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River Ganga Water as Reservoir of Microbes with Antibiotic and Metal Ion Resistance Genes: High Throughput Metagenomic Approach

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Abstract – The large-scale use of antibiotics and trace elements contributes to their progressive environmental release and eventually to the spread of bacterial antibiotic resistance genes (ARGs) and metal ion resistance genes (MRGs). A high-throughput genomic sequencing of the microbial population was conducted in water and sediments in the Ganges River harbouring resistance genes. The results showed that a large range of resistance genes with high sediment abundance are present in the river. Beta-lactam, multidrug/efflux and elfamycin were the extremely dominant ARGs. In water and sediment, ARGs such as (tuf, parY, ileS, mfd) were very abundant. In water and sediments, the most abundant metal resistance gene was the MRGs subtype acn. Most of the types of ARGs showed a strong positive correlation (p ⁇ 0.05) with the types of MRGs in the river area, indicating that their distribution and transfer could be related. The taxonomic classification showed that the two most common phyla were Proteobacteria and Actinobacteria in water and sediments. The most abundant genera were Arcobacter, Terrimicrobium, Acidibacter and Pseudomonas. This study indicates that the production of resistance genes and their subsequent proliferation and accumulation in environmental bacteria is driven by antibiotics and metals. The present genomic study highlights the value of such studies and draws attention to the mitigation of contaminants associated with the distribution of ARGs and MRGs in the river setting.

Keywords: Antibiotic Resistance Genes (ARG); Antibiotics; River Ganga

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INTRODUCTION

The global threat to natural biodiversity and human health is the degradation of the marine ecosystem. The growing human population has increased the use of pesticides and other agro-inputs to achieve global food security (Ehrlich and Harte, 2015). In addition to routine use in the agricultural sector, pesticides are used to control vector-borne diseases (VBD) such as malaria, especially in developing countries (Van den Berg et al., 2012), including India, for household purposes. Yet, inevitably, the indiscriminate use of pesticides in agriculture and households results in a large amount of their residues in an ecosystem that gradually washes out into natural streams such as rivers, wetlands, and ultimately into the sea, causing the natural marine biodiversity to be enormously disturbed. It has been documented that significant quantities of pesticide residues found in wetlands, rivers, and oceans can cause countless adverse effects on aquatic organisms such as endocrine disruption, growth reduction, etc (Carvalho et al., 2009). The use of organ chlorine (OC) pesticides has long been banned in many countries, but due to their high prevalence, they are still present in natural streams, sediments, marine flora, and fauna. Several aquatic environments, including rivers, wetlands and seas, were periodically monitored on an ongoing basis to determine the extent of OC and its risk assessment for aquatic life Because of their high lipophilicity properties; they are readily absorbed by the river sediments and the marine fauna. In the fish, Drapane africana (2237–6368 µg/kg) compared to Mochokus niloticus (1006–3288 µg/kg) in the Niger River of Nigeria, high concentrations of OC have been identified Gene networks involved in largemouth bass reproduction and immune function are documented to disrupt OC-contaminated Lake Apopka sites . The widely used pesticides found in the natural stream belong to the organophosphate, carbamate, pyrithroid, etc. category, other than OC. The immune function and structural integrity of Cyprinus carpio L fish is stated to be compromised by chlorpyrifos and other organophosphate pesticides. By way of oxidative stress and apoptosis (Jiao et al., 2017). A pyrithroid insecticide, lambda-cigalothrin, has been reported to reduce the free amino acid content of fish in the muscles, liver, and brain of the Alazani River.

Several physical and chemical approaches are being used to extract these harmful xenobiotics from natural habitats due to the toxic impact of pesticide residues

on native flora and fauna, such as landfills, recycling, pyrolysis, etc. However, bioremediation using various microorganisms has been found to be the most feasible strategy, and has been shown to operate in many polluted sites. The presence of organophosphate hydrolase (OPH) enzymes in these microorganisms has been reported to be capable of detoxifying them by cleavage of phosphate ester (P-O, P-F, P-CN, and P-S) bonds The yeast (Saccharomyces cerevisiae) is capable of incorporating the poorly hydrolyzed P-S class of organophosphate into the ribosomal operon of the same gene, encoding the wild-type OPH (enhanced variant enzyme S308L-OPH) Likewise, because of the existence of the opdA gene, the bacterial strain, Bacillus pumilis, was confirmed to be capable of bioremediation of methyl parathion, a P-S type OP pesticide (Ali et al., 2012). It is documented that several microorganisms belonging to the Bacillus, Brevibacillus, Ochrobactrum, Pseudomonas, Serratia, and Sphingobium genera are capable of degrading different pyrithroid pesticides through metabolic activity (Several genes have been identified with parathyroid degrading ability, viz. In Klebsiella sp, estP. ZD112 pye3 from the soil met genome (Li et al., 2008), pytH from Sphingobium sp. JZ-1 pytZ, and pytY (from the Ochrobactrum anthropi YZ-1 genomic library) (Zhai et al., 2012). Not only bacteria, but also several fungi with the ability to remediate pesticides via a catabolic or co-metabolic process have also been identified. It has been documented that fungi belonging to the genera Aspergillus, Candida, Cladosporium, Tricoderma, etc., degrade various pyrethroid pesticides such as cyfluthrin, bifenthrin, deltamethrin, etc.

A good number of microbial species maintain and die in the natural aquatic environment, which can degrade these harmful xenobiotic residues in situ. Met genomic studies have therefore been carried out extensively in recent years to classify these possible microorganisms and their functional role. A sediment micro biome in the Deepwater Horizon oil spill and the effect of oil deposition on microbial communities in surface sediments were seen in met genomic studies (Mason et al., 2014). The microbial cultures of two thermal pools in Kamchatka, Russia have been revealed by met genome-assembled genomes (MAGs) (Wilkins et al., 2019). Similarly, the main bacterial species were characterized using shotgun met genomics in the Daphnia magna micro biota . The met genomic analysis identified antibiotic resistance genes (AMRs) from the River Yamuna sediments (Das et al., 2020). The possible microbial population involved in the biodegradation of phenanthrene, diesel, and hexadecane in the mangrove sediment was established using the met genomics method and the degrading bacteria belonged to the Bacillus sp., Pseudomonas sp., Acinetobacter sp., and Staphylococcus sp. genera. met genomic approach was also used to demonstrate the role of IS1071, a xenobiotic degradation insertion component flanks, in the formation and distribution of gene cluster bacterial catabolic pathway The biodegradation pathway of DDT, HCH, and atrazine was studied via a met genomics approach in freshwater and marine sediments. The study described 69 genera with major populations belonging to the genus Plesiocystis sp., Anaerolinea sp., Jannaschia sp., and Mycobacterium sp., capable of degrading these persistent pesticides, and found the existence of various genes, viz. Encoding atzB, hdg, and hdt for ethylaminohydrolase, dehalogenase, and hydratase, respectively (Fang et al., 2014). A functional met genomic analysis of rumen samples of Holstein dairy cows was also reported for the degrading enzyme carbamate pesticide (Ufarte et al., 2017). The met genomics method was also used to explain the underlying mechanism of in situ biodegradation and to predict the propensity for microbes to degrade in the soil of Queensland, Australia (Jeffries et al., 2018). There were, however, not many studies on the occurrence of the native microbial community with bioremediation potential in the Ganga and Yamuna river ecosystems. With this context, the present study emphasizes the identification and relative abundance through the met genomics approach of potential bioremediation microbes capable of degrading contaminants in these river sediments. The research also aims to examine the diversity and relative abundance of these bioremediation microbes at the various contaminated and non-polluted Ganga and Yamuna River sites.

DNA isolation

Sediment samples were processed for the DNA isolation using FastDNA spin kit (MP Biomedicals, LLC) following the manufacturer's instructions. Briefly, 500 mg of sediment sample from each site is used to isolate DNA. After following the standard protocol and steps, binding matrix was gently resuspended into 50-100 µl of DES (DNase/pyrogen free water). Next, eluted DNA after centrifugation was stored at 4°C into the clean catch tubes. Finally, 50 µl of eluted DNA from each sample were pooled and concentrated in rotary vacuum evaporator up to half of its total volume. Next, agarose gel electrophoresis and Nanodrop-UV spectrophotometer were used for the quantity and purity check. Quantity of the pooled DNA sample was about 45ng/uL.

Library preparation and Illumina sequencing

Illumina library was prepared using the NEXT Flex DNA library protocol and preparation guide at the Genotypic Technology's Genomics facility. Very briefly, S220 system from Covaris Inc., USA was used to shear DNA and generate ~200-500 bp fragments. Further, Agilent Bioanalyzer was utilized to see fragment size distribution. Next, HighPrep beads (MagBio Genomics, Inc, USA) used to clean fragmented DNA and further end-repair, A-tailing and adaptors ligation was performed through NEXT Flex DNA Sequencing kit following the manufacturer's protocol. Adaptor ligated fragments were cleaned and subjected to PCR using primers from NEXT Flex DNA Sequencing kit. Qubit fluorometer and the Agilent Bioanalyzer were used for the quantification and size distribution of the prepared library respectively

according to the manufacturer's protocol. Finally, Illumina NextSeq sequencing platform is utilized for the sequencing of prepared library.

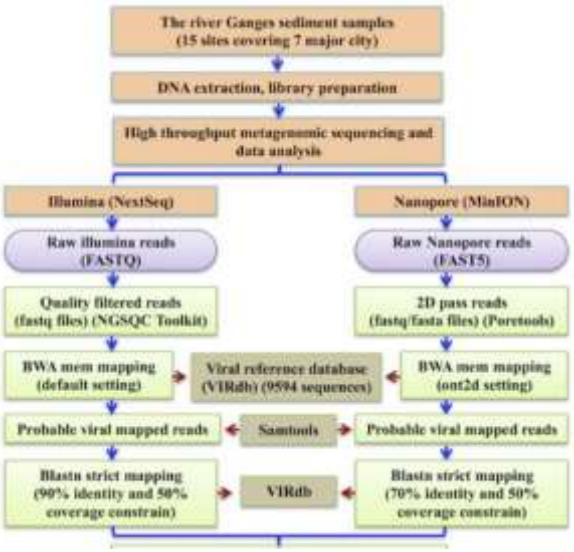
Library preparation and Nanopore sequencing

DNA fragments of size >5kb was extracted from low melting point agarose gel. Gel purified DNA (~300 ng, Qubit, Invitrogen) was used for the library preparation. Further, barcoded using PCR reaction (LongAmpTaq 2x New England Biolabs, USA), then 1.2x AmPure beads (Beckmann-Coulter, USA) was used to clean, and end-repaired through NEBnext ultra II end repair kit (New England Biolabs, USA). Next, 1x AmPure beads used to clean end-repaired DNA. Then, NEB blunt/TA ligase (New England Biolabs, USA) utilized to perform adapter ligations (HPA and HPT) for 15 minutes each. Further, 50 ul of MyOneTM streptavidin C1 beads (Invitrogen, USA) was used to clean library mix and eluted in 25ul of elution buffer. In total, ~198 ng of sequencing library was used for the sequencing employing MinION Mk1b (Oxford Nanopore Technologies, UK) with SpotON flow cell ID FAB49007 (R9.4) on MinKNOW 1.1.21 in a 48hr sequencing protocol. Metrichor V.2.43.1 was used for the base calling.

Met genomic sequence data processing and analysis for phage identification

Illumina sequencing data from the Ganges sediment was subjected to the quality control to remove low quality reads (quality score<30) and filtering using NGSQC toolkit . Correspondingly, met genomic-sequencing data (FAST5) from Nanopore (MinION) sequencing platform was converted and extracted into more readable and useful FASTA and FASTQ formats through implementing pore tools . Finally, all quality filtered data from Illumina and 2D read dataset from Nanopore was utilized for the viruses/phage profiling and further analysis.

For this, a local database (VIRdb) was constructed with lineage information by utilizing all viral/phage reference sequences (9594). For the identification of probable viral reads and to reduce the search space, first BWA software package with MEM algorithm is employed for illumine data (with default settings) and with predefined (-x) ont2d setting for the nanopore sequencing data. For same, viral reference sequence database (VIRdb) was indexed using bwa index function. Further, probable viral mapped reads from both the sequencing data were extracted from sequence alignment map (SAM)/binary alignment map (BAM) files using the SAM tools view function. For virome classification and distribution analysis, probable viral reads were subjected to the blastn mapping with the stringent measures of 90 percent identity and 50 percent coverage constrain for the illumine data and for the nanopore data 70 percent identity and 50 percent coverage criteria was used with max target 1 (best hit only) to avoid the chance of getting false positive hits or to gain more confident virome distribution. The complete workflow used for the virome profiling and analysis in the study is illustrated in Figure 1. Virome distribution was shown using the krona plot for interactive visualization.



Metal contamination and its correlation with microbial community

High levels of metals were detected in large number of samples that may be generated and introduced owing to the industrial discharges. High concentrations 70 and 100 µg/L of trace elements such as Mn, Fe, Co, Cu were recorded in Bhagirathi and Alaknanda (the upstream of Ganga) respectively as compared to 60 µg/L at Narmada Udgam. Their concentration further gradually increased in the downstream samples of Ganga (over 2500µg/L at Jajmau), however there was no signicant change in Narmada. Cauvery has a relatively lower concentration of trace elements, except Cu (with 15µg/L total trace elements). n the river Gomti, the level of both trace and toxic metals were relatively high at all the sampling sites, i.e. the total level of trace and toxic elements up to 208 µg/L and 150µg/L respectively. The samples of Ganga from Bithoor (G6) and Jajmau (G7) showed the highest concentration of all the elements except Cd, which was highest in Gomti river In Narmada and Cauvery, chromium (Cr) and arsenic (As) were mostly present at downstream while trace of lead (Pb) and cadmium (Cd) were present at all sites. Interestingly, the level of As was higher at all sites of Ganga in comparison to other rivers (Fig 8). Consequently, metal and antibiotic resistant bacteria identied as Acinetobacter baumannii species from the Bhagirathi river and Alcaligenes faecalis species from Cauvery river were isolated (data not shown) and characterized. It is signicant to note that high levels of metal resistant genes (MRGs) were detected in pristine water samples as compared to downstream polluted samples. The highest MRGs were detected in C1 sample of Cauvery, followed by G2 in Ganga and N2 of Narmada river (Fig. 9A), which are upstream locations of the rivers. Maximum MRGs was detected

for copper followed by chromium (Cr), arsenic, zinc and iron. Most of the MRGs were located on bacterial chromosome (91.5%), whereas rest (8.5%) were found to be located in plasmids The highest number of plasmids located MRGs were recorded in Cauvery C1 (22.32%) and Ganga G2 (14.32%) samples, whereas all other samples have low percentage, ranging from 4 to 10% Besides MRGs, biocides and other chemical resistance genes for ethidium bromide, rhodamine 6G, acriavine and triclosan were also found in abundance in the pristine sample as compared to their respective downstream samples The PCoA plot of bacterial data at species level with respect to the metal concentration using the phyloseq package3 in MicrobiomeAnalyst depicted a signicantrelation of trace metals with respective microbial communities (Fig. 10) revealing their direct correlation with the presence of trace metals.

Dissolved Oxygen

DO levels in surface water body demonstrate the capacity to help oceanic life? The high DO esteems implies the pace of oxygen recharging in water is more prominent than the oxygen usage. Sufficient DO is essential for acceptable water quality. DO levels between 5.0 and 8.0mg/l are agreeable for endurance and development of amphibian living beings. In the current examination, the DO substance were gone from 7.1-7.5mg/l (pre-storm seasons) and 7.7-8.2mg/l (post-rainstorm seasons) at site I. The worth reaches from 6.8-7.2mg/l (pre-rainstorm seasons) and 7.2-7.7mg/l (post-storm season) at site II. The low worth was found in pre-storm period because of diminished dissolvability of oxygen in mid-year months and the other way around.

Biological Oxygen Demand (BOD)

Body is the measure of oxygen needed by the living beings (microorganisms) in the usage or adjustment of natural issue. All in all, the BOD is the measure of oxygen needed by the microorganisms during their development in wastewater. It is significant pointer of the natural contamination status of a water body. The Unpolluted water has BOD estimation of 3mg/l or less and modern wastewater has BOD esteem 25000mg/l. The estimation of BOD in the current examination was most elevated nearby I when contrasted with site II (found in Table 2). The estimation of BOD was more in pre-rainstorm seasons and demonstrates that the waterway can be somewhat dirtied at various Ghats. The higher qualities Chloride (Cl-1) is one of the significant anions found in water and are for the most part joined with calcium, magnesium or sodium. Chlorides are drained from different rocks into soil and groundwater by enduring. The chloride particle is profoundly versatile and is shipped to shut bowls. The primary wellspring of chloride in surface water and groundwater is because of barometrical precipitation, creature takes care of, septic tanks, utilization of inorganic manures and landfill leachate. In this investigation, the chloride substance were gone from 158-164mg/l (pre-storm seasons), and 184-194mg/l (post-different sources. Practically comparable outcomes were seen by (Bhargava, 1982; Rao, 1992; Shukla, 1989) for the Ganga stream water.

CONCLUSIONS

This is the first report on the identification of bioremediation microbes in sediments of the river Ganga and Yamuna in India, using a met genomic approach. The present study unraveled the in-depth insights on the relative abundance of important native bacteria and fungi convoluted in bioremediation in these rivers and their functional properties for possible biotechnological applications. The identified *Nitrobacteria humburgensis* could reduce heavy metals like zinc, cadmium, mercury, and lead. Similarly, *Rhodobacter spheroids* could reduce arsenic, *Shewanella putrefactions* for iron, *Bacillus cereus* for molybdenum and *Alcaligenes faecalis* for PAHs. The identified fungus like *Aspergillums flaves* could reduce lead, cadmium, chromium, and nickel, and *Aspergillus nidulans* for arsenic, etc., It was found that these bioremediation bacteria and fungi were more abundant in the sediments of a polluted stretch of the river. Several identified protein domains, which are actively involved in environmental bioremediation processes, could be explored for genome-scale engineering for industrial applications in the future.

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