J. Bio-Sci. 32(2): 61-69, 2024 **ISSN 1023-8654**
http://www.banglajol.info/index.php/JBS/index
DOI:https://doi.org/10.3329/jbs.v32i2.75758

INVESTIGATION ON THE DISTRIBUTION OF ANTIBIOTIC RESISTANT BACTERIA IN DRY FISH OF CHALAN BEEL AREA FROM NORTHWESTERN PART OF BANGLADESH

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Abstract

Dried fish, a valuable animal protein source, may pose health risks due to microbial contamination influenced by water quality, hygiene practices, handling, packaging, and transportation. This research focuses on conducting an in-depth analysis of the occurrence and antibiotic resistance patterns of bacteria, identified through 16S rRNA sequencing, in different dried fish varieties obtained from the *Chalan beel* area in Northwestern Bangladesh. For the purpose of this study, homogenization of collected samples was carried out and resulting homogenates were refrigerated at a temperature of -20°C. Then the samples were subjected to a series of 10-fold dilutions and spread over 1.5% nutrient agar supplemented with Tetracycline. Thereafter, plates were incubated at 37°C for a duration of 24 hours to form bacterial colonies. Twelve aseptically collected dried fish samples had a total viable count ranging from 2.0×10^2 to 6.5×10^5 CFU/ml, with tetracycline influence at 32 µg/ml (7×10^2 to 5.4×10^3 CFU/ml) and 64 µg/ml (3.0×10^2 to 2.1×10^3 CFU/ml). Notably, Tengra fish (*Mystus* sp.) exhibited the highest viable count 6.5×10^5 CFU/ml (Control), while Darikana (*Esomus danricus*), Chela (*Salmostoma phulo*), Moa (*Amblypharyngodon mola*), and Guchi (*Mastacembelus pancalus*) fish showed the lowest count (3.0×10^2 CFU/ml) at a Tetracycline concentration of 64 µg/ml. Moreover, microbial analysis using 16S rRNA gene sequencing identified twelve unique bacteria belong to genera. The most prevalent genus appears to be Enterobacter, identified by 6 strains. These include *Enterobacter hormaechei* strains UCHE20, RB13, AUH-ENM30 and 20710, *Enterobacter ludwigii* strain JCR-38 and *Enterobacter* sp. M3. Other identified genera included Klebsiella (with *Klebsiella variicola* and *Klebsiella pneumoniae*), Proteus (*Proteus mirabilis*), Macrococcus (*Macrococcus caseolyticus*), Acinetobacter (*Acinetobacter johnsonii*), and Bacillus (*Bacillus* sp.). This study provides valuable insights into the complex microbial dynamics in dried fish, emphasizing the need for enhanced food safety and public health measures.

Key words: Chalan *beel,* Dried fish, Food safety, Microbial contamination, 16s rRNA sequencing.

Introduction

The rise and dissemination of bacteria resistant to antibiotics present a notable risk to public health, as well as posing concerns for food safety and environmental well-being (Serwecińska 2020). For many years, antibiotics have been utilized in both human and animal medicine. In humans, they are employed for treating infectious diseases, while in animals, they are used not only for health treatment and protection but also as growth enhancers in the animal industry (Xu et al. 2015). The extensive utilization of antibiotics can exert selection pressure on microorganisms in the environment, potentially leading to the escalation of antibiotic resistance in the microorganisms (Xu et al. 2015). The global health issue of antibiotic resistance has arisen due to the improper use and excessive consumption of antibiotics in both human and animal healthcare,

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coupled with insufficient infection control practices in medical facilities (Islam et al. 2023). It is widely recognized that antibiotics, even at minimal concentrations, present a considerable threat to both environmental and human health (Rodriguez-Mozaz et al. 2015).

In the past ten years, there has been a growing focus on the proliferation of bacterial resistance in the natural environment. This heightened attention has arisen from the mounting apprehension within the medical and scientific communities regarding the swift rise of antibiotic-resistant bacteria (ARB), encompassing resistance to novel antibiotics and pharmaceuticals considered as last-resort options (Serwecińska 2020). The dissemination of antibiotic resistant genes (ARGs) and bacteria resistant to antibiotics may pose health risks to both humans and animals (Kemper 2008). Resistant organisms have the potential to enter the human body either directly or indirectly (Rosal et al. 2010). Moreover, resistant genes are present in diverse environmental media and are widely disseminated through horizontal gene transfer (HGT) mechanisms in the environment (Rosal et al. 2010, Zhou et al. 2011). The World Health Organization (WHO) has categorized the rise and expansion of antibiotic-resistant bacteria as one of the three major challenges to public health in the 21st century (Rodriguez-Mozaz et al. 2015).

Chalan Beel, situated in the northwestern region of Bangladesh, plays a crucial role in sustainable the livelihoods of local communities. It consists of a network of depressions connected by diverse channels, creating an expansive water body during the rainy season (July-November), with a coverage of approximately 400 km^2. In the dry winter and summer periods, the water area contracts to a range of 52-78 km^2, resembling a collection of beels in various sizes. This transformation provides valuable alluvial crop land during the post-monsoon season (Sayeed et al. 2014). It supports the well-being of approximately five million individuals through the provision of fish and aquatic resources, agricultural produce, and grazing areas for livestock (Ahashan et al. 2008). It serves as a significant source of fish, contributing substantially to both domestic consumption and international trade (Sayeed et al. 2014) and the present fish output in Chalan Beel stands at 11,000 metric tons (Ahashan et al. 2008).

Dry fish, commonly known as "*shutki*," is a popular food item extensively enjoyed in Bangladesh (Kubra et al. 2020), undergoes a traditional preservation process that involves drying in the open air. As the largest wetland in the country, Chalan Beel generates a substantial quantity of fish annually (Samad et al. 2009). Conventional drying methods are typically basic, and adherence to proper hygiene is infrequent. In the rainy season, characterized by elevated humidity levels, traditional approaches often fail to achieve thorough drying. Under such circumstances, stored dried fish may reabsorb moisture, rendering them vulnerable to bacteria, fungi, or insect infestation (Samad et al. 2009). Despite the cultural and economic significance of dried fish in the area, there is a lack of scientific knowledge regarding the microbial safety of this product, particularly concerning antibiotic resistance. The aim of this research is to perform a comprehensive investigation into the presence, diversity, and patterns of antibiotic-resistant bacteria using 16S rRNA sequencing found in various types of dried fish sourced from the *Chalan beel* area in Northwestern Bangladesh. Additionally, the study seeks to identify the phylogenetic placement of these bacterial strains.

Materials and Methods

Collecting samples and conducting exploratory activities

Dried fish samples were obtained from different dried fish producer in Chalan beel area (Naogaon, Atrai, Natore, Singra, Gurudaspur and Ullapara) of Rajshahi division between January and February in Bangladesh. All the collected samples were carefully sealed in sterile zip look bags and then stored in an

icebox. Subsequently, the samples were transported to the Laboratory within the Department of Zoology at the University of Rajshahi, Bangladesh. They were promptly preserved at a temperature of -20°C for subsequent experiments. Then the samples (10 g) cut into small pieces and vortex 90 ml of distilled water. Prior to dilution and plating, homogenization was carried out for a duration of 1 to 2 minutes. The resulting homogenates were then carefully collected in sterile containers and refrigerated at -20°C until they were required. The list of dried fish species found in Chanal Beel is mentioned in Table 1 (Nahiduzzaman et al. 2020). Among them twelve dried fish species were used for conducting this investigation.

Table 1: List of dried fish species found in Chanan Beel.

Sl. No.	Local name	English name	Scientific name
1	Punti	Barb	*Puntius* sp.
2	Raikhor	Reba carp	*Cirrhinus reba*
3	Phul chela	Minnow	*Salmostoma phulo*
4	Silver carp	Exotic carp	*Hypophthalmicthys molitrix*
5	Darkina	Flying barb	*Esomus danricus*
6	Mola	Mola carplet	*Amblypharyngodon mola*
7	Taki	Spotted snakehead	*Channa punctatus*
8	Shoal	Snakehead	*Channa striatus*
9	Guchi	Striped Spiny eel	*Mastacembelus pancalus*
10	Tara baim	Lesser Spiny eel	*Mastacembelus aculeatus*
11	Gutum puiya	Guntea loach	*Lepidocephalus guntea*
12	Bou mach	Bangla loach	*Botia dario*
13	Icha chingri	Freshwater prawn	*Macrobrachium* sp.
14	Chanda	Elongate glassy perchlet	*Chanda nama*
15	Kholisha	Goramy	*Colish faciatus*
16	Tengra	River catfish	*Mystus* sp.
17	Kakila	Needlefish	*Xenentodon cancila*
18	Chapila	Shad	*Gudusia chapra*
19	Bele	Tank goby	*Glossogobius giuris*
20	Baspata	Gangetic ailia	*Ailia coila*
21	Boal	Helicopter catfish	*Wallago attu*

The assessment of colony forming units (CFUs).

Measuring the number of viable bacteria (CFUs) holds significant importance in this experiment, serving as a crucial measurement to assess the presence of precursor cells within a specific cell population. To determine the count of viable bacteria, dried fish samples were mixed with sterile phosphate buffered saline (PBS) and subjected to a series of 10-fold dilutions. Enumeration was carried out on plates containing nutrient broth with 1.5% nutrient agar, supplemented with Tetracycline (TC). Plates without antibiotics were used as the control group. Subsequently, these plates were incubated at 37°C, and after 24 hours, the viable bacterial colonies were counted using the following equation. The standard formula of CFU (Reasoner 2004) is given below:

$$\text{CFU} = \frac{\text{Number of colonies on agar plate}}{\text{Total dilution of tube} \times \text{amount plated}}$$

DNA isolation and PCR amplification

DNA was extracted from bacterial strains using the phenol-chloroform technique, as outlined by Wright et al. (2017). Following extraction, the 16S rRNA gene was amplified using polymerase chain reaction (PCR) in a Thermocycler. The PCR was performed in a 50 µL reaction mixture that included 2 µL of the DNA template, 25 µL of 2 × PCR Master Mix, and 2 µL of each primer (20 pmol/µL). The primers used in this study were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and U1492R (5'-GGTTACCTTGTTACGACTT-3'), as noted by James in 2010 (James 2010). The PCR process began with an initial denaturation of DNA at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and elongation at 72°C for 1 minute, ending with a final extension at 72°C for 5 minutes. The PCR product, measuring 5 µL, was then run on a 1.5% agarose gel at 90 volts for 45 minutes. Gel visualization was carried out using UV transillumination, and the findings were carefully documented.

DNA sequencing and evolutionary analysis

A detailed sequencing of the 16S rRNA gene, covering 1500 base pairs and incorporating both reverse and forward reactions, was conducted using a genetic analyzer at the Central Dogma Lab, Invent Technologies Ltd., Dhaka. This sequencing process employed both forward and reverse primers. The sequences were then meticulously edited to remove PCR primer binding sites, with manual adjustments made using by MEGA software. For identifying the sequences of the genes, an automated comparison was made with bacterial sequences available in the databases (http://www.ncbi.nlm.nih.gov/) using BLAST analysis. Following this, a phylogenetic tree was constructed utilizing the neighbor-joining method.

Results

Number of viable cells

The viable bacterial count fluctuated across varying concentrations of Tetracycline in this investigation. Specifically, at a dosage of 32 µg/ml, the count ranged from 7×10^2 to 5.4×10^3 CFU/ml, signifying a variability within this spectrum (Table 2). Similarly, with an elevated concentration of 64 µg/ml, the range extended from 3.0×10^2 to 2.1×10^3 CFU/ml (Table 2). Additionally, the untreated control group, notably the Tengra fish, displayed the highest viable bacterial count at 6.5×10^5 CFU/ml (Table 2). Conversely, the lowest count of 3.0×10^2 CFU/ml was recorded in Darikana, Chela, Moa, and Guchi fish species under the influence of 64 µg/ml Tetracycline (Table 2).

Table 2: Viable count of Tetracycline resistant bacteria in dried fish from Chalan Beel area.

Sample name	CFU/ml (Control)	Average CFU (CFU/ml) dose 32 μg/ml	Average CFU (CFU/ml) dose 64 μg/ml
TAKI	2.0×10^5	1.9×10^3	6.0×10^2
CHINGRI	6.1×10^5	3.8×10^3	9.0×10^2
PUTI	2.3×10^5	3.2×10^3	2.1×10^3
TENGRA	6.5×10^5	4.0×10^3	7.0×10^2
KHALISA	4.9×10^5	3.5×10^3	1.4×10^3
DARKINA	2.7×10^3	1.3×10^3	3.0×10^2
CHELA	1.8×10^5	5.4×10^3	3.0×10^2
BOAL	1.8×10^5	8.0×10^2	4.0×10^2
CHANDA	3.1×10^5	1.6×10^3	9.0×10^2
KHAYRA	3.7×10^5	1.2×10^3	9.0×10^2
MOA	2.7×10^4	1.5×10^3	3.0×10^2
GUCHI	1.7×10^5	7.0×10^2	3.0×10^2

DNA isolation and amplification

Our in-depth research resulted in the isolation of twelve distinct bacterial strains across six genera from diverse samples of dried fish. The utilization of agarose gel electrophoresis facilitated the identification of a wide-ranging array of bacterial strains (Fig. 1). Subsequent to isolation, the PCR products of these strains, each approximately 1500 base pairs long, underwent direct sequencing. The resultant sequencing data demonstrated significant similarity to known bacterial genera, ranging from 97.04% to 100.00%. Table 3 meticulously catalogues the names of the bacterial species alongside their respective strain IDs and the percentages of observed homology.

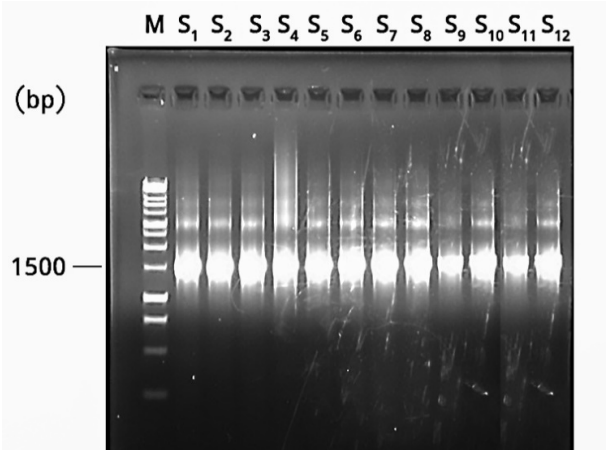

Fig. 1: Analysis of DNA from twelve isolated bacteria using gel electrophoresis.

Table 3: Molecular identification of resistant bacteria obtained from dried fish species.

Strain ID	Identified bacteria	Query coverage (%)
M-KHY-64	*Enterobacter hormaechei* strain UCHE20	99.15
M-DAR-64	*Klebsiella variicola*	98.37
M-CHL-32	*Enterobacter hormaechei* strain RB13	97.04
M-CHN-64	*Enterobacter ludwigii* strain JCR-38	98.66
M-TAK-64	*Enterobacter hormaechei* strain AUH- ENM30	98.25
M-KHY-64	*Enterobacter hormaechei* strain 20710	100.00
M-BOL-32	*Proteus mirabilis* strain HH140	98.68
M-KHL-32	*Macrococcus caseolyticus* strain GCF1S3	98.22
M-MOA-64	*Enterobacter* sp. M3	97.42
M-TNG-32	*Acinetobacter johnsonii* strain I22	98.56
M-DAR-32	*Klebsiella pneumoniae* strain KPN6328	99.12
M-MOA-32	*Bacillus* sp. strain 201709CJKOP-109	100.00

Additionally, the creation of phylogenetic trees, as depicted in Fig. 2 informed by BLAST analysis, illuminated the evolutionary pathways of these isolates by pinpointing related sequences among different species or organisms. Examination of the microbial isolates from the dried fish samples unveiled the presence of twelve unique bacteria, encompassing *Enterobacter hormaechei* strains (UCHE20, RB13, AUH-ENM30, 20710), *Klebsiella variicola*, *Enterobacter ludwigii*, *Proteus mirabilis*, *Macrococcus caseolyticus*, *Enterobacter* sp., *Acinetobacter johnsonii*, *Klebsiella pneumoniae*, and *Bacillus* sp.

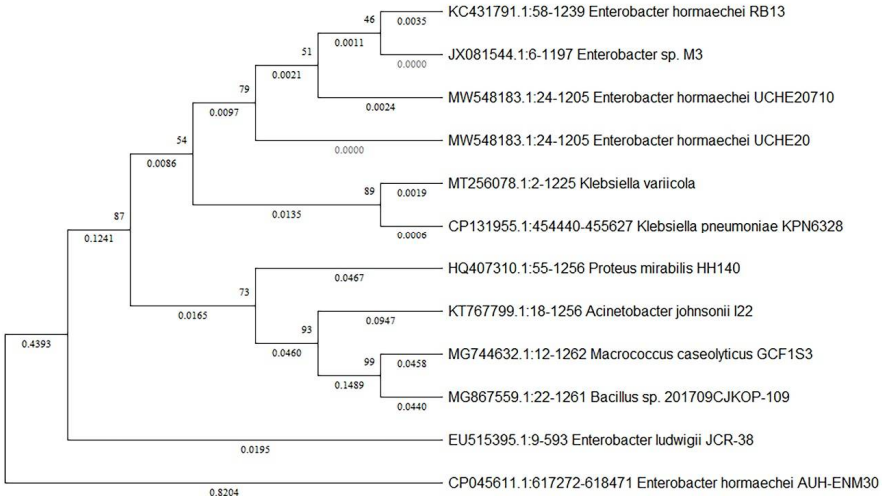

Fig. 2: Evolutionary relationships among bacteria isolated from dried fishes.

Discussion

This research aimed to explore the pattern of antibiotic resistant bacteria in different dried fish varieties obtained from the Chalan Beel area in Rajshahi, Bangladesh. Fish are highly prone to microbial contamination due to their soft tissues and the aquatic surroundings. Numerous bacteria inhabit the slime surface, gills, and intestines of living fish. Following the death of fish, many of these bacteria transform into potential contaminants, exploiting the breakdown of the defense system and proliferating as they invade the flesh (Nur et al. 2020). One of the primary factors leading to the subpar quality of fish in retail trade is inadequate hygiene during handling, improper storage practices, physical damage, and exposure to dirty water and microorganisms (Nur et al. 2020).

In our investigation, the viable bacterial population showed variability across different concentrations of Tetracycline, with counts ranging from 7×10^2 to 5.4×10^3 CFU/ml at 32 μg/ml and from 3.0×10^2 to 2.1×10^3 CFU/ml at 64 μg/ml. Among the tested groups, the highest count, reaching 6.5×10^5 CFU/ml, was observed in the Tengra fish (control), whereas the lowest count, at 3.0×10^2 CFU/ml, was recorded in Darikana, Chela, Moa, and Guchi fish exposed to 64 μg/ml of Tetracycline. The observed variance in bacterial counts across species and concentrations implies differing susceptibility levels to Tetracycline's antimicrobial properties among the tested fish populations. Our results are consistent with the findings from the study conducted by Begum et al. (2021) involving 18 types of dried fish, the Total Viable Count (TVC) ranged from 1.28 × 107 CFU/ml to 3.74 × 109 CFU/ml. The highest microbial load was detected in Lottya, while the lowest TVC was observed in the Ruhi dried fish sample. Another experiment showed that the Total Plate Count (TPC) of dried Jat Punti showed variations based on their mother and origin . The retail market sample recorded the highest TPC at 8.58 ± 0.46 × 106 CFU/ml, while the control sample exhibited the lowest TPC at 2.20 ± 0.15 × 105 CFU/ml (Hussain et al. 2016). Therefore, the findings of this study demonstrated that diverse samples of dried fish displayed a significant variation in Total Viable Count (TVC), surpassing the acceptable threshold.

Our thorough investigation led to the identification of twelve bacterial strains belong to six different genera from a variety of dried fish samples. Analysis of these microbial isolates from the dried fish samples uncovered the existence of twelve distinct bacteria, including strains of *Enterobacter hormaechei* (UCHE20, RB13, AUH-ENM30, 20710), *Enterobacter ludwigii*, *Enterobacter* sp., *Klebsiella variicola*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Macrococcus caseolyticus*, *Acinetobacter johnsonii*, and *Bacillus* sp. The results of this investigation are in accordance with the outcome of the study carried out by Nur et al. (2020), which identified four types of bacteria, namely *E. coli*, *Salmonella* spp., *Staphylococcus* spp., and *Pseudomonas* spp., in both raw and dried fish samples.

Conclusion

This study aimed to investigate the distribution of antibiotic resistant bacteria from diverse dried fish samples from Chalan Beel area, Rajshahi, Bangladesh. The study found that 100% of the samples of dried fish collected from this area were contaminated by Tetracycline resistant bacteria. The viable bacterial counts ranged significantly from as low as 3.0×10^2 CFU/ml in fish treated with Tetracycline to a high of 6.5×10^5 CFU/ml in the control group (Tengra fish). Microbial analysis using 16S rRNA gene sequencing identified 12 unique bacteria from 6 genera. As a precautionary measure, further investigations are crucial for identifying the sources of contamination of Tetracycline resistant bacteria which may seriously impact human health.

Acknowledgements

I would like to express my sincere thanks to my respected supervisor Dr. Md. Habibur Rahman, Professor Dept. of Zoology, University of Rajshahi, to Director, Prof. Dr. Jahan Ara Khanam and other faculty members of Institute of Biological Sciences, University of Rajshahi. I am thankful to Dept. of Zoology, University of Rajshahi for giving me laboratory facilities.

Conflict of interest: The authors declare no conflicts of interest related to the publication of this article.

References

Ahashan MR, Sayeed MA, Nahiduzzaman M, Sultana M, Barman D and Hossain MA (2008). Fish marketing system in the Chalan Beel area in north-west Bangladesh. Journal of Agroforestry and Environment 2(2): 47-51.

Begum MD, Muniruzzaman M, Salauddin M and Rahman MM (2021). Detection and antibiogram study of bacteria isolated from dried and cooked fish. Veterinary Sciences: Research and Reviews 7(2): 134-142.

Hussain MA, Sayeed MA, Kabir ML, Sumon TA, Das S and Ahmed MA (2016). Determination of microbiological quality of dried Jat Punti (*Puntius sophore*) collected from Sylhet district, Bangladesh. International Journal of Natural and Social Sciences 3(3): 72-79.

Islam MA, Mondal SK, Islam S, Akther Shorna MN, Biswas S, Uddin MS, Zaman S and Saleh MA (2023). Antioxidant, cytotoxicity, antimicrobial activity, and in silico analysis of the methanolic leaf and flower extracts of *Clitoria ternatea*. Biochemistry Research International 2023(1): 8847876.

James G (2010). Universal bacterial identification by PCR and DNA sequencing of 16S rRNA gene. PCR for Clinical Microbiology: An Australian and International Perspective 2010: 209-214.

Kemper N (2008). Veterinary antibiotics in the aquatic and terrestrial environment. Ecological Indicators 8(1): 1-3.

Kubra K, Hoque MS, Hossen S, Husna AU, Azam M, Sharker MR and Ali MM (2020). Fish drying and socio-economic condition of dried fish producers in the coastal region of Bangladesh. Middle-East Journal of Scientific Research 28(3): 182-192.

Nahiduzzaman M, Flowra FA, Hossen MN, Awal MR, Rahman MA and Akter S (2020). Traditional fish drying method practiced by the farmers of *Chalan beel* (Singra Upazila) and their socio-economic status. International Journal of Fisheries and Aquatic Studies 8: 184-190.

Nur IT, Ghosh BK and Acharjee M (2020). Comparative microbiological analysis of raw fishes and sun-dried fishes collected from the Kawran bazaar in Dhaka City, Bangladesh. Food Research 4(3): 846-851.

Reasoner DJ (2004). Heterotrophic plate count methodology in the United States. International Journal of Food Microbiology 92(3): 307-315.

Rodriguez-Mozaz S, Chamorro S, Marti E, Huerta B, Gros M, Sànchez-Melsió A, Borrego CM, Barceló D and Balcázar JL (2015). Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Research 69: 234-242.

Rosal R, Rodríguez A, Perdigón-Melón JA, Petre A, García-Calvo E, Gómez MJ, Agüera A and Fernández-Alba AR (2010). Occurrence of emerging pollutants in urban wastewater and their removal through biological treatment followed by ozonation. Water Research 44(2): 578-588.

Samad MA, Galib SM and Flowra FA (2009). Fish drying in Chalan Beel areas. Bangladesh Journal of Scientific and Industrial Research 44(4): 461-466.

Sayeed MA, Hashem S, Salam MA, Hossain MA and Wahab MA (2014). Assessment of Chalan beel ecosystem diversity through remote sensing and geographical information systems. International Journal of Innovation and Applied Studies 7(1): 353.

Serwecińska L (2020). Antimicrobials and antibiotic-resistant bacteria: a risk to the environment and to public health. Water 12(12): 3313.

Wright MH, Adelskov J and Greene AC (2017). Bacterial DNA extraction using individual enzymes and phenol/chloroform separation. Journal of Microbiology and Biology Education 18(2): 1-3.

Xu J, Xu Y, Wang H, Guo C, Qiu H, He Y, Zhang Y, Li X and Meng W (2015). Occurrence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river. Chemosphere 119: 1379-1385.

Zhou LJ, Ying GG, Zhao JL, Yang JF, Wang L, Yang B and Liu S (2011). Trends in the occurrence of human and veterinary antibiotics in the sediments of the Yellow River, Hai River and Liao River in Northern China. Environmental Pollution 159(7): 1877-1885.

(Manuscript received on 18th June 2024 and revised on 16th July 2024)