal site, Dharampur on northern side of Damodar River, Asansol, West Bengal, India, were enumerated, followed by determination of resistance for antibiotics and heavy metals. Maximum resistance is shown against ampicillin (95%) and minimum against ciprofloxacin (5%). Most of the isolates exhibited an increasing order of tolerance for the metals (µg/mL) copper (200), cadmium (200), iron (400) and chromium (400), with minimum inhibitory concentration (MIC) ranging from <50 to 1600 µg/mL. A total of 100 bacteria have been successfully isolated from internal organs of *Clarias batrachus* (Aeromonas species (20%); *Escherichia coli*, (45%); *Bacillus* species (4%); *Pseudomonas aeruginosa* (6%), *Staphylococcus aureus* (18%) and coagulate-negative *Staphylococci aureus* (7%)). In terms of antibiotic susceptibility testing, each isolate was tested against 10 antibiotics. The multiple antibiotic resistance (MAR) index of the isolated bacterial ranged from 0.2-0.7. These observations indicate that the bacteria isolates are from a high risk source where antibiotics are frequently used, possibly from sewage effluents. Significant occurrence of bacterial population in organs of fish with high incidence of resistance for antibiotics and heavy metals may pose risk to fish fauna and public health.

KEY WORDS: *CLARIAS BATRACHUS*, ANTIBIOGRAM, HEAVY METAL RESISTANCE, MULTIPLE ANTIBIOTIC RESISTANCE.

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INTRODUCTION

Clarias batrachus, commonly called asian catfish is a threatened (Hossain et al., 2006; Ahmad et al., 2012; Roy et al., 2019), and critically endangered species (Binoy, 2010). It's a promising hardy fish, excellent nutritional profile and market price is high (Hossain et al., 2006; Goswami 2007; Debnath 2011).Tham et al., (2009) have reported heavy metal inhibitions by AchE from *C. batrachus*. Heavy metals are ubiquitous and persist as environmental pollutants that are introduced into the environment through anthropogenic activities, like

mining and smelting, also well as through irrigation and other sources of commercial waste. However, untreated or partially treated wastewaters introduce an enormous amount of contaminates particularly heavy metals into agricultural lands (Wang and Tao, 1998; Boateng et al., 2019). The existence of heavy metals within the environment represents a big and long-term environmental hazard since they're not biodegradable and have a tendency to accumulate in living organisms (Kobya et al., 2005; Liao et al., 2008; Genchi et al., 2020).

Indiscriminate use of various antibiotics has caused development of resistance for various antimicrobials and chemotherapeutic agents among the gut flora of homeotherms. Use of antibiotics will exert more selective pressure and resistant pathogens are going to be encontered more frequently (MacMillan, 2001; Priyadarshini et al., 2020). Resistance to antibiotics and metals occurs simultaneously when the genes specifying resistant phenotypes are located together an equivalent genetic element like a plasmid, transposon, or integron (Chapman 2003; Frost et al., 2005; Venner et al., 2009; McMillan et al. 2019). This present study was conducted to evaluate the antibiotic and heavy metal tolerance of bacteria obtained from internal organs of *Clarias batrachus*.

MATERIAL AND METHODS

Clarias batrachus were collected from the sewage fed water areas near IISCo slag disposal site, Dharampur on northern side of Damodar River at an altitude of 75 meters. Longitudinally it is at 86°55' East and latitudinally at 23°40' North Asansol, West Bengal, India. Total of 180 fish samples were collected from June to August 2019. 60 samples were collected at the first visit and was repeated three times. Fish were collected in sterile plastic bags with labelling from sites. Fish samples were transported to the laboratory in cooler box and tested on the same day. Fish samples were sacrificed to dissect out aseptically the liver, spleen, kidney and gills (Pathak and Gopal, 2005) placed in labelled bottle containing peptone water and buffered peptone water. The contents were homogenized and the homogenate from peptone water was streaked using a sterile loop on blood agar and MacConkey agar. Streaked plates were incubated at temperature (37 °C) aerobically for 24 hours. Homogenate in buffered peptone water was incubated for 24 hours at 37 °C. After incubation, 1 ml of the homogenate tranferred into Rappaport Vassiliadis (RV) agar and icubated for 24 hours at 37 °C. On third day, loopful of the sample containing bacteria was streaked on Xylose Lysine Deoxychocolate (XLD) agar and incubated for 24 hours at 37 °C (Markey et al. 2013).

The isolated bacteria were identified using morphological characteristics, Gram staining and biochemical tests (oxidase, motility, indole, citrate, lysine decarboxylase, urease. Triple sugar iron). The scheme of Cowan and Steel (1993) was followed for characterization and identification of strains, and the results were interpreted using Bergey's Manual of Systematic Bacteriology (Staley et al. 1989). Autoclaved fish and uninoculated media were used as negative controls. Different strains of bacteria obtained from MTCC Chandiagrh, India (Aeromonas 646, *Bacillus niacini* MTCC 8323, *Escherichia coli* MTCC 739, *Pseudomonas aeruginosa* MTCC 2453, *Staphylocccus aureus* MTCC 2940) were used as positive control.

Antibiotic susceptibility testing was conducted consistent with Kirby and Bauer disk diffusion method (Bauer et al., 1966) by employing commonly used antibiotics (namely 30 µg of amikacin, 30 µg of amoxicillin, 10 µg of ampicillin, 30 µg of chloramphenicol, 5 µg of ciprofloxacin, 10 µg of gentamycin, 5 µg of levofloxacin, 10 µg of sparfloxacin, 25 µg of streptomycin and 30 µg of tetracyline). The colonies were transferred into agar plate. The swab was then streaked in three, different directions over the surface of plate of Mueller-Hinton Agar such that a uniform well spread out inoculum is achieved. After 18 hours of incubation at a specific temperature (37±1 °C) the plates were examined and the diameters of the inhibition zone was measured to the nearest millimeter. Inhibitions were measured and the result interpreted using Clinical Laboratory Standard Institute (2017).

The strain isolated was tested to four metals by Agar dilution method (Malik and Ahemad, 2006; Kinare and Shingadia, 2014). Stock solutions of 104 µg/mL were prepared by dissolving the precise quantities of the follwing metal salts: $CdCl_2$ (SRL), $K_2Cr_2O_7$ (SIGMA-ALDRICH), $CuSO_4$. 5H2O(SIGMA-ALDRICH) and $FeCl_3$ (MERCK) in water and sterilized. 20 mL of agar was poured into petri plates and therefore the volume of metal stock solutions was calculated by the formula: C1 × V1 = C2 × V2, where C1 is the metal concentration avaiable, V1 is the volume of stock solution used, C2 is that the concentration of metal in agar, and V2 is that the volume of agar. Then the isolated strains were streaked onto the medium-containing increasing concentrations of metal salts using sterile loops. Then, plates were sealed and incubated at 30 °C for five days. Plate-containing only agar was also inoculated and incubated to act as control. Rock bottom concentration of every metal at which no growth occurred in comparison to the control plates was considered as MIC value.

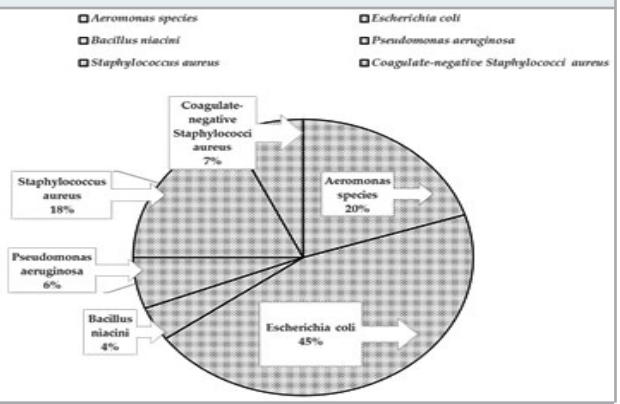
The multiple antibiotic resistance (MAR) index of isolates against tested antibiotics were determined as described by Krumperman (1983) using the formula: a/b, where "a" represents the number of antibiotics against which a particular isolate was resistant and "b" the total number of antibiotics used for test.

RESULTS AND DISCUSSION

The bacteria isolated were Aeromonas species, *Escherichia coli, Bacillus niacini, Pseudomonas aeruginosa*, *Staphylococcus aureus*, and coagulate-negative *Staphylococci aureus* which were identified using morphological properties, gram staining, and series of biochemical tests. Total 100 bacteria were isolated from

liver, spleen, kidney and gill. The bacterial load, i.e., total viable count, was found to be 5.62×10^4, 4.12×10^4, 2.30×10^4 and 1.76×10^4 c.f.u./mL in liver, spleen, kidney and gill of the experimental fish, respectively. The percentage prevalance of the isolated bacteria were *Aeromonas* species (20%); *Escherichia coli*, (45%); *Bacillus* species (4%); *Pseudomonas aeruginosa* (6%), *Staphylococcus aureus* (18%) and coagulase-negative *Staphylococci aureus* (7%). Prevalence rate of isolated bacteria are plotted in (Figure 1).

Figure 1: Prevalance rate of the isolated bacteria



The antibiotic resistance among random bacterial isolates from all four organs has shown a full range of resistance (0–100%) for ten common antibiotics of therapeutic and prophylactic uses among human beings, and in fish aquaculture. Resistance was found to be maximum among the isolates from spleen, kidney, and liver, while it was minimum among those from gill. Maximum average resistance was exhibited for ampicillin (95%) and tetracycline (75%) and minimum for ciprofloxacin (05%) (Table 1).

The heavy metal resistance percentage among bacterial isolates from fish organs presented in Table 2. The maximum tolerance, in general, was observed for chromium and iron (400 µg/mL), while it was minimum for copper and cadmium (200 µg/mL). The MIC value of isolates is presented in Table 3. Test isolates were also found to be tolerant to different concentrations of various toxic heavy metals as evidenced by their MICs ranging from <50 to 1600 µg/mL (Table 3). Except some gill isolated bacteria (ADCG14-ADCG21) the MAR index of all isolates show values higher than 0.2 (Table 4). All the isolate (except ADCG14-ADCG21) were multidrug resistant (resistant to three or more drugs). The trend of MAR index is alarming for the bacterial species isolated from the site.

Table 1. Antibiotic resistance (%) among bacterial isolates from fish organs

Antibiotics (µg/mL)	Fish organs				Average resistance
	Liver	Spleen	Kidney	Gill	
Amikacin (AMK) (30)	90	80	60	30	65
Amoxycillin (AMX) (25)	90	80	70	40	70
Ampicillin (AMP) (10)	100	100	90	90	95
Chloramphenicol (CHL) (30)	80	50	50	20	50
Ciprofloxacin (CIP) (5)	10	10	00	00	05
Gentamycin (GEN) (10)	50	40	20	10	30
Levofloxacin (LVX) (5)	50	40	10	00	25
Sparfloxacin (SPX) (10)	60	50	30	20	40
Streptomycin (STR) (25)	20	10	10	00	10
Tetracyline (TET) (30)	100	80	70	50	75

Table 2. Heavy metal resistance (%) among bacterial isolates from fish organs

Heavy metals (µg/mL)	Fish organs				Average resistance
	Liver	Spleen	Kidney	Gill	
Copper (200)	10	00	00	00	2.5
Chromium(400)	100	100	80	70	87.5
Cadmium (200)	30	20	10	00	15
Iron (400)	90	100	80	50	80

Table 3. MIC values for different heavy metals among bacterial isolates from fish organs

Heavy metals	MIC values (µg/mL) for different Fish organs			
	Liver	Spleen	Kidney	Gill
Copper	< 50	-	-	-
Chromium	1600	1600	800	400
Cadmium	100	50	< 50	-
Iron	1600	800	800	400

Contamination of river water with municipal sewage and industrial effluent results in the occurrence of pathogenic microorganisms, particularly fecal bacteria and toxic metals, above their maximum permissible limits (Chatterjee et al., 2010, Iloms et al., 2020). Fish in such water are exposed to these bacteria and metals, which bioconcentrate in different organs of fish. It has been observed to be maximum in liver and minimum in gills. Thus, it appears from these findings that soft tissues in massive organs are more prone to bioconcentration of bacteria, leading to incidence of infectious diseases among the aquatic fauna. This may be due to availability of more nutrients and lack of exposure to the surroundings.

Table 4. Code number assigned with organs from which the bacteria is isolated, antibiotic resistance Profile and multiple antibiotic resistance index of the isolated bacteria from site

Sl No	Code No assigned to isolate	Organs from Isolated	Antibiotic resistant Profile	Resistant to number of Antibiotics	MAR Index
1	ADCL1	Liver	AMK,AMX,AMP,TET,CHL,GEN	6	0.6
2	ADCL2	Liver	AMK,AMX,AMP,TET, CHL,LVX	6	0.6
3	ADCL3	Liver	AMK,AMX,AMP,TET, CHL,GEN,SPX	7	0.7
4	ADCL4	Liver	AMK,AMX,AMP,TET, CHL,LVX,SPX	7	0.7
5	ADCL5	Liver	AMK,AMX,AMP,TET, CHL,GEN,SPX	7	0.7
6	ADCL6	Liver	AMK,AMX,AMP,TET, CHL,LVX,SPX	7	0.7
7	ADCL7	Liver	AMK,AMX,AMP,TET, CHL,GEN,SPX	7	0.7
8	ADCL8	Liver	AMK,AMX,AMP,TET, CHL,LVX,SPX	7	0.7
9	ADCL9	Liver	AMK,AMX,AMP,TET, CHL,GEN,SPX	7	0.7
10	ADCL10	Liver	AMK,AMX,AMP,TET, CHL,LVX,SPX	7	0.7
11	ADCL11	Liver	AMK,AMX,AMP,TET, CHL,GEN,SPX	7	0.7
12	ADCL12	Liver	AMK,AMX,AMP,TET, CHL,GEN,SPX	7	0.7
13	ADCL13	Liver	AMK,AMX,AMP,TET, CHL,LVX,SPX	7	0.7
14	ADCL14	Liver	AMK,AMX,AMP,TET, CHL,LVX,SPX	7	0.7
15	ADCL15	Liver	AMK,AMX,AMP,TET, CHL,LVX,SPX	7	0.7
16	ADCL16	Liver	AMK,AMX,AMP,TET, CHL,LVX	6	0.6
17	ADCL17	Liver	AMK,AMX,AMP,TET, CHL,LVX	6	0.6
18	ADCL18	Liver	AMK,AMX,AMP,TET, CHL,GEN	6	0.6
19	ADCL19	Liver	AMK,AMX,AMP,TET, CHL,GEN	6	0.6
20	ADCL20	Liver	AMK,AMX,AMP,TET, CHL,GEN	6	0.6
21	ADCL21	Liver	AMK,AMX,AMP,TET, CIP,GEN,STR	7	0.7
22	ADCL22	Liver	AMK,AMX,AMP,TET, CIP,STR	6	0.6
23	ADCL23	Liver	AMK,AMX,AMP,TET,CIP,LVX,STR	7	0.7
24	ADCL24	Liver	AMP,TET,LEV,GEN,STR,SPX	6	0.6
25	ADCL25	Liver	AMP,TET,LEV,GEN,STR,SPX	6	0.6
26	ADCS1	Spleen	AMP,AMK,AMX,TET,GEN,LVX	6	0.6
27	ADCS2	Spleen	AMP,AMK,AMX,TET, CHL,SPX	6	0.6
28	ADCS3	Spleen	AMP,AMK,AMX,TET, GEN,LVX	6	0.6
29	ADCS4	Spleen	AMP,AMK,AMX,TET, CHL,SPX	6	0.6
30	ADCS5	Spleen	AMP,AMK,AMX,TET, CHL,SPX	6	0.6
31	ADCS6	Spleen	AMP,AMK,AMX,TET, GEN,LVX	6	0.6
32	ADCS7	Spleen	AMP,AMK,AMX,TET, CHL,SPX	6	0.6
33	ADCS8	Spleen	AMP,AMK,AMX,TET, GEN,LVX	6	0.6
34	ADCS9	Spleen	AMP,AMK,AMX,TET, CHL,SPX	6	0.6
35	ADCS10	Spleen	AMP,AMK,AMX,TET, GEN,LVX	6	0.6

Table 4 Continue

36	ADCS11	Spleen	AMP,AMK,AMX,TET, CHL,SPX	6	0.6
37	ADCS12	Spleen	AMP,AMK,AMX,TET	4	0.4
38	ADCS13	Spleen	AMP,AMK,AMX,TET	4	0.4
39	ADCS14	Spleen	AMP,AMK,AMX,TET	4	0.4
40	ADCS15	Spleen	AMP,AMK,AMX,TET, CHL,SPX	6	0.6
41	ADCS16	Spleen	AMP,AMK,AMX,TET, GEN,LVX	6	0.6
42	ADCS17	Spleen	AMP,AMK,AMX,TET, CIP,STR	6	0.6
43	ADCS18	Spleen	AMP,AMK,AMX,TET	4	0.4
44	ADCS19	Spleen	AMP,AMK,AMX,TET,CHL,SPX	6	0.6
45	ADCS20	Spleen	AMP,AMK,AMX,TET, CIP,STR	6	0.6
46	ADCS21	Spleen	AMP,CHL,SPX,CIP,STR	5	0.5
47	ADCS22	Spleen	AMP, CHL,SPX, GEN,LVX	5	0.5
48	ADCS23	Spleen	AMP, CHL,SPX, GEN,LVX	5	0.5
49	ADCS24	Spleen	AMP, CHL,SPX, GEN,LVX	5	0.5
50	ADCS25	Spleen	AMP, CHL,SPX, GEN,LVX	5	0.5
51	ADCK1	Kidney	AMK,AMX, AMP,TET	4	0.4
52	ADCK2	Kidney	AMK,AMX, AMP,TET	4	0.4
53	ADCK3	Kidney	AMP, CHL, SPX,TET	4	0.4
54	ADCK4	Kidney	AMK,AMX, AMP,TET	4	0.4
55	ADCK5	Kidney	AMP, CHL, LVX,TET	4	0.4
56	ADCK6	Kidney	AMK,AMX, AMP,TET	4	0.4
57	ADCK7	Kidney	AMK,AMX, LVX,TET	4	0.4
58	ADCK8	Kidney	AMK,AMX, LVX,TET	4	0.4
59	ADCK9	Kidney	AMX, AMP, GEN,TET	4	0.4
60	ADCK10	Kidney	AMK AMP, GEN,TET	4	0.4
61	ADCK11	Kidney	AMK,AMX, GEN,TET	4	0.4
62	ADCK12	Kidney	AMX, AMP, GEN,TET	4	0.4
63	ADCK13	Kidney	AMK,AMX, AMP,TET	4	0.4
64	ADCK14	Kidney	AMK AMP, CHL, SPX	4	0.4
65	ADCK15	Kidney	AMK, AMP, CHL,TET	4	0.4
66	ADCK16	Kidney	AMX, AMP, CHL,TET	4	0.4
67	ADCK17	Kidney	AMX, AMP, CHL,STR	4	0.4
68	ADCK18	Kidney	AMX, AMP, CHL,SPX,TET	5	0.5
69	ADCK19	Kidney	AMP, AMP, GEN,STR	4	0.4
70	ADCK20	Kidney	AMK, AMP, CHL, STR	4	0.4
71	ADCK21	Kidney	AMK, AMP, CHL, SPX,TET	5	0.5
72	ADCK22	Kidney	AMX, AMP, CHL, SPX	4	0.4
73	ADCK23	Kidney	AMK,AMP, CHL, SPX	4	0.4
74	ADCK24	Kidney	AMX, AMP, CHL, SPX,TET	5	0.5
75	ADCK25	Kidney	AMK, AMP, CHL, SPX	4	0.4
76	ADCG1	Gill	AMK, AMP,TET	3	0.3
77	ADCG2	Gill	AMX, AMP, TET	3	0.3
78	ADCG3	Gill	AMK, AMP, TET	3	0.3
79	ADCG4	Gill	AMX, AMP, TET	3	0.3
80	ADCG5	Gill	AMK, AMP, TET	3	0.3
81	ADCG6	Gill	AMX, AMP, TET	3	0.3
82	ADCG7	Gill	AMX, AMP, TET	3	0.3
83	ADCG8	Gill	AMK, AMP, TET	3	0.3
84	ADCG9	Gill	AMX, AMP, TET	3	0.3
85	ADCG10	Gill	AMX, AMP, TET	3	0.3
86	ADCG11	Gill	AMK, AMP, TET	3	0.3
87	ADCG12	Gill	AMX, AMP, TET	3	0.3
88	ADCG13	Gill	AMK, AMP, TET	3	0.3
89	ADCG14	Gill	AMK, AMP	2	0.2
90	ADCG15	Gill	AMX, AMP	2	0.2
91	ADCG16	Gill	AMX, GEN	2	0.2

Table 4 Continue

92	ADCG17	Gill	AMX, GEN	2	0.2
93	ADCG18	Gill	AMP, GEN	2	0.2
94	ADCG19	Gill	AMP, SPX	2	0.2
95	ADCG20	Gill	AMP, CHL	2	0.2
96	ADCG21	Gill	AMP, CHL	2	0.2
97	ADCG22	Gill	AMP, CHL, SPX	3	0.3
98	ADCG23	Gill	AMP, CHL, SPX	3	0.3
99	ADCG24	Gill	AMP, CHL, SPX	3	0.3
100	ADCG25	Gill	AMK, AMP, SPX	3	0.3

Bioconcentration of aquatic bacteria such as coliforms, streptococci, and aeromonads in gut, liver, and muscles of tilapia fish grown in a sewage-contaminated pond has also been noticed (Fattal et al., 1993; Wamala et al., 2018). The resistance exhibited for ciprofloxacin, levofloxacin, streptomycin and gentamycin is a signal of the effectiveness of broad-spectrum antibiotics of the present generation. With these observations it appears that the source of the problem of antibiotic resistance in riverine ecosystems is fecally contaminated water, and fish populations in them plays important role in creating resistance. Antibiotic resistance patterns in the bacterial population in an aquatic ecosystem have been found to be useful in identifying non point sources of fecal pollution (Wiggins et al., 1999; Labrador et al., 2020).

The occurrence of resistance for common antibiotics is, further, an indication of indiscriminate use of these antibiotics, leading to constraint in antimicrobial therapy for infectious diseases. The loss of antibiotic susceptibility among the aquatic bacteria has been observed to be affected to a considerable extent by the physicochemical qualities of water and seasonal variations (Pathak et al., 1993). In addition to assessment of loss of antibiotic susceptibility, the test isolates were also found to be tolerant to different concentrations of various toxic heavy metals as evidenced by their MICs. The isolates from visceral organs, i.e., spleen, kidney, and liver, exhibited maximum resistance for ampicillin, tetracycline, and amoxicillin with the highest tolerance for iron and chromium; while the isolates from gills showed minimum resistance for ampicillin and tetracycline with rather low tolerance for cadmium and copper.

These observations indicate that visceral organs provide better conditions for bacterial growth and biological activity than exposed organs such as gill. Increase in the MIC of toxic metals as well as antibiotic resistance among aquatic bacterial populations is also an indication of risk to the safety of the aquatic ecosystem, fish fauna, and ultimately human health. MAR values were ranging from 0.2 to 0.7. MAR indexes of the present work revealed that bacteria from locally raised fish may has been exposed to test antibiotics. McPhearson et al., 1991 reported that the MAR index of bacteria from a catfish pond, near a river where antibiotic was commonly used as treatment, was as high as 0.76.

Currently, local fish farmers employ amoxicillin to treat fish diseases. The present results proven that amoxicillin is no longer effective, since maximum bacterial isolates were sensitive to it. It appears that the emergence of resistance is also influenced by the physicochemical characteristics of water and several environmental factors including hospital and *aquaculture waste disposal* (Rhodes et al., 2000; Iwu et al., 2020) along with the form and bioavailability of metals in the ecosystem. Resistance also develops from a non-specific mechanism with gene regulation of plasmids and chromosomes which may be heritable due to the presence of a resistance factor (R-factor) among the aquatic bacterial population (Silver and Walderhaug, 1992). The infections caused by the pathogenic bacteria with R-plasmids may pose a risk of therapeutic problems to public health and fish population. Thus, the water bodies with antibiotic and metal resistant bacteria serve as an environmental reservoir and source for the development of this trait among opportunistic pathogens and constitute a significant public concern.

Therefore, such studies should be considered for the selection of antibiotics in dealing with water-borne diseases, particularly among fishermen and fish consumers. These findings indicate that sewage and industrial pollution are responsible for the emergence of bacterial resistance and deterioration of water quality, along with risk to biodiversity of the hydrobionts and the human health.

Conflict of Interest: We declare that we have no conflict of interest

Ethical Approval: This article is not under consideration or published elsewhere. Ethical clearance for the study was obtained from IAEC, Approval No. 17/IAEC (05)/ RNLKWC/2019, Dated 27.07.2019.

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