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Research Article

Whole Metatranscriptome Analysis - An Indicator of Anthropogenic Activities in a Sub Himalayan Endorheic River, a Life Line of Northern India

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Abstract:

Runoff from surface water as well as varied anthropogenic activities carry along multiple contaminants, pollutants and allochthonous microbes from soils, plants and multiple other sources to natural water bodies. Microbial communities in freshwaters are known to impact both environment as well as human health. Transcriptome sequen cing has been a powerful tool in understanding genome structure and function identifying genetic networks underlying cellular, physiological, biochemical and biological systems and establishing molecular biomarkers that respond to diseases, pathogens and environmental challenges. We present in this study metagenomic and metatranscriptomic analysis of river Ghaggar's microbial communities. Transcriptome sequencing of water samples collected from river Ghaggar using Illumina platform resulted in a total of 5.7 GB of high quality read sequence. The sequence assembly resulted in 39899788 transcript reads that predicted 141766 genes which have been considered for the final downstream analysis to assess the functional capacities of microbial communities present in the samples. Taxonomic annotation of the genes using Kaiju revealed prevalence of protists, algae, fungi as the most abundant organisms. Functional profile of the sample concluded the existence of a metabolically active micro-ecosystem generated through anthropogenic activities along the course of the river. The sampling site, Rakhigarhi chosen for the present study was significant not only because it collated all the inputs of the sewage treatment plants (STPs) and the agricultural run offs along the river, but also the fact that it was a major centre of Harappan site settlements dating back to 2600BC-1800BC. The results of this research highlight the deteriorating water quality of this river Ghaggar, which has been a lifeline of northern India since antiquity.

Key words: Environment, Freshwater, Microbiome, Metatranscriptomic, River Ghaggar

Introduction

Freshwater resources have been globally affected by multiple stressors such as water abstraction, intensive farming, land use, climate change etc and the importance of assessing the potential risks on stream ecosystems as well as the consumers, is becoming imperative.

Ghaggar river is one such major water body originating at Parwanoo, Himachal Pradesh, with a length of around 320 KM with a catchment area of 42,200 sq. km. It is an ancient river with archaeological sites dating back to 5500 BC scattered along its banks[1]. Once a perennial river it now is endorheic and feeding three agriculturally dominant states and a union territory of Northern India. However along its course, it receives discharge from various cities, STPs and agricultural runoffs[2]. Villages alongside the river have been bearing the brunt of its deteriorating quality as revealed by the medical reports, where farmers have been known to suffer of skin and stomach ailments due to the usage of the river water. As drinking water is the most significant source of Fluorosis apart from other sources including cigarette smoke and industrial pollution, daily consumption of contaminated river water was possibly the reason for patients complaining of fluorosis.

Also, *Porphyromonas gingivalis*, and *Treponema* sp., causative agents of pyorrhoea found in the results of WMA prove the reports published in 2018 on indiawaterportal.org. Many cases of patients with complaints of itchy skin, premature greying of hair (including children), diarrhoea and stomach-related ailments have been reported in the basin, with farmers being the worst-affected [3–8]. Industries, agriculture and varied human activities have been thriving on this river and ungratefully contributing to this deterioration [9–11]. The present study is one of the first attempts to analyze the microbiome of this endorheic river of India using metatranscriptomics. The site chosen for the study is significant, since it lies in the downstream region of Rakhigarhi; Haryana located 29°17'35"N 76°6'51"E. This is a significant location since it collates inputs of almost all the treatment plants as well as the fact that Rakhigarhi, was a major centre of Harappan site in the Indian subcontinent boasting one of the oldest settlements dating back to 2600BC-1800BC in the Ghaggar river basin [1,12]. The metagenomics and metatranscriptomics tool used in the study have provided new revelation about microbial ecosystems' functioning and the relationships that microorganisms maintain with and within their environment[13–17].

We present a study focused on simultaneous metagenomic and metatranscriptomic analysis of river Ghaggar's microbial communities, encompassing the prokaryotes, lower eukaryotes and the viruses unlike most studies that are focused with respect to either eukaryotic / prokaryotic population in a given niche [14,18,19]. Our study focuses on metagenomics together with metatranscriptomic research revealing the complete community profile of prokaryotic and eukaryotic microorganisms living in association within an economically, commercially and archeologically significant niche.

Materials and Methods

2.1 Site description, Sample collection and Characterization

Rakhigarhi village in Hissar district of Haryana, India is a downstream catchment area lying in Ghaggar river basin. Samples were collected in a 1 L sterile container for microbial analysis and in 250ml glass stoppered bottled for measuring of Dissolved Oxygen. For microbial analysis samples were collected from multiple points at the site 29°17'35"N 76°6'51"E as per standard procedures and pooled [20,21]. The physico-chemical parameters like conductivity and pH were recorded on site using Equip-tronics conductivity meter EQ-660A and Contech pH meter respectively. The Dissolved Oxygen (DO) was fixed on site, and the samples refrigerated till further use. Chemical parameters like Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Total Dissolved Solids (TDS), Total Hardness (TH), and Total Alkalinity (TA) were measured using standard procedures [22].

2.2 Sample preparation and Total RNA extraction for Metatranscriptome analysis

The samples were pooled (numbered as 1.9723) and centrifuged at 5000 rpm for 15 minutes at 4°C using Eppendorf refrigerated centrifuge 5810R. The mass obtained was then re-suspended in minimum volume (2ml) of sterile Phosphate Buffered Saline, and subjected to total RNA extraction using Trizol method. The quality of the total isolated RNA was checked on 1% denatured agarose gel and quantified using Qubit fluorometer.

2.3 Next Generation Sequencing (NGS): Whole Metatranscriptomic Analysis (WMA)

2.3.1 Library construction

Library was prepared using Illumina TruSeq stranded mRNA Library Preparation Kit and as per its described protocol [23]. mRNA was enriched from total RNA followed by fragmentation. The fragmented mRNA was converted into first-strand cDNA, followed by second-strand generation, A-tailing, adapter ligation and finally ended by limited number of PCR amplification of the adaptor-ligated libraries. Library quantification and qualification was performed using DNA High Sensitivity Assay Kit. The amplified library was analyzed in Bioanalyzer 2100 (Agilent Technologies) using High Sensitivity (HS) DNA chip as per manufacturer's instructions [24].

2.3.2 Cluster Generation and Sequencing

After obtaining the Qubit concentration for the library and the mean peak size from Bioanalyser profile, libraries were loaded into Illumina platform for cluster generation and sequencing. Paired-End (PE) sequencing allowed the template fragments to be sequenced in both the forward and reverse directions. The library molecules bind to complementary adapter oligos on paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the frag-

2.4 Bioinformatics Analysis

2.4.1 High Quality Data Statistics & Metagenome Assembly

The data statistics of data received for sample 1.9723 was taken using in-house shell script. De novo assembly of high quality paired end (PE) reads was accomplished using CLC Genomics Workbench 6.0 at default parameters (Minimum contig length: 200, Automatic word size: Yes, Perform scaffolding: Yes, Mismatch cost: 2, Insertion cost: 3, Deletion cost: 3, Length fraction: 0.5, Similarity fraction: 0.8). The statistical elements of the assemblies were calculated by in house perl scripts. (See table 1, 2 and 3)

Table 1: Data statistics of samples

Sample name	# Reads (R1)	# Reads (R2)	Total Reads	Total Data (Gb)
1.9723	19949894	19949894	39899788	5.7

Table 2: Metagenome Assembly Statistics

Assembly element	Sample 1.9723
Number of scaffolds	160137
Total genome length in bp	93055740
Average scaffold size	581.100
N50	605
Max scaffold size	11402

Table 3: Statistics of predicted genes

Gene element	Sample 1.9723
Number of gene	141766
Total gene length (bp)	78320487
Average gene length	552.46
Max gene length	11220

2.4.2 Gene Prediction, Taxonomic and Functional Annotation

Genes were predicted using Prodigal (v2.6.3) [25] (Table 4) and the predicted Gene from scaffolds were then taken for taxonomic and functional analysis using Kaiju [26] and Cognizer respectively. The final sequences were uploaded to the Kaiju web server (http://kaiju.binf.ku.dk) with the default parameters. Cognizer was used to assess the functional capacities of microbial communities present in the samples. Cognizer is a comprehensive stand-alone framework which is enabled to simultaneously provide COG, KEGG, Pfam, GO and FIG fams annotations to individual sequences constituting metagenomic datasets [27].

Results & Discussions

3.1 Site description

Rakhigarhi village located in the Hissar district of Haryana is a major centre of Harappan site of Pre-Indus Valley Civilization settlement dating to as early as 4600 BC. Situated in the Ghaggar-Hakra river plain at a distance of some 27 km from the seasonal Ghaggar river[1]**.** Expanding human population, industrialization and intensive agricultural and industrial practices have intensified the anthropogenic effects on this river.

3.2 Sample collection and analysis

Results of the physico-chemical analysis of the samples collected are presented in Table 4. The samples were collected in equal volumes throughout the day in sterile bottles during the monsoon (Month of August) season. All the values of the physico-chemical parameters like pH, conductivity, BOD, COD, and TDS observed were higher in comparison to those prescribed by W.H.O.[28] and to those reported in earlier studies [10,29,30,31].

Table 4: Physico-chemical parameters of the pooled sample

Sampling point	pH	Conductivity (µmho/cm)	TDS (mg/l)	COD (mg/l)	BOD (mg/l)	Total Hardness as $CaCO_3$ in mg/l	Total Alkalinity as $CaCO_3$ in mg/l
29°17'35"N 76°6'51"E	7.3±0.2	905±1.33	512±10	28±0.5	5±0.3	272±2.33	295±1.85

Where, mg/l stands for milligrams per litre; TDS, Total dissolved solids; COD, Chemical oxygen demand; BOD, Biological oxygen demand and hardness and alkalinity are expressed as mg/l equivalents of Calcium carbonate ($CaCO_3$).

3.3 Community composition

Biodiversity was assessed from ribosomal sequences through KAIJU. Dominance of Eukaryotes was evident (Fig 1) in the data with the top 50 hits (46% protists, 28% fungi and 26% algae) showing complete absence of prokaryotes. Most of the eukaryotes were affiliated with Chlorophyceae, Oligohymenophorea, Agaricomycetes, Heterolobosea sp.

Protists are considered as chief bacterial grazers which help in managing bacterial population, and also act as protective reservoirs in freshwater, marine ecosystems and soils [32]. Studies have confirmed that certain protists can however safe-guard bacterial pathogens from numerous environmental countercurrents as well as give an idealistic or perfect environment for bacterial replication. For example, Mycobacterium avium isolates capable of surviving within free-living amoeba are said to be well protected from the harmful effects of antimicrobials which has resulted in their increased virulence [33,34]. Salmonella enteritidis isolates that survived Tetrahymena grazing exhibited enhanced acid-resistance property along-with resistance to calcium hypochlorite[35,36]. Legionella pneumophila which are found residing in pellets excreted by Tetrahymena tropicalis also exhibited an increased gentamicin resistance and ability to thrive in nutrient-deficient environments [37]. Free-living amoebae may behave as parasites under certain circumstances like Acanthamoeba sp. Naegleria fowleri known as agents of diseases in humans and animals are found to be dominant in the river. These protozoans are also known to be associated with cutaneous lesions and sinusitis in immune-compromised individuals sometimes leading to leading to severe brain pathologies [38–41]. Acanthamoeba sp. has also known to contain diverse bacterial endosymbionts (Legionella and Legionella-like pathogens) that are similar to human pathogens, implicated in outbreaks of pneumonias in debilitated hosts [42,43].

The data also revealed the presence of *Ichthyophthirius multifiliis* a known ectoparasite of freshwater fish which is known to cause white spot disease, or Ich; one of the most common and persistent diseases in fish [44]. Many of the algal genus like *Leptolyngbya, Synechococcus, Mastigocladus laminosa* detected in this study are known to be responsible for algal blooms [45]. Also Nitrogen fixing heterocystous cyanobacteria BGA; *Nostoc, Calothrix* and diatoms; *Epithemia*, *Rhopalodia* known to be bioindicators of nitrate levels [46] were detected in the study. Algae belonging to genus *Prototheca* and *Euglena* detected in the taxon were indicative of high sewage levels in the river [45]. *Protheca wickerhamii*, a green algae that lacks chlorophyll has also known to be associated with human and animal diseases [47]. *Alexandrium fundyense* a species of dinoflagelates detected in the samples have been known to cause Paralytic shellfish poisoning [48,49]. A few other significant algae found in the taxon are *Microcystis sp.,*are known to be bioindicators of a pre-existing high nutrient status [45]. The presence of *Microcystis aeruginosa*is also impactful since it is known to produce neurotoxins and hepatotoxins, such as microcystin and cyanopeptolin [50,51].

The absence of prokaryotes in the first 50 hits could be attributed to grazing by the predominant protozoans. Conversely, prokaryotes sequences were much more abundant when MG-RAST metagenome analysis server was used for assessing the allochthonous inputs in the river sample. These results revealed that about 74% of the reads were assignable to a habitat, with 0.58% of them assigned to human gut, 0.328% assigned to anthropogenic habitat, 0.0043% assigned to soil and other aquatic ecosystems, 0.004% assigned to plants habitat and 26% assigned as others (farm, canopy, cow shed, microbial, continental slope, marine, forest etc.). Around 39 strains of *Mycobacterium sp.* were detected. *M. leprae*, *M. tuberculosis* are causative agents of leprosy, tuberculosis. Multidrug-resistant non-tuberculous mycobacteria (NTM); *M. abscessus* [52] also featured in the data. *Actinobacteria* over *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Cyanobacteria* and unclassified viruses at the phylum level that have been observed in the samples are associated with the human gut [53,54].

Prominent groups related to water environment were observed as *Proteobacteria, Firmicutes, Actinobacteria, Cyanobacteria, Arthropoda and Chordata.* This emphasized bacteria in particular as the active microbiota from anthropogenic inputs. A number of functional genes also indicated the presence of viruses frequently reported as potential pathogens. Here, the presence of dsDNA and ssRNA viruses and retroviruses, known to infect with more or less specificity arthropods (*Baculoviridae, Poxviridae*), animals and humans (*Herpesviridae, Retroviridae, Siphoviridae*), aquatic organisms like fish and amphibians (*Phycodnaviridae*) was observed. The inventory of riverine prokaryotic and eukaryotic genes and transcripts obtained in this study unravels a world of microbial taxa and functions and effects in a significant river ecosystem.

Fig 1. Bubble plot showing the relative taxonomic abundance of sample 1.9723

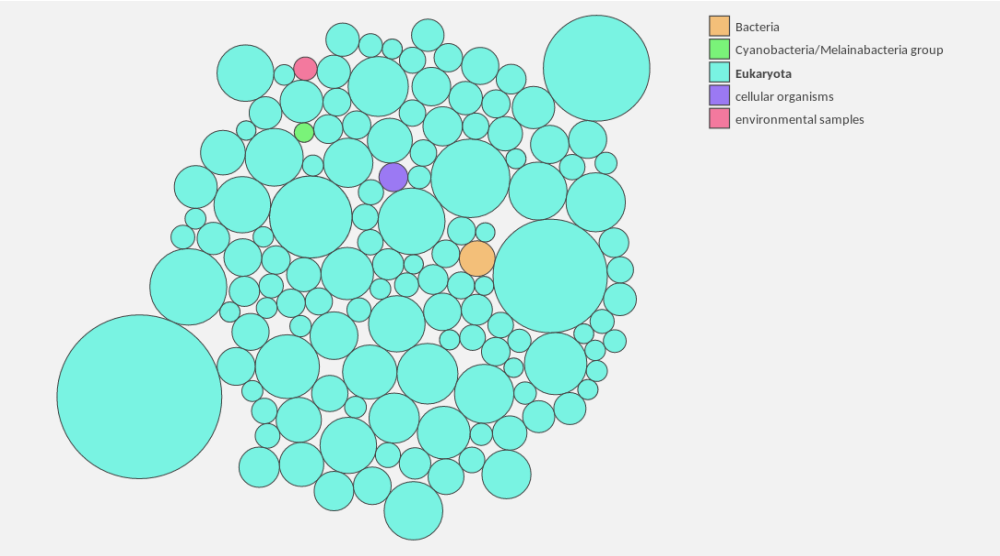


Fig 1: Bubble size indicates taxon abundance relative to its maximum abundance (largest bubble size). The size of the circle is scaled logarithmically to represent the number of sequences assigned directly to the taxon.

Figure 2: Bar chart showing the taxonomic abundance of 1.9723 at phylum level.

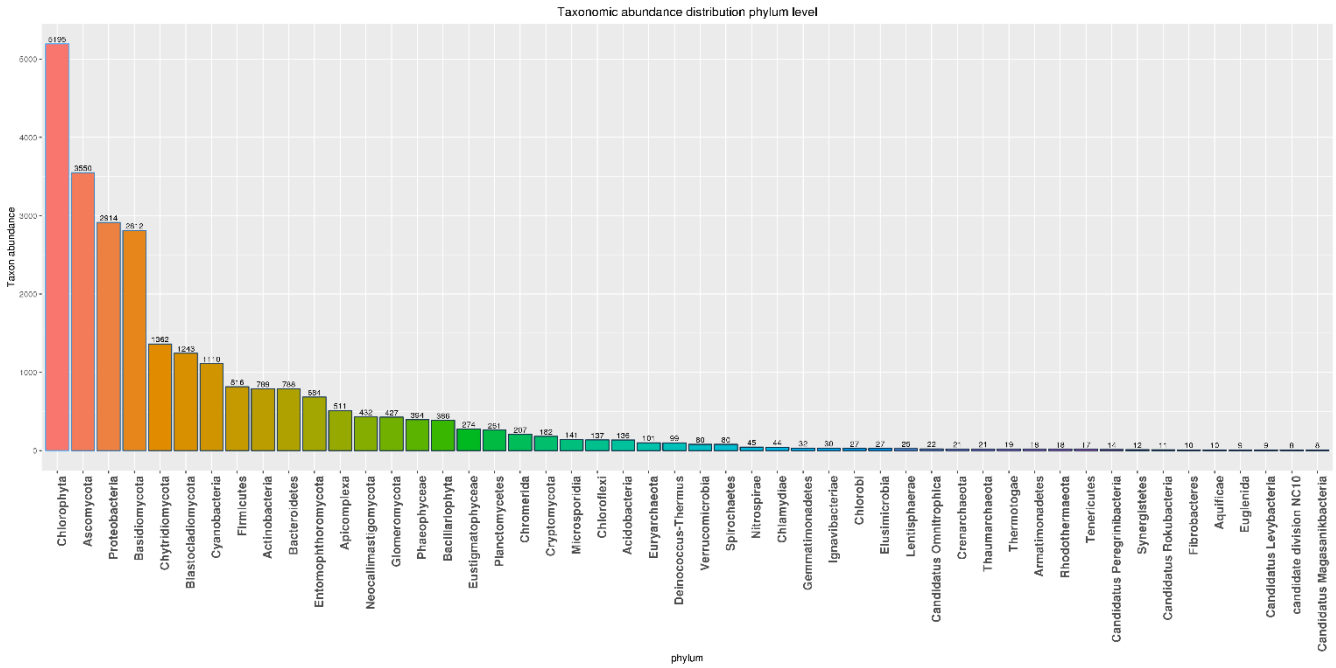


Figure 3: Bar chart showing the taxonomic abundance of sample 1.9723 at family level.

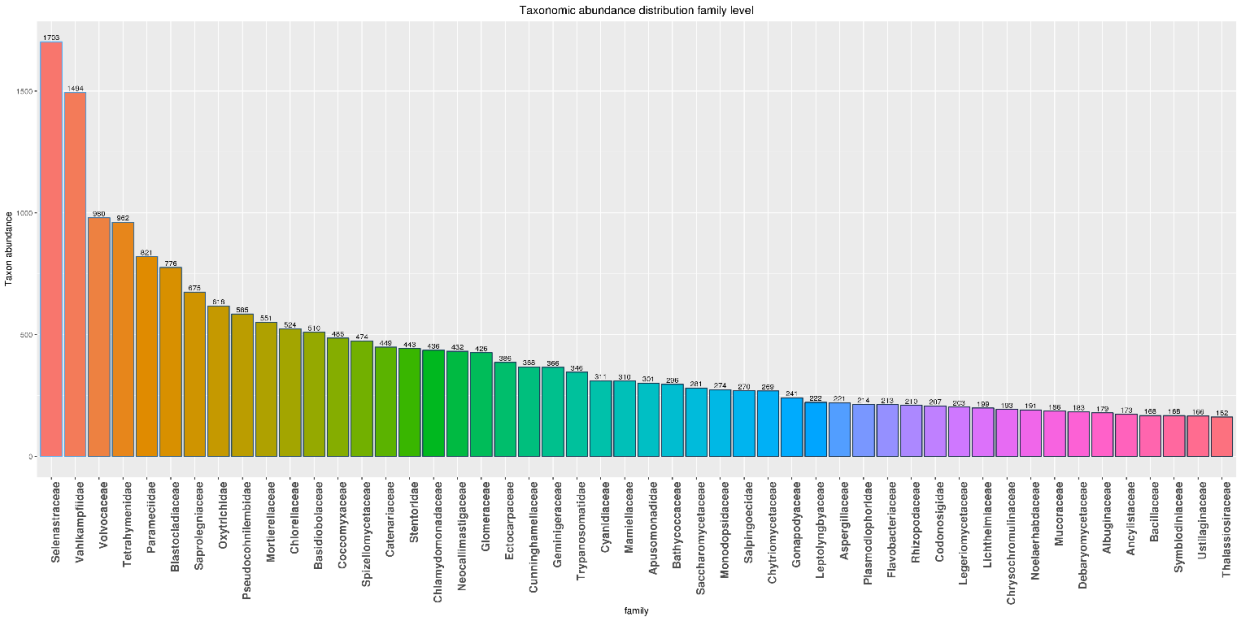


Figure 4: Bar chart showing the taxonomic abundance of sample 1.9723 at genus level.

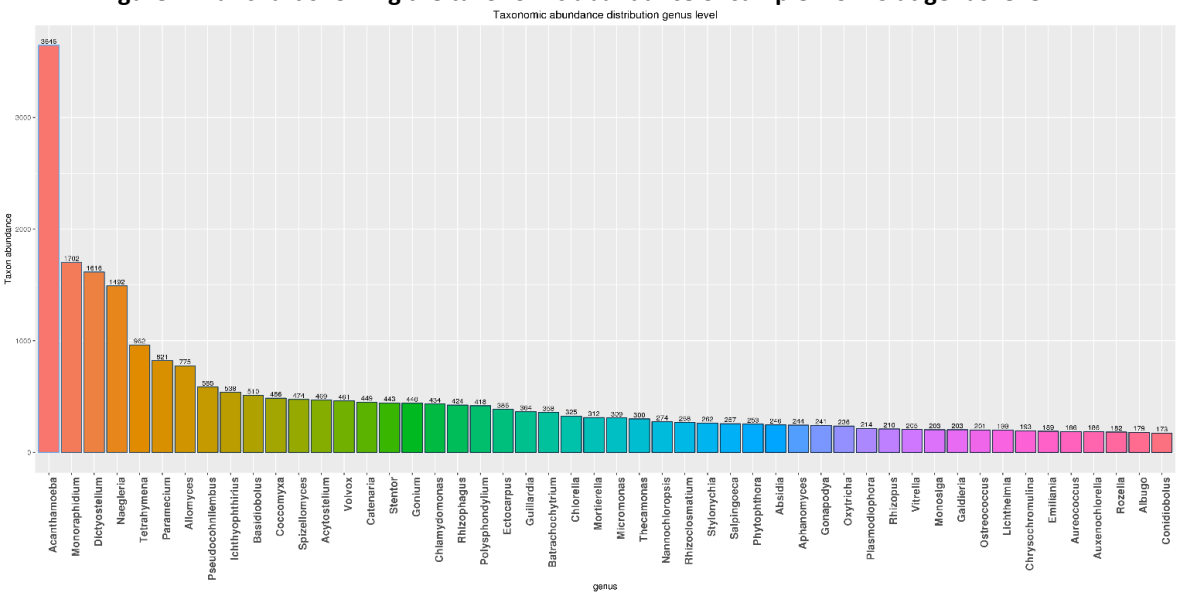


Figure 5: COG Functional Category Hits Distribution of sample 1.9723

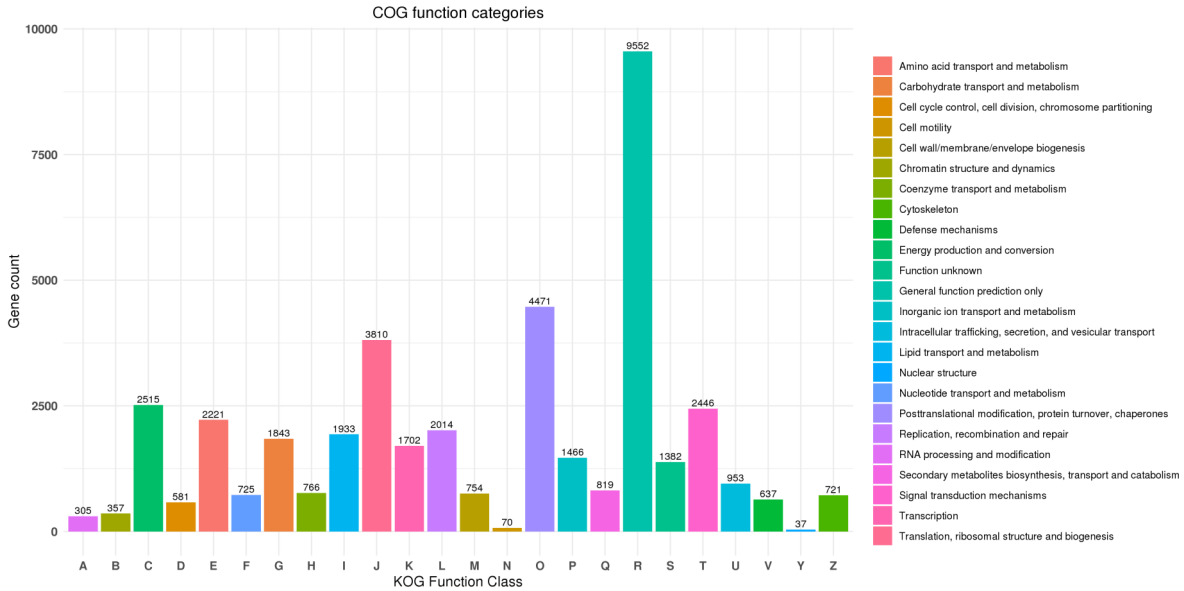
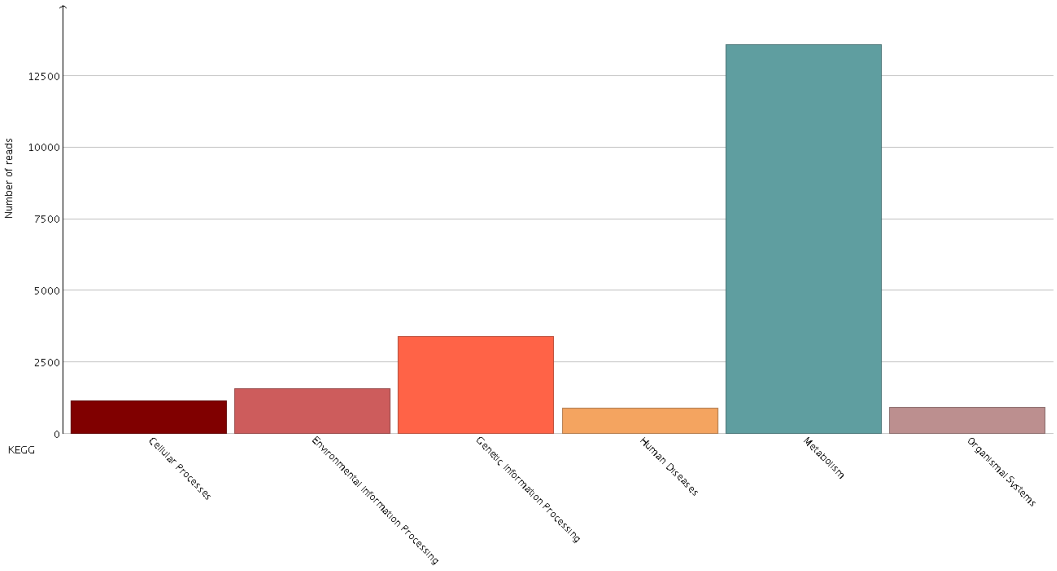


Figure 6: KO Functional Category Hits Distribution of sample 1.9723



3.4 Functional analysis

Functioning analysis of the community was explored by identifying protein sequences associated with the gene ontologies (GO). The relative distribution of GO terms' among the datasets was variable, thus highlighting metabolic requirements within community's capabilities. Results overall, indicated predominance of metabolic processes oriented towards energy production (catabolism) and transports, followed by genetic information processing as per the KEGG Orthology.

Enzymes important in detoxification metabolism processes such as catalase (E.C. 1.11.1.6; GO: 0004096), Cytochrome P450 (GO:0005506, GO:0016705, GO:0020037, GO:0055114), Glycosyl transferases, related to UDP- glucuronosyl transferase (GO:0005975, GO:0016758, GO:0030259) were seen abundance in the functional analysis. The expression of Serine/ threonine protein kinase (STPK) (GO:0004672, GO:0005524, GO:0006468, GO:0005515) observed could be indicative of the fact that STPKs sense a wide range of signals and coordinate multiple cellular processes to mount an appropriate response.

Expression of tricarboxylic acid cycle (TCA) enzymes like succinate dehydrogenase (GO:0003997, GO:0005777, GO:0006635, GO:0055114), citrate synthase (GO:0044262, GO:0046912), phosphoenol pyruvate carboxykinase (ATP) (GO:0004612, GO:0005524, GO:0006094), and pyruvate dehydrogenase (GO:0008152, GO:0016746) corresponding to the oxidative breakdown of carbohydrates, lipids and amino-acids into CO_2 were evident in the analysis indicating a metabolically active predominantly aerobic consortium. Enzymes of pentose phosphate shunt pathway and related enzymes like Glutathione S-transferase, omega (EC 2.5.1.18; GO: 0005515), ribose phosphate diphosphokinase (GO:0000287, GO:0004749, GO:0009165), fructose-bisphosphate aldolase, (GO:0004332, GO:0006096) phosphogluconate dehydrogenase (NADP + dependent, decarboxylating) (GO:0004616) and fructose 1,6-bisphosphate-1-phosphatase (GO:0042132) were detected, along with ADP binding activity (GO:0043531). Pentose phosphate shunt is a pathway that plays a pivotal role in supplying redox equivalents as well as sugars that are used in the biosynthesis of amino acids as well as nucleic acids, once again indicative of an active metabolic consortium. Gene products involved in the generation of pathogenesis (GO:009407) were also expressed along with expression of gene products (GO:0005524, GO:0016887) related to ABC-type multidrug transport system, ATPase component, antibiotic transport system ATP-binding protein.

The diverse metabolically active eukaryotic and prokaryotic microflora, their escalating antibiotic resistance, and the nurturing pathogens in the river are all in all indicative of a micro ecosystem that could severely affect the flora and fauna dependent on it.

Conclusion:

World Health Organization (2019)[28] reports that economic growth of a country is limited by one third due to lack of clean water. Literature cites not only emergence but also escalation of the growing problem of antibiotic-resistant bacteria to two-thirds of the rivers studied [55]. The analysis of river samples has seen a transition from fundamental physico chemical, microbial analysis to metatranscriptomics, in a view to understand its ecosystem as well as its impact on health as well as environment. Sampling locations, frequency and seasonal variations however are the major limitations in such studies.

The composition of microbial communities as revealed by WMA in the present study was significant enough to emphasize the deteriorating water quality of the Ghaggar River. The ill-effects of anthropogenic inputs as revealed by the thriving microbial communities affect not only the health of the population which depends on it but would damage artifacts present at this major archaeological site. Our metatranscriptomic approach gives insights into tracking the functioning of microbes within the river sample, their physiological traits and potential impact on health and environment. These findings might also prove helpful in identifying specific target genes for futures investigations. However a different depiction could have been drawn under different climate conditions like during seasonal shifts, in case of pollution due to natural emissions, etc., which

Data Availability

The sequence files generated in this study were deposited to NCBI's Sequence Read Archive under BioProject accession no. PRJNA530022 with the sample accession number SAMN11290911.

Acknowledgements:

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