
Exploring The Microbiome of River Ganges and Harnessing the Potentials of Novel Phages Under One Health

By

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Under the supervision of

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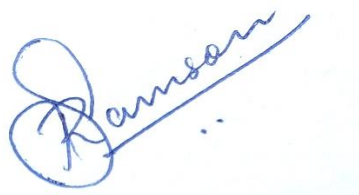
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Signature of the Supervisor

Dr.Mahesh S.Dharne

Date: 28/05/24

Place: CSIR-NCL, Pune



DEDICATION

*This Thesis is dedicated to
Almighty God -My Source of
Strength, Grace and Wisdom
My Beloved Parents, and
Husband*

*Thank you for being a constant
source of inspiration and
motivation, and for your love,
unwavering support, and
encouragement along the way*



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ABBREVIATIONS

AMR	Antimicrobial Resistance	Pmax	PowerMax Soil Kit
ARGs	Antimicrobial Resistance Genes	LA	LongAmp
ESKAPEE	<i>Enterococcus faecium</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella pneumoniae</i> , <i>Acinetobacter baumannii</i> , <i>Pseudomonas aeruginosa</i> , and <i>Enterobacter</i> species <i>Escherichia coli</i>	SDGs	Sustainable Development Goals
MRGs	Metal Resistance Genes	CFU	Colony Forming Units
MDR	Multiple Drug Resistance	PFU	Plaque Forming Units
DNA/RNA	Deoxyribose/Ribose Nucleic Acid	ANOVA	Analysis of Variance
ϕ	Bacteriophage/phage	WW	Wastewater
KEGG	Kyoto Encyclopedia of Genes and Genomes	OD	Optical density
QIIME 1	Quantitative Insights Into Microbial Ecology	ATCC	American Type Culture Collection
PCA	Principal Component Analysis	CLSI	Clinical and Laboratory Standards Institute
PCR	Polymerase Chain Reaction	ANI	Average nucleotide identity
CTAB	Cetyl–trimethyl–ammonium bromide	SDG	Sustainable Development Goal
MSDP	MO BIO Powersoil Total RNA/DNA isolation kit	FE-SEM	Field emission scanning electron microscopy
BGDP	HipurA Bacterial Genomic DNA Purification Kit	CLSM	Confocal Laser Scanning Microscope
DNA_XR	DNA Xpress Reagent	HR-TEM	High-Resolution Transmission Electron Microscopy

APV	All Prep Viral DNA/RNA kit	SM Buffer	SodiumMagnesium Buffer
PL	PureLink™ Viral RNA/DNA Mini Kit	MOI	Multiplicity of Infection

SYNOPSIS REPORT

Synopsis Report

		Synopsis of the Thesis to be submitted to the Academy of Scientific and Innovative Research for Award of the Degree of Doctor of Philosophy in Sciences/ Engineering	
Name of the Candidate		Pardhe Rachel Samson	
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Research Supervisor/ Co-supervisor		Dr. Mahesh Dharne	

Introduction

River Ganges originates as River Bhagirathi from the Himalayan glacier- Gomukh and takes the name Ganges after the confluence of Bhagirathi River and Alaknanda River at Devprayag in Uttarakhand, India (Khairnar, 2016). Most of the studies pertaining to the River Ganges in the past have emphasized the deteriorating water quality and the alterations in the microbial diversity as an attribute of elevated anthropogenic activities (Bhardwaj et al., 2010; Farooquee et al., 2008; Srivastava et al., 1996). Little is known about microbial communities in the Ganges River and their spatial-temporal dynamics. Freshwater rivers have a direct impact on society and human health, water of varying sizes, productivity, and geological history. Taken together with the seasonal dynamics of many freshwater systems, we expect these river environments to harbor a great deal of undiscovered microbial diversity. The whole metagenome approach is specialized to capture a broader arena of life, including all types of nucleic acids, i.e., from plants, animals, microbes, viruses, and plankton. The Bioinformatics approach is expected to indicate the mapping of the microbiome across various databases. This information is crucial in identifying

the untapped microbial diversity of River Ganga across its pristine stretch. Therefore, in this study, we employed shotgun metagenomics nanopore sequencing to study microbial community structure and its predictive functions from the ecologically underexplored sediment samples along a ~1750 km river transects, in upstream/ pristine stretch and the anthropogenically impacted/ the lower stretch before and after the monsoon. Additionally, the River Ganges has a rich source of bacteriophages, which inhibit several pathogenic microorganisms. However, the knowledge of the bacteriophage diversity of the River Ganges and their application under one health remains elusive (Samson et al., 2024).

The raw (untreated) WW comprises various pathogenic bacteria. The key pathogens in sewage that cause environmental and health problems are *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Adenovirus*, and *Norovirus*. The contamination of these microbes in the environment occurs through intestinal and extra-intestinal routes (Jang et al., 2017). Therefore, biological indicators are to be more focused to reflect the connotation of sewage recycling better and provide an answer to the current water-environmental sanitation practices. The current WW treatment includes physical, chemical, and biological methods for eliminating microbial contamination. (Qian et al., 2022). However, the rise of antibiotic resistance in bacteria has considerably challenged the treatment of microbial pollution in WW. Therefore, to tackle the existing antibiotic resistance crisis, there is a need to safeguard human health by shielding animal and environmental health through the 'one health' approach (Garvey, 2020).

Given this, the present study was initiated to explore the microbiome of the River Ganges and explore the untapped phage diversity from the pristine stretch of the River Ganges to isolate novel phages and explore their potential for use as biocontrol agents against *E. coli* and *Pseudomonas aeruginosa*, facilitating improved environmental health.

Statement of the problem

- Limited information on the microbiome of the Ganges River
- Increasing environmental AMR and lack of existing practices to tackle them
- Unexplored pristine stretch of the Ganges for isolation of potential phages to improve environmental health.

Methodology

Sampling and DNA extraction of the lower stretch

The sediment samples (depth ~20 cm) from fifteen locations at two different seasons were collected in triplicates (n=90) (n denotes the number of samples) from the left bank (LB), center (M), and right bank (RB) of the river, in December 2017 and June 2018. These locations include Haridwar, Bijnor, Kanpur, Prayagraj, Varanasi, Patna, and Bhagalpur. Water-saturated sediment samples were collected in triplicates. Subsequently, the samples (LB, M, and RB) were pooled together location-wise, and the total DNA was extracted using RNeasy PowerSoil Total RNA Isolation Kit (Qiagen) and RNeasy PowerSoil DNA Elution Kit (Qiagen).

Sampling and DNA extraction of the upper stretch

Sediment samples (300-500gm) were collected in four seasons (pre-monsoon June 2018, June 2019) (post-monsoon October 2018 and February 2020). A total of 12 sediment samples were collected from 6 locations (Gomukh, Gangotri, Harshil, Maneri, Tehri, Rishikesh) under study in each season. The samples were collected in sterile polypropylene containers, retained on ice, transported to the laboratory, and stored at 4°C for further microbiological processing. DNA was extracted from the 10 gm of sediment samples using DNeasy PowerMax Soil Kit (Qiagen) as per the manufacturer's protocol. The quality and concentration of DNA for each sample were assessed by Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific) and qubit fluorometer (Thermo Fisher Scientific) quantification using a qubit HS assay kit (Thermo Fisher Scientific, Q32853). Until further downstream processing, the DNA was stored at -20°C. Amplification of DNA samples from the upper stretch was carried out with PCR expansion (EXP-PCA001-Oxford Nanopore Technologies) + Long Amp (LA) (NEB) as per the manufacturer's protocol.

Metagenomic DNA sequencing and annotation pipeline

Total DNA from upper and lower stretches was sequenced by nanopore sequencing technology using the MinION sequencing platform. The fastq files obtained were processed for quality check, followed by their upload to the MG-RAST (Metagenomic Rapid Annotation using Subsystem Technology) server. Annotation was done against the RefSeq and SEEDsubsystems database for functional diversity analysis. Additionally, another metagenome classifier-Kaiju v1.7.3 (for lower stretch) and Kraken-2 for upper stretch, was used to ascertain a higher sensitivity and precision to the taxonomic reads. For ascertaining ARGs, deep ARG tool was used for both stretches.

Isolation, enrichment, and propagation of phage ERS-1

Water-saturated sediment samples of the Ganges River from the Harshil location was used as the source of phage isolation using respective hosts (*E.coli* ATCC 8739,) The sediment was allowed to settle briefly while the sediment-laden water (~40 mL) was equilibrated with 10 mL of SM buffer for 1 hour in an incubator at 37°C with a speed of 180 rpm. The resulting mixture was removed and centrifuged at 6000×g for 10 mins. The supernatant (~40 mL) was then passed through a combination of 0.45µm 0.22µm of Polyether sulfone (PES) membrane-based syringe filters (Hi-Media), respectively, and 10 mL of filtrate from each set of the syringe filters was used for enrichment. The enrichment flasks were incubated at 37°C for 24 h with shaking (180rpm). The presence of lytic phages in the samples was confirmed by observing clear zones (plaques) against the bacterial lawn through the qualitative spot assay. The quantitative enumeration of phages in the enriched lysate was done using the soft agar overlay method. Each set was titrated in triplicate three independent times to ascertain the phage titer. Purification and concentration of ϕ ERS-1 was carried out with Amicon® Ultra-15 centrifugal filter with Ultracel100 kDa membrane (Merck Millipore).

Isolation, enrichment, and propagation of phage PRS-1

Water-saturated sediment samples of the Ganges River from the Maneri (30°45'14"N 78°33'34"E) location were used as the source of phage isolation against the primary host *P. aeruginosa* (ATCC 15692). The sediment was allowed to settle briefly while the sediment-laden water (~40 mL) was equilibrated with 10 mL of SM buffer for 1 hour in an incubator at 37°C with a speed of 180 rpm. The resulting mixture was removed and centrifuged at 6000×g for 10 mins. The supernatant (~40 mL) was then passed through a combination of 0.45µm 0.22µm of Polyether sulfone (PES) membrane-based syringe filters (Hi-Media), respectively, and 10 mL of filtrate from each set of the syringe filters was used for enrichment. A total of 5 mL of log-stationary phase culture of *P. aeruginosa* PAO1 (host) was added to 20 mL of the filtrate obtained from the previous step. The enrichment flasks were incubated at 37°C for 24 h with shaking (180rpm). The presence of lytic phages in the samples was confirmed by observing clear zones (plaques) against the bacterial lawn through the qualitative spot assay. The quantitative enumeration of phages in the enriched lysate was done with the soft agar overlay method. Each set was titrated in triplicate three independent times to ascertain the phage titer. Purification and concentration of ϕ PRS-1 was carried out with Amicon® Ultra-15 centrifugal filter with Ultracel100 kDa membrane (Merck Millipore).

Morphological features of ERS-1 and PRS-1

The morphology of ϕ ERS-1 and PRS-1 was visualized using Jeol JEM-F2100 high resolution-transmission electron microscope (HR-TEM) at 200 kV and imaged with a Xarosa emsis camera coupled to the microscope. Morphological classification of phage was done following the guidelines recommended by the International Committee on Taxonomy of Viruses (ICTV).

Phage adsorption and one-step-growth curve for ERS-1 and PRS-1

The host cell culture at the exponential phase was mixed with the fresh phage lysate at an MOI of 0.1 and incubated at 37°C, 100 rpm for 30 mins. After 5 minutes, an aliquot was drawn and centrifuged to pellet the adsorbed fraction of the phage each time. At the same time, the supernatant was filtered using a 0.2 μ m PES syringe filter to obtain the fraction of unadsorbed phages. One one-step growth curve assay was carried out with fresh phage lysate of ERS-1 and PRS-1 at a MOI of 0.1 was added to the host cells (10^8 cells/mL) and allowed to adsorb for 20 mins at 37°C, 100 rpm. The adsorbed phages were centrifuged at $10,000 \times g$ for 5 min, and subsequently, the bacteria-phage complex (pellet) was resuspended in 2 mL of SCDB and incubated at 37°C, 100 rpm for 100 mins. A 100 μ L aliquot was collected every 10 minutes, followed by plaque assay to determine the phage count (PFU/mL) at each time interval.

Evaluation of pH and temperature stability for ERS-1 and PRS-1

Briefly, the pH of the SM buffer was adjusted to 3, 5, 7, 9, and 11, followed by adding 100 μ L of the phage suspension to 900 μ L of SM buffer with respective pH. The samples were incubated for four hours at 37°C, 100 rpm in a dry bath, followed by plaque assay. For determining the thermal stability, suspensions of 100 μ L of the phage in 900 μ L of SM buffer were made and incubated at a diverse set of temperatures (4, 15, 25, 37, 45, 55, and 65°C) for four hours at 100 rpm followed by measuring the phage titer with plaque assay.

DNA extraction, Genome Sequencing, and analysis for ERS-1 and PRS-1

A high-titer phage stock was used to isolate DNA from both phages. The DNA was extracted using PureLink Viral RNA/DNA Mini Kit (Thermo Fisher Scientific). The phage DNA concentration was quantified. Library preparation of the samples for whole genome sequencing was carried out with Ligation Sequencing Kit (SQK-LSK114) and Native Barcoding Kit (SQK-NBD 114.24) as per the manufacturer's instructions. The library was loaded onto flow cell R10.4.1(Oxford Nanopore Technologies) using the MinION Mk1C sequencing platform. The genome was assembled using Flye, a *de novo* genome assembler v 2.9.1. Further, the phage

genome annotation of the putative proteins was predicted with Rapid Annotation using the Subsystem Technology (RAST) annotation server (<https://rast.nmpdr.org>). VipTree was used to understand genome-wide similarities between the isolated phages and other reference viral genomes (Nishimura et al., 2017). Whole genome Average nucleotide identity (ANI) to ascertain genetic relatedness between ERS-1, PRS-1, and other closely related phages was computed with OAT v0.93.1 (Orthologous Average Identity Tool) software.

Time-dependent evaluation of cell viability against E. coli biofilm by ERS-1

In-vitro efficacy of both the phages was assessed against their respective hosts in biofilm control through inhibition and disruption assays using Film Tracer™ LIVE/DEAD® Biofilm Viability kit (Invitrogen). The experiment was carried out and divided into two sets as follows:

Set I: Biofilm Inhibition (ϕ ERS-1 treatment at 0 hr. followed by imaging the replicates at 6, 12, and 24 hrs.).

Set II: Biofilm Disruption (ϕ ERS-1 treatment after 24 hrs. on pre-formed biofilm and imaging the replicates at 6, 12, and 24 hrs.).

Statistical analysis of the results was performed in GraphPad Prism v9.5.1 using RM-one-way ANOVA with multiple comparisons and a p-value of 0.05.

ERS-1 Bacteriophage-based biocontrol for reduction of bacterial counts from wastewater

A total of 1 L (n=3) of untreated wastewater samples were collected in a sterile bottle by grab-sampling method. The samples were brought immediately to the lab and processed for experimental setup. The raw sewage (untreated wastewater) samples were divided into test and control groups. For the test group (100 mL aliquot of the raw sewage was challenged with 1 mL of high titer phage stock of ϕ ERS-1 (10^{17} PFU/mL), while the control group was devoid of any treatment. The experiment was carried out in triplicates, and the flasks from each group were incubated at 37°C for 24 hours, followed by recording the results as CFU/mL, while the reduction in the bacterial counts was calculated in the form of log reduction and percent reduction. A time-kill assay was performed to harness the broad-spectrum potentials of ϕ ERS-1 in improving the reusability of wastewater.

Set I: Unenriched group (Test: 100 mL raw sewage +1 mL ϕ ERS-1), (Control: 100 mL raw sewage).

Set II: Coliform enriched group (Test: 100 mL raw sewage +10 mL Mc Conkey broth + 1 mL ϕ ERS-1), (Control: 100 mL raw sewage + 10 mL Mc Conkey broth).

For each time point, the bacterial count was determined as CFU/mL, and the efficacy of ϕ ERS-1 in reducing the bacterial counts was calculated through log reduction and (%) reduction.

Antibiofilm Potentials of ϕ PRS-1

Quantification and Visualization of Biofilm Inhibition and Disruption of ϕ PRS-1

A biofilm inhibition and disruption assay was performed to understand ϕ PRS-1's effect in inhibiting and disrupting the biofilm formed by PAO1. In this experiment, the biofilm of the host culture was formed in a 35 mm treated tissue culture dish (Hi-Media) that supports cell adherence followed by immediate addition of the phage (inhibition) and addition of phage after 24 hours on the preformed biofilm (disruption).

Time-dependent evaluation of cell viability of *P. aeruginosa* PAO1 biofilm

In-vitro efficacy of ϕ PRS-1 was assessed against *P. aeruginosa* PAO1 ATCC 15692 in biofilm control through inhibition and disruption assays using Film Tracer™ LIVE/DEAD® Biofilm Viability kit (Invitrogen). The experiment was carried out and divided into two sets as follows:

Set I: Biofilm Inhibition (ϕ PRS-1 treatment at 0 hr. followed by imaging the replicates at 6, 12, and 24 hrs.).

Set II: Biofilm Disruption (ϕ PRS-1 treatment after 24 hrs. on pre-formed biofilm and imaging the replicates at 6, 12, and 24 hrs.).

Statistical analysis of the results was performed in GraphPad Prism v9.5.1 using RM-one-way ANOVA with multiple comparisons and a p-value of 0.05.

In-vitro cytotoxicity evaluation of ϕ PRS-1 on L929, HUVEC, and Vero cell lines

Microscopic observation of the cells was carried out, and after achieving more than 80% confluency, the spent media was decanted from the cell culture flask. Subsequently, 200 μ L of 1X Trypsin-EDTA solution (HiMedia) and an equal volume of DMEM were added to the flask and incubated for 2-3 mins in a CO₂ incubator (Thermo Fischer Scientific). The contents were then transferred into a sterile 1.5 mL microfuge tube (Tarsons) and centrifuged at 6000 rpm for 2 mins. The supernatant was decanted, and the cells were resuspended in a fresh 1 ml medium. After that, the cells were taken as 10 μ L cells + 10 μ L trypan blue + 80 μ L PBS in separate sterile 200 μ L microfuge tubes, and the number of cells was numbered using a Neubauer counting chamber. 10⁴ cells were seeded in each well of a 96-well microtiter plate and incubated for 24 hours in a CO₂ incubator. The next day, the seeded plate was microscopically examined for cell

confluency. After achieving >80% confluency, the spent media was decanted from the plate and washed twice with 1X PBS pH 7.4 (Hi-Media). Subsequently, phage lysates were added to the wells and incubated for 24-48 hours to assess the cytotoxicity. After 24 hours, the plate was visualized microscopically for morphological changes in the cells and incubated further for up to 48 hours. The cytotoxicity was assessed using the MTT method. A total of 1mg/mL of MTT stock was prepared, and ~30 µL of MTT was added in all the wells such that the final concentration was 0.5 mg/mL/well. The plate was incubated for 3 hours, followed by removal of all the contents from the well and adding 99% DMSO. The resulting suspension was incubated for 5 mins, and the absorbance was recorded at 570 nm (Fig 15, 16). The percent cell viability was calculated using the standard:

$$\% \text{ Cell viability} = \frac{\text{Average O.D. of the Sample}}{\text{Average O.D. of the control}} \times 100$$

Results and their interpretation

Bacterial community and resistome composition of the lower stretch

A total of 152 bacterial phyla and 3256 bacterial genera were observed at all the locations in both seasons. *Proteobacteria* (45.89 ±4.16%), was the most prevalent phylum, followed by *Actinobacteria* (9.64 ±1.46%), *Chloroflexi* (9.36 ±2.19%), *Acidobacteria* (8.54 ±3.37%), and *Bacteroidetes* (7.35 ±3.39%) in all the samples across both the seasons. A seasonal comparison between all the samples was made to understand compositional changes in the bacterial taxonomy. The overall bacterial taxonomy remains similar at the phylum level across both seasons (p>0.99; ANOVA with BH correction). At the genus level, *Streptomyces* (17-32%) dominated at all the locations except for G13US, JG14 US, J/G14DS, G15Y, JG16US, J/G16DS, where a predominance of *Nitrospira* (19-32%)—nanoARG-based analysis. The analysis revealed the prevalence of genes extending resistance to a total of 28 classes of antibiotics. The most abundant ARGs across all the locations were those that offer resistance to multidrug/multiple antibiotics (MDR) (39.67 ±3.68%), followed by bacitracin (7.70 ±1.37%); peptide (7.65 ± 0.95%); tetracycline (6.86 ± 1.23%) and glycopeptide (5.3± 1.14%). categories of ARGs were investigated for their gene name (subtypes). A higher prevalence of genes *rpoB2* (13.32 ± 2.86%), *ompR* (9.98 ± 1.83%), *bcrA* (9.08 ± 2.05%), *tetA* (48) (7.86 ± 1.89%), multidrug ABC transporter (6.08 ± 1.61%), *patA* (5.95 ± 1.33%), *vanR* (4.98 ± 1.43%) and *bacA* (4.57 ± 1.33%) were abundant in all the samples.

Virome of the lower stretch

Analysis revealed the presence of bacteriophages from *Siphoviridae*, *Myoviridae* and *Podoviridae* families against antibiotic and metal-resistant bacteria such as *Pseudomonas*, *Burkholderia*, *Stenotrophomonas*, *Cupriavidus*, *Pandoraea*, and *Ralstonia*. to understand the phage-related and host-phage associated functions, predictive *in-silico* functional annotation of the datasets was carried out using Subsystems and KEGG databases in MG-RAST. The prevalence of hits against phage-related proteins indicates a strong presence of a host-phage relationship. Interestingly, our study also found hits for sequences related to phage-associated lytic activities (lysozyme, endolysin, N-acetylmuramoyl-L-alanine amidase, endopeptidases).

Bacterial community and resistome composition of the upper stretch

Analysis of the bacterial diversity through MG-RAST revealed a predominance of *Bacteroidetes* (40-43%), followed by *Proteobacteria* (29-31%), *Firmicutes* (13-17%) and *Actinobacteria* (5-7%) during post-monsoon (October 2018 and February 2020). A significant increase was observed in the relative abundance of *Proteobacteria* (75-79%), while a substantial decrease was observed in phylum *Bacteroidetes* (4-6%) in all the samples during pre-monsoon (June 2018 and June 2019). Similarly, at the genus level, during the post-monsoon, a significant increase in the relative abundance of genus *Bacteroides* was observed in February 2020 (60-65%) and October 2018 samples (90-94%). For the pre-monsoon samples, a predominance of *Thiobacillus* (16-18%), *Burkholderia* (13-18%), *Sideroxydans* (14-16%), *Pseudomonas* (9-12%) and *Polaromonas* (6-8%) was observed. The resistome comprised of Multidrug ABC transporters that contribute to drug efflux pumps- increased in the pre-monsoon samples (glacier melting, tourism). *kdpE*, *mdtB*, *adeF*, *baeR* genes are involved in controlling the virulence loci in pathogenic *Escherichia* and *Shigella* sp. While the *macB*, *mexK*, *mexW*, *mexB*, *arnA*, *adeF*, *baeR* genes are responsible for Antibiotic efflux in *P. aeruginosa*, *P. fluorescens*, PAO1.

Virome of the upper stretch

Majority of viral diversity comprises of bacteriophage families containing more lytic viruses i.e. *Podoviridae* and *Myoviridae*. The presence of phages against putative pathogens spanning the ESKAPEE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, *Enterobacter* sp., *Escherichia coli*) of bacteria opens a window of opportunity for their bioprospection. Additionally, phage-related functions were present in their structure, life cycle, assembly, and exit. Also, the presence of phage proteins plays a key role in attachment, breaking of bacterial cell wall, phage DNA replication, phage

gene expression, capsid formation, assembly, and release. Therefore, a higher relative abundance of bacteriophage families in the upper stretch over other viral families could be correlated with one of the "non-putrefying" properties. The metagenomic data serves as a blueprint to tap into the unexplored phage diversity and isolate potent phages to tackle AMR under one health.

Isolation, identification, and growth parameters of ERS-1

The plaque morphology of ϕ ERS-1 clear plaques encircled in a translucent halo. The morphological architecture of ϕ ERS-1 comprised a long head showing an icosahedral symmetry, a collar, a contractile tail, a small baseplate with short spikes, and six long terminal (tail) fibers. An increase in the temperature from 37°C to 45°C resulted in a significant loss in the phage titer (ANOVA, $p < 0.001$). The pH stability of ϕ ERS-1 was determined from pH 3 to 11. From the phage titration, pH 7 was the optimum pH for sustaining phage stability. On either side of the pH scale, it was observed that the phage titer reduced from 10^{17} PFU/mL to 10^{10} PFU/mL (pH 3) and 10^{12} PFU/mL (pH 5, pH 9), respectively. However, a phage titer of 10^9 PFU/mL is considered a high titer. This suggests that ϕ ERS-1 continues to have an infectious nature for *E. coli* even at a diverse pH range.

Genome analysis, annotation, and proteome of ϕ ERS-1

The genome length of ϕ ERS-1 consists of 175,242 bp. The genome of ϕ ERS-1 clustered with some *Escherichia* phages, followed by *Shigella* phages, all belonging to *Tequatrovirus*, a genus of the *Straboviridae* family, according to ICTV. Further, from the orthoANI comparison of the genome of ϕ ERS-1 and closely related *Tequatrovirus* members, it was observed that ϕ ERS-1 has an ANI of 94% with *Escherichia* phage LH2, indicating the close relatedness of its genome.

Time-dependent evaluation of E. coli biofilm inhibition and disruption by ERS-1

The CLSM analysis of ϕ ERS-1 treated *E. coli* showed red fluorescence at 6, 12, and 24 hours, indicating the dead biomass of *E. coli* cells, as compared to the control (devoid of ϕ ERS-1 treatment). The presence of live biomass was ascertained through CFU counts of viable cells. Results indicated a significant inhibitory effect on biofilm formation at 6 hrs., which continued up to 24 hours (ANOVA, $p < 0.0001$). Previous studies have demonstrated the antibiofilm effect of *Tequatrovirus* against *E. coli* and *Salmonella*, with bacterial reduction from 2-5 logs within 6-24 hours (Liao et al., 2022; Zhou et al., 2022). However, in our study, we found that ϕ ERS-1 was capable of reducing the abundance of *E. coli* cells from $6.14 \log_{10}$ CFU/mL at 6 hours to $8.22 \log_{10}$ CFU/mL at 24 hours, with a ~100% reduction in the total viable counts. In natural

environments, most biofilms are in their mature state, and therefore, to detach the biofilm, disruption of EPS polymers followed by planktonic cell dispersal is essential (Shrestha et al., 2022). It was observed that the number of dead/ non-viable cells increased in the phage-treated group for 24 hours. This indicates a gradual disruption of *E. coli* biofilm by ϕ ERS-1. The total viable counts at each time point revealed that ϕ ERS-1 was capable of disrupting the pre-formed biofilm layer of *E. coli* with a reduction of 2.4 log₁₀ CFU/mL at 6 hours, followed by 2.70 log₁₀ CFU/mL at 12 hours, and 3.88 log₁₀ CFU/mL at 24 hours.

ERS-1 Bacteriophage-based reduction of bacterial counts from wastewater

The initial screening results showed a significant reduction of the bacterial load of 2.27 log₁₀ CFU/mL in the ϕ ERS-1 treated group of the raw sewage after 24 hours (p<0.001). Further, a time-kill assay was performed to evaluate the magnitude of reduction in the viable coliform load in control and ϕ ERS-1-treated groups. Interestingly, in the set of ϕ ERS-1 treated raw sewage, after 24 hours, a 2- 2.4 log₁₀ reduction was observed compared to the control group. Notably, in the coliform enriched set of raw sewage, 4.2 log₁₀ CFU/mL reduction was observed in the ϕ ERS-1 treated group. This indicates that ϕ ERS-1 could be used as a green approach to reduce the coliform counts. For phage PRS-1, it was observed that the genome clustered with *Pseudomonas* phages, all belonging to the *Pbunavirus* group, under unclassified family according to the ICTV. The genome showed an ANI of 90% with the closely related phages, indicating novel species under *Pbunavirus* genome.'

Isolation, identification, and growth parameters of PRS-1

Twelve phages were isolated against PAO1, of which the ϕ PRS-1 showed a maximum inhibitory effect up to 48 hours. The morphological architecture of ϕ PRS-1 comprised a long head showing an icosahedral symmetry, a collar, a contractile tail, a small baseplate with short spikes, and six long terminal (tail) fibers. Additionally, the percentage of free phages in the unadsorbed fraction decreased rapidly at 15 mins (~70%), and only ~10 % of free phages were observed from 20-30 mins. Phage PRS-1 showed a latent period of 30 mins and a burst size of 25 ($\pm$ 5) phage particles per infected cell of *P. aeruginosa* PAO1 (host). The temperature stability of ϕ PRS-1 was determined from 4°C to 75°C. Interestingly, it was observed that the phage titer remained relatively stable from 4°C to 45°C. Additionally, a significant decline was observed in the phage titer at 55°C to 65°C (ANOVA, p<0.001). The pH stability of ϕ PRS-1 was determined from pH 3 to 11. From the phage titration, pH 7 was the optimum pH for sustaining phage stability. On

either side of the pH scale, it was observed that the phage titer reduced in pH 3 and 5, and 9, respectively.

Genome analysis, annotation, and proteome of ϕ PRS-1

The genome length of ϕ PRS-1 consists of 65,628 bp. Analysis of ORFs revealed that they mainly comprise hypothetical proteins followed by phage proteins involved in defining phage structure, DNA replication, repair, packing, and lysis of the host. Analysis of amino-acid sequences in the whole genome of ϕ PRS-1 and closely related phages was performed using VipTree. Further, depending on the S_G scores of tblastx, a subset of 9 closely related phages and one distantly related phages was chosen to construct a rectangular phylogenetic tree (**Fig 2C**). The genome of ϕ PRS-1 clustered with *Pseudomonas* phages, all belonging to *Pbunavirus*, a class of *Caudoviricetes*, according to ICTV. Further, from the orthoANI comparison of the genome of ϕ PRS-1 and closely related *Pbunavirus* members, it was observed that ϕ PRS-1 has an ANI of ~89.72- 90% with related *Pseudomonas* phages indicating novelty of its genome and therefore ranked as unclassified *Pbunavirus* PRS-1(**Fig 2D**).

Time-dependent evaluation of PAO1 biofilm inhibition and disruption by PRS-1

The CLSM analysis of ϕ PRS-1 treated *P. aeruginosa* PAO1 showed red fluorescence at 4, 8, 12, and 24 hours, indicating the dead biomass of *P. aeruginosa* PAO1 cells, as compared to the control (devoid of ϕ PRS-1 treatment). The presence of live biomass was ascertained through CFU counts of viable cells. Results indicated a significant inhibitory effect on biofilm formation at 4 hrs., which continued up to 24 hours (ANOVA, $p < 0.0001$). A gradual disruption of *P. aeruginosa* PAO1 biofilm by ϕ PRS-1 was observed. The total viable counts at each time point revealed that ϕ PRS-1 was capable of disrupting the pre-formed biofilm layer of *P. aeruginosa* PAO1 with a reduction of 3.2 log₁₀ CFU/mL at 6 hours, followed by 4.27 log₁₀ CFU/mL at 12 hours, and ~7 log₁₀ CFU/mL at 24 hours. These values indicate that ϕ PRS-1 can disrupt biofilm with a percent reduction of viable cells up to 99% with 6 hours of treatment. After 24 hours, the percent reduction of *P. aeruginosa* PAO1 cells observed in the biofilm was 99.99%.

In-vitro cytotoxicity evaluation of ϕ PRS-1 on L929, Vero, and HUVEC cell lines

Evaluation of cytotoxicity of ϕ PRS-1 was done with an aim to understand the extent of cytotoxic effects of the phage preparation on the cells due to the presence of bacterial endotoxins. The results showed no observable difference in the cellular morphology of the ϕ PRS-1 preparation i.e., either the enriched filtrate, high titer stock, PEG precipitated stock, endotoxin removed

(ETR) prep, vehicle control (only SM buffer) when images after 48 hours of incubation in both L929 as well as Vero cell lines. Further, to ascertain the cell viability, an MTT assay was performed with control and ϕ PRS-1-treated cells. Interestingly, it was observed that all of the phage preparations showed a cell viability between 95-99%, which was comparable to the growth control set. This indicates that the phage PRS-1 is a potent candidate and shows no apparent cytotoxic effects on these cell lines under standard conditions.

Conclusion

The present study is the first report revealing the total microbial diversity and their associated resistome together with the bacteriophage diversity and functions of River Ganges along its 1750 km stretch. Additionally, this study demonstrates the isolation and characterization of a novel phage ϕ ERS-1 and PRS-1 from an untapped location of the Ganges River. The strength of our research lies in the novelty of phages and their bacteriolytic and antibiofilm potentials in reducing the bacterial load of environmentally relevant pathogens in their planktonic cells and biofilm form. The data also shows the efficacy of the newly isolated, high titer ϕ ERS-1 in controlling the bacterial (coliform) load in the sewage water and possibly improving the water quality. Our approach of using polyvalent phage as a green alternative for bacterial biocontrol of sewage water highlights the importance of the potential applications of such broad-spectrum bacteriophages to improve the quality of the effluent and disposal of sludge in the environment. The ϕ PRS-1 is a new species of the Pbunavirus genus lacking any putative AMR-associated, virulence-related, or lysogenic genes, as evident from genome characterization. The ϕ PRS-1 at its 0.0000001 MOI could reduce the abundance of planktonic cells of PAO1 by 8.14 log₁₀ CFU/mL within 24 hours, which is the first report from a single, non-engineered phage. The antibiofilm biofilm data of ϕ PRS-1 against PAO1 showed a log₁₀ reduction of 9.83 (inhibition) and 7.01 (disruption) after 24 hours. Additionally, the in-vitro cytotoxic studies indicate no apparent cytotoxic effects exerted by this phage. These results serve as a foundational basis for the use of ϕ PRS-1 in the disinfection of surfaces contaminated with MDR- *P. aeruginosa* in the environments.

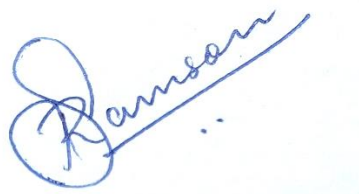
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Chapter 1

Introduction

River Ganges: The Lifeline of India, and A Hub of Ecological Harmony And Microbial Diversity- A Literature Background

1.1 The Ganges Basin

River Ganges (Ganga) is the national River of India and is among the crucial glacier-fed rivers of the Himalayan ranges. The Ganges originates as River Bhagirathi (the source stream of the Ganges) from the Gomukh, the snout of the Gangotri glacier. However, the Ganges proper emerges at Devprayag in Uttarakhand, India, from the confluence of two of its source streams, namely the Bhagirathi River and the sediment-laden-Alaknanda River (Penn, 2013). The pristine stretch of the Ganges is marked from its origin, Gomukh, to Rishikesh and is characterized by limited civilization scattered along its catchment area. The lower stretch (anthropogenically impacted/dynamic stretch) of the Ganges is marked by its debouchment from Himalayan regions to Gangetic plains, with dense civilization along its catchment area (Bhutiani et al., 2014). It traverses ~2500 km and drains into the Bay of Bengal in the Sunderban Delta. The Ganges basin is one of the most fertile and densely populated regions in the world (Agarwal, 2015a). During its course of run from Gomukh to the Bay of Bengal, the Ganges encounters around fifteen tributaries, which account for 60% of its total water, making it the third largest river in the world in terms of the amount of water discharged (Zhang et al., 2018). The melting of Himalayan permafrost gives rise to the sediment load, which gushes out alongside the waters, thereby contributing to the remarkable physical properties of the water at Gomukh (Khairnar, 2016). This majestic riverine system is referred to as a lifeline for a vast multitude of populations, as it fulfills various sustenance needs of people and also serves as an embodiment of spirituality and culture (Bhutiani et al., 2016; Dutta et al., 2020).



Fig. 1: Illustrative Infographics of the Ganges River Basin. (Image source: <https://www.worldbank.org/en/news/feature/2015/03/23/india-the-national-ganga-river-basin-project>).

1.2 Literature background of the pollution load of the Ganges and its relevance to the Environmental AMR

Several anthropogenic activities along the basin and at the shore of this river have contributed most to its pollution. Besides, the discharge of various untreated wastes, such as industrial and sewage, has further altered the water quality of this riverine system (Jani et al., 2018). Recently, Naqvi et al. reported dumping over 50 MLD (million liters per day) of hazardous waste and 140 MLD of domestic waste in Kanpur (Haider Naqvi SK, 2020). Another study by Srivastava et al. mentioned that Ganges' water was unfit for bathing and drinking at Haridwar and Rishikesh (Srinivas et al., 2019). Additionally, Ganges water at other locations such as Bihar, Kanpur, Varanasi, *Triveni Sangam* in Allahabad, Ghazipur, Buxar, Patna, Bhagalpur, and Howrah-Shivpur in West Bengal was neither potable nor suitable for bathing (Dutta et al., 2020). Yamuna River is one of the most polluted rivers in India due to the domestic

and industrial effluents, untreated sewage, wastewater, and other toxic substances (Misra, 2010; Rani et al., 2013). Of the total water of the Ganges, 16% is contributed by the Yamuna River. As a result of this, the pollution levels of the Ganges are further amplified. Previous studies on the Ganges are restricted to its water quality, increased water pollution due to human intervention, discharge of domestic and sewage waste, rapid industrialization, the influx of tourists, and religious and cultural practices on the banks of the river (Bhardwaj et al., 2010; Farooquee et al., 2008; Srivastava et al., 1996).

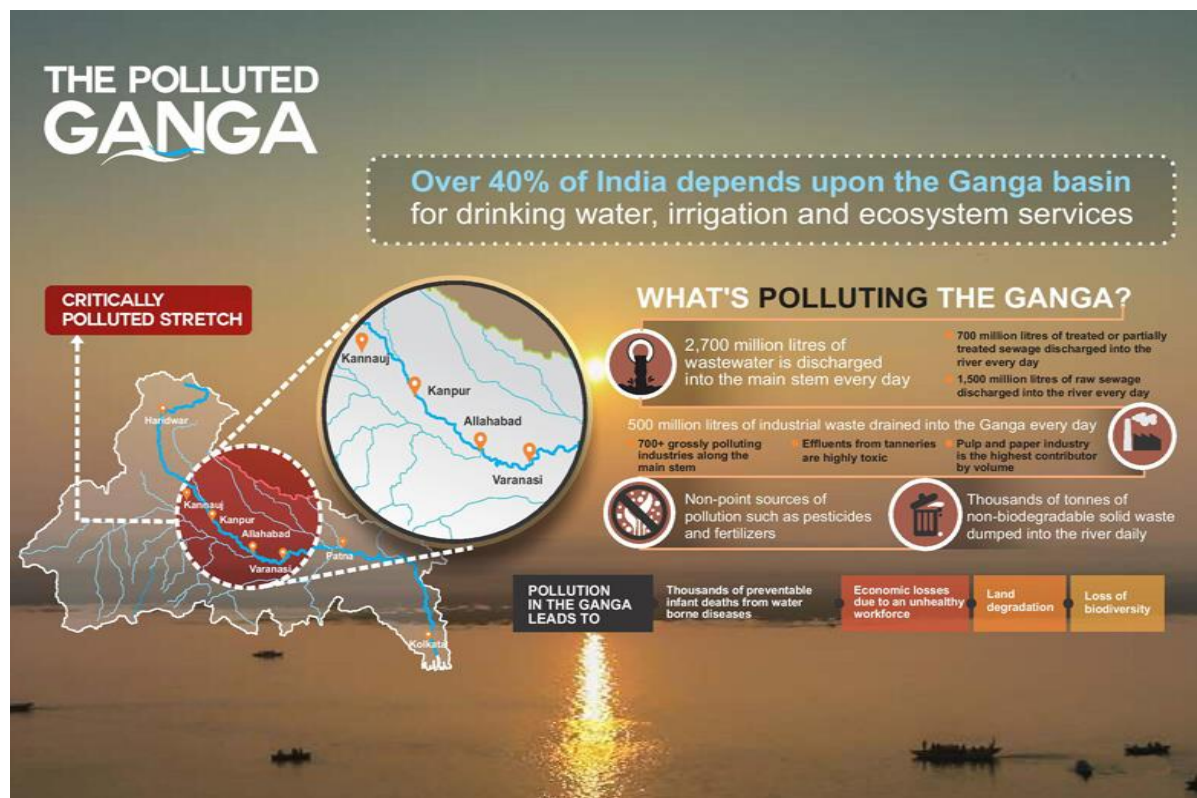


Fig. 2: Illustrative Infographics of the critically polluted stretch of the Ganges River. (Image source: <https://www.worldbank.org/en/news/feature/2015/03/23/india-the-national-ganga-river-basin-project>).

Another critical contaminant of river health is antimicrobial resistance genes (ARGs). The World Health Organization (WHO) has stated that ARGs are a new pollutant. Excessive metals and antibiotics in any environment impose selection pressure on the microbial communities and eventually alter the native diversity (J. Chen et al., 2019). Human activities

primarily influence freshwater ecosystems such as rivers and lakes and, therefore, serve as hotspots for the proliferation and evolution of ARGs, MRGs, and the bacteria trafficking them. These include several significant reservoirs that drain into the rivers, such as discharges from hospitals, sewage treatment plants, industries, and agricultural/ domestic/ urban runoff (Li et al., 2016). The key bacterial contributors to environmental antimicrobial resistance (AMR) are the pathogens belonging to the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (which may include *Escherichia coli*) group. The environmental dimensions of AMR have been illustrated (Fig. 3), while the prevalence of members from the ESKAPE group in different water sources has been illustrated in (Fig. 4).



Fig. 3: Potential sources and sinks for antimicrobial resistance in the environment.

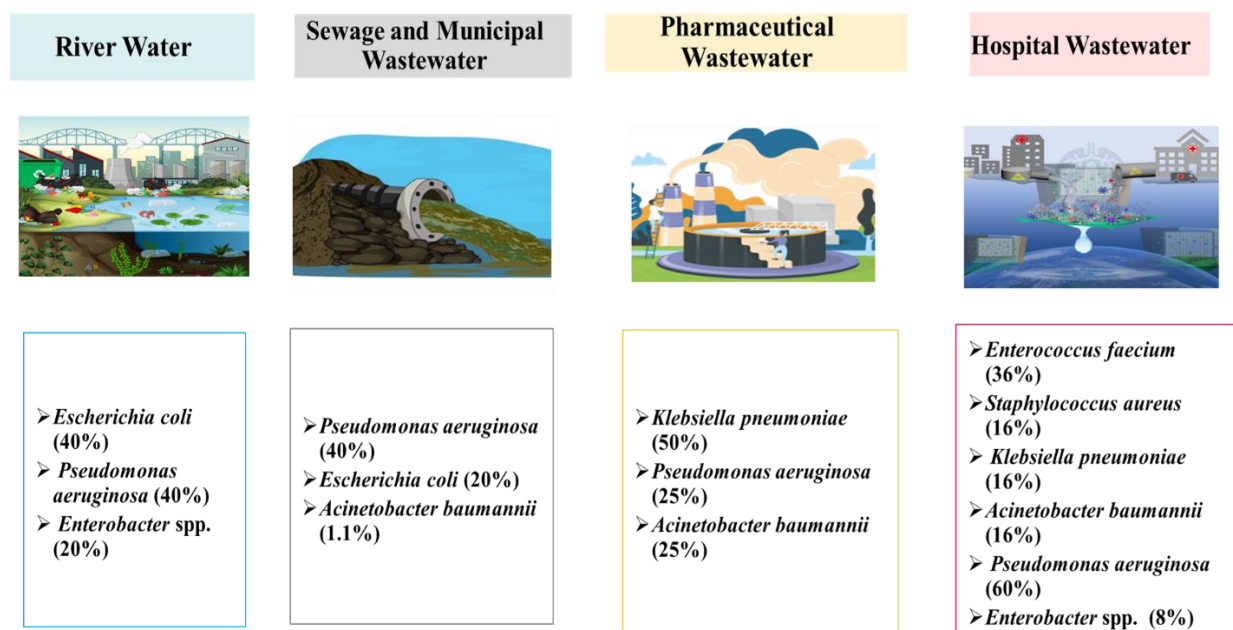


Fig. 4: An illustration summarizing the key bacterial contributors to antimicrobial resistance in aquatic environments based on various literature sources (De Oliveira et al., 2020; Denissen et al., 2022; Ferdinand et al., 2023; Graham et al., 2019).

In recent studies, the water quality deterioration of urban rivers has become an important issue, as they are pivotal in the sustainable development of cities. However, significant changes in the water quality across urban settlements have been attributed to the copious release of anthropogenic pollutants and fecal contamination, which results in the emergence of ARGs, MRGs, and the associated resistant bacteria (H. Chen et al., 2019; Wang et al., 2019). A few metagenomics-based studies have emphasized the presence of ARGs and MRGs in this riverine system (Behera et al., 2020b; Kumar et al., 2021; Reddy and Dubey, 2019; Zhang et al., 2018). Additionally, several studies on the Ganges River have documented water pollution due to excessive amounts of heavy metals such as Mn, Zn, Cr, Cu, Ni, Cd, Pb, and Hg (Boral et al., 2020; Pal et al., 2018). Others have reported selective isolation of fecal coliforms and antibiotic-resistant bacteria (Ali et al., 2021; M Vortmann, 2015). These studies indicate that the overall health of the Ganges River is declining and highlight the need for continuous monitoring and targeted interventions to improve management practices. However, to date, there is a lack of holistic understanding of the microbiome and associated resistome

along the anthropogenically dynamic segment of the Ganges River. This information will be crucial and would serve as a baseline data for risk assessment and monitoring studies.

1.3 The Concept of One Health and Water Security for The Ganges- A Perspective

The Ganges is not just a river; it embodies a complex web of interdependencies where the concepts of water security and river health converge to shape the destiny of millions (Trivedi, 2010). Water security is a key component of environmental health and entails ensuring reliable access to safe and clean water for human consumption, sanitation, and ecosystem functioning. The Ganges River plays a vital role in providing water security for millions of people living along its banks. Still, growing water demand, coupled with pollution and climate variability, challenges its sustainability (Trivedi, 2010). Recent reports show that the health of the Ganges is threatened by various environmental stressors, including pollution, habitat degradation, and climate change, which directly impact water security and microbial diversity (Dwivedi et al., 2020; Kattel, 2022; Poschlod et al., 2020; Trivedi, 2010).

One Health, as an integrated approach, recognizes the interconnectedness of human health, animal health, and environmental health, highlighting the need for collaborative efforts to address complex challenges (Mackenzie and Jeggo, 2019). Environmental health considerations within the One Health framework emphasize the importance of preserving the ecological integrity of the Ganges River basin to safeguard human and animal health. Pollution from industrial waste, agricultural runoff, and untreated sewage poses significant risks to water quality, leading to the proliferation of waterborne pathogens and harmful algal blooms. Mitigating these threats requires holistic strategies that promote sustainable land use, water management, and pollution control measures. Water security, defined by the United Nations as "the capacity of a population to safeguard sustainable access to adequate quantities of acceptable quality water for sustaining livelihoods, human well-being, and socio-economic development" (UNESCO and IWSSM, 2019), forms the cornerstone of sustainable development in the Ganges basin. The importance of environmental health in one health has been illustrated in **(Fig. 5)**. Achieving water security entails more than just ensuring the availability of water; it necessitates a holistic approach that integrates ecological, social, and economic considerations within the broader framework of river health (Opperman et al., 2020).

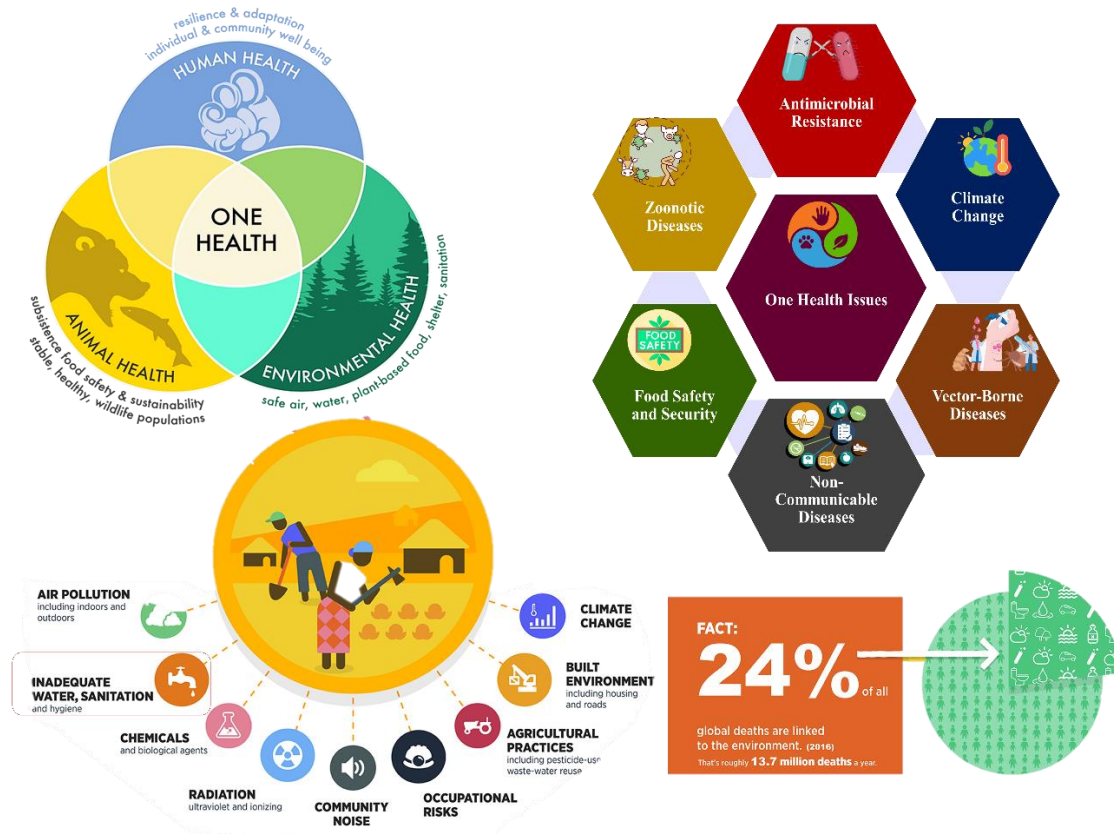


Fig. 5: The concept of one health and the importance of environmental health. (Information Source for this image: <https://www.cdc.gov/onehealth/resource-library/one-health-graphics.html>, <https://www.who.int/activities/environmental-health-impacts>)

River health serves as a metaphor akin to the well-being of humans or animals, intricately tied to human satisfaction and the river's resilience. Monitoring and understanding the degradation of rivers necessitates an examination of their physical, chemical, and biological attributes. However, human interventions have emerged as a central concern, contributing to the continuous decline of river ecology and environmental integrity (Kuehne et al., 2023). Safe and high-quality water can harbor a remarkable diversity of microorganisms (Mishra et al., 2021). Microbial communities play a pivotal role in aquatic ecosystems, regulating nutrient cycles, enhancing productivity, providing biotechnological resources, and responding to environmental changes. Their metabolic activity profoundly influences water chemistry, highlighting the importance of understanding their role in these environments (Gupta et al.,

2017; Ibarbalz et al., 2016). Due to their rapid growth and metabolism, microbes are sensitive to changes in environmental conditions, including fluctuations in organic and inorganic substances and pollutants (Behera et al., 2020b).

The quality of water in the Ganges River is a matter of grave concern, with pollution from industrial effluents, agricultural runoff, urban sewage, and solid waste posing significant risks to human health and ecosystem integrity. The presence of various heavy metals, pathogens, pesticides, and other contaminants in the river disrupts the balance of human-animal-environmental health (Agarwal, 2015b; Behera et al., 2020a; Biswas et al., 2015; Paul, 2017a). Therefore, addressing water pollution requires robust regulatory frameworks, investment in wastewater treatment, and community-based initiatives aimed at pollution prevention and control (Bashir et al., 2020; Kuehne et al., 2023; Mitra et al., 2022). Integrating traditional knowledge systems with scientific expertise can enhance our understanding of the complex dynamics of river systems and inform context-specific interventions (Yanou et al., 2023).

1.4 Special Properties of the Ganges River and Details of the Bacteriophages

The Ganges River has been revered for its non-putrefying (self-cleansing) and special healing properties for centuries (Khairnar, 2016). The River Ganges has been linked to the discovery of bacteriophages since the ancient past. The experimental foundation of the antibacterial property of the water from the Ganges River against *V. cholerae* was demonstrated by Ernst Hankin in 1896. In 1918, Félix d'Hérelle termed the factor conferring this exceptional property to the Ganges as 'bacteriophage' (Abedon et al., 2011). It is well-known that despite tremendous anthropogenic activities and their related pollution load, the Ganges can rejuvenate itself rapidly (Paul, 2017b; Reddy and Dubey, 2019; R. Samson et al., 2019; Zhang et al., 2018). This fact has also been witnessed amidst the COVID-19 lockdown, which provided a window period for the restoration of the Ganges River in the absence of any human intervention. The result, with a span of 2-3 months, was evident in the remarkable clean water, even at the most polluted sites (Dutta et al., 2020). The reason behind this is still unknown; however, it is attributed mainly to its bacteriophages.

Bacteriophages (phages) are viruses that infect bacteria. Their broad genetic plasticity, ubiquitous presence, genomic versatility, self-replicating ability, and diverse environmental

and evolutionary adaptability make them the most abundant entity in the biosphere (Shen and Loessner, 2021). Phages direct the microbial community ecology, diversity, and virulence, as their predation significantly affects the bacterial population, associated functions, and community composition. Phages can infect their cognate bacterial host in a diverse range of environments, such as water, sediments, soils, sludge, and mucosal surfaces of the human body (Batinovic et al., 2019). Bacteriophages have a distinct morphology composed of an icosahedral protein capsid enclosing the core genetic material (DNA/RNA). This nucleocapsid is attached to a contractile sheath (tail) encompassing the baseplate and/or tail fibers. These tail fibers assist in the attachment of bacteriophages to specific receptors on the bacterial cell surface (Sanz-Gaitero et al., 2021). A significant variation is seen between the phages in infecting their bacterial hosts. Specific unique recognition receptors (lipopolysaccharide (LPS), teichoic acids, and porins) on bacterial cell surfaces determine the host range of phages. An interplay between the host receptors and receptor-binding proteins (RBP) of phages is crucial in shaping the microbial community structure in different environments (García et al., 2019; Israel, 1978).

Phages have two life cycles known as lytic and temperate (lysogenic). The lytic cycle of phage replication is marked by a hijack of host replicative machinery to produce and release progeny of virions, which further re-initiate the lytic cycle in the new host (Weinbauer, 2004). Alternatively, the lysogenic cycle is characterized by dormancy of the phage upon genome incorporation, followed by simultaneous replication of phage due to the integration of the phage genome into the host (prophage) or as an extrachromosomal genetic material (circular or linear plasmid). The lysogeny is switched to the lytic cycle under stress response signals, reactive oxygen species (ROS), DNA damage, and altered metabolism from the bacterial host (Howard-Varona et al., 2017) (**Fig. 6**). A recent study has shown communication between temperate phages for release of 'arbitrium' (pro-lysogenic hexapeptide) by the predecessor phage, which enables an offspring phage to opt for either the lytic or lysogenic cycle (Erez et al., 2017). Additionally, prophage integration confers several survival benefits to the lysogen, known as prophage-mediated superinfection exclusion. This collectively offers the lysogen homotypic defense (against closely related phages) and heterotypic defense (against distant relatives of phages) (Samson et al., 2013).

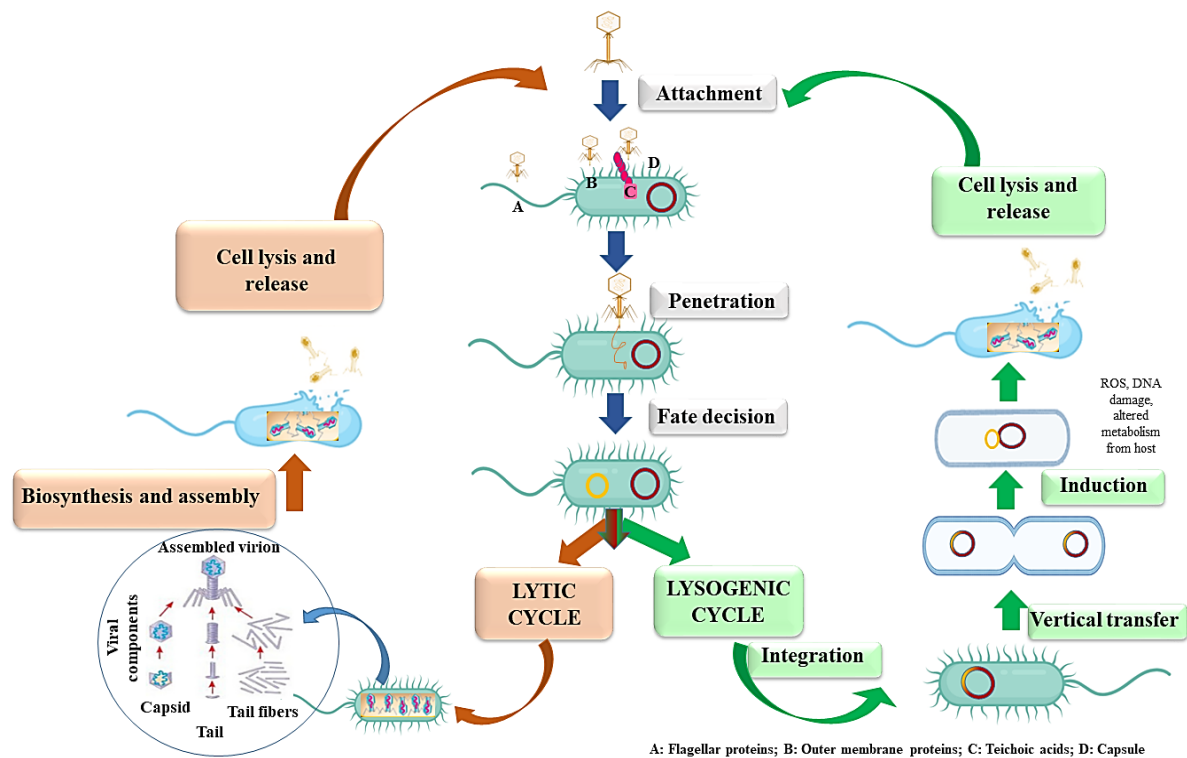


Fig. 6: Illustration of the two stages of the life cycle of a bacteriophage.

Frederick Twort and Felix d'Herelle independently discovered bacteriophages in 1915 and 1917, respectively (D'Herelle, 1917; Twort, 1915). Since then, the era of phage research has been instrumental in advancing genetics and molecular biology. The study of phages has provided insights into the evolution of genomes, bacterial co-evolution, gene expression, mechanism of DNA transfer, etc. (Harada et al., 2018). However, the molecular interaction of phages with bacteria (host) in the natural environment and human body remained elusive for several years, limiting the perception of their applications (Chibani-Chennoufi et al., 2004). Next-generation sequencing technologies have broadened our understanding of molecular mechanisms of host-phage interaction and a repertoire of bacteriophage applications (Batinovic et al., 2019; O'Sullivan et al., 2016).

The current scenario for tackling the existing AMR crisis involves a global drive to identify, develop, advance, and expand novel antimicrobial agents and other therapeutic alternatives (Principi et al., 2019). Bacteriocins, antimicrobial peptides, and bacteriophages (phages) are emerging as feasible alternatives to antibiotics in combating multidrug resistance among bacterial pathogens (Garvey, 2020). Phages are bacterial viruses that, in their natural

state or upon engineering, offer many applications that bridge several interdisciplinary fields. Certain important traits of phages, such as their ease of production, eco-friendly nature, cost economy, genome plasticity, and lack of polydispersity, make them dynamic building blocks for various applications (Islam et al., 2021). These applications include the use of phages in medicine (phage therapy, phage-based vaccination, surface disinfection, biofilm control); biotechnology (gene transfer methods for plants and animals, gene delivery phage display-based selection for biological affinity molecules, biosensing devices, biocontrol of plant pathogens); nanotechnology; food bio-preservation and safety; ecology; microbiome studies, etc. Additionally, the biological potentials of lytic bacteriophage in phage-derived products have been used in phage therapy, food processing, and production industries (Moye et al., 2018; Sharma et al., 2018). Therefore, phages in whole or phage-derived products have numerous functions that can further be tailored for desired applications.

Considering the current trend of research with bacteriophages, the most explored area is the therapeutic use of bacteriophage, followed by food and agriculture. There are very few reports wherein phages have been used for environmental applications, which has been summarized in **Table 1**.

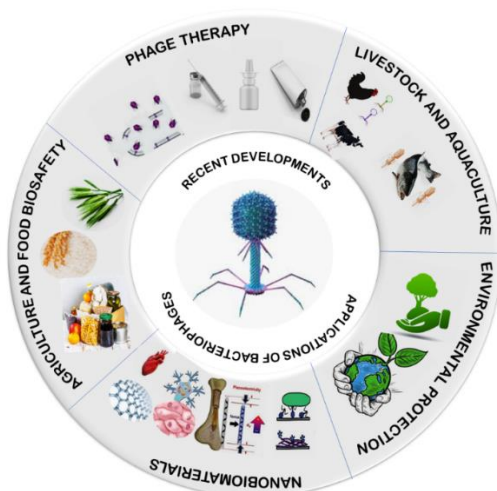


Fig. 7: Graphical representation of various applications of bacteriophages.

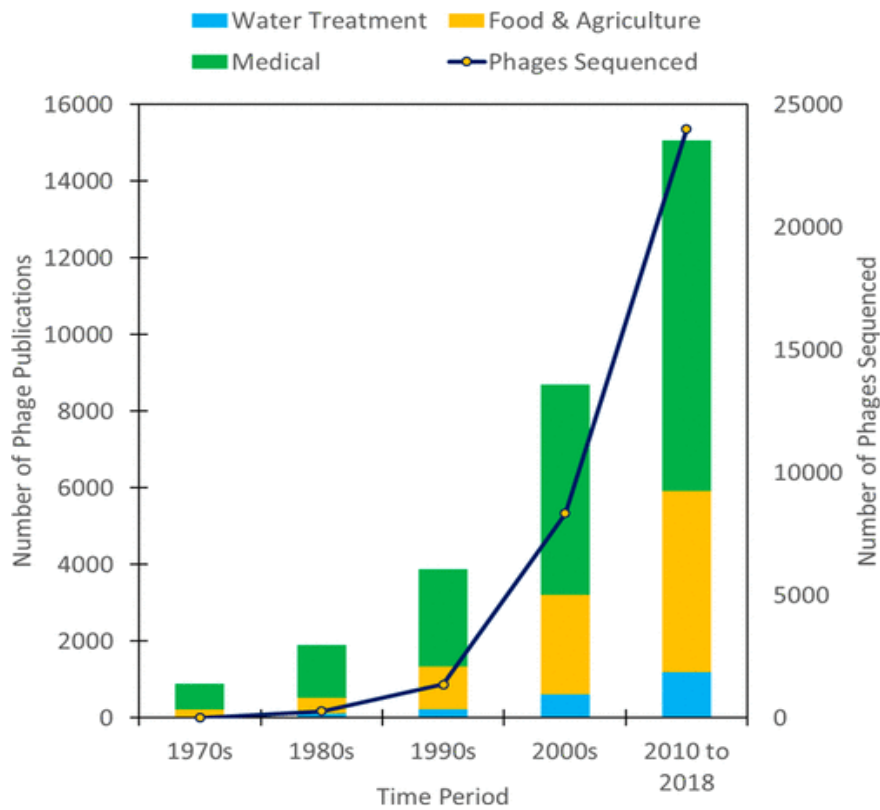


Fig. 8: The trend of bacteriophage research (Adapted from Mathieu et al., 2019) (Mathieu et al., 2019).

Table 1: The environmental use of bacteriophages

Sr. no.	The aspect of environmental use	Bacteriophage used	Bacteria targeted
1	Reduction of Pathogenic load	Coliphages against <i>E. coli</i> RRL-3704	<i>Salmonella</i> sp., <i>Shigella</i> sp., <i>Vibrio</i> sp. and <i>Pseudomonas</i> sp.
2	Biomass Bulking in Activated Sludge Process	Lytic Phage against <i>Sphaerotilus natans</i>	<i>Sphaerotilus natans</i>
3	Reduction of Pathogenic load	Cocktail of 3 phages (sww65, sww275, and sww297) against <i>Salmonella</i>	<i>Salmonella</i> sp.

4	Reduction of Pathogenic load	Phage against <i>E. coli</i>	<i>E. coli</i>
5	Removal of bacterial biofilms from surfaces	Phage against <i>Pseudomonas aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
6	Removal of bacteria from filtration systems	Phage against <i>P. aeruginosa</i>	<i>P. aeruginosa</i>
7	Reduction of Pathogenic load	A mixture of two lytic coliphages against <i>Klebsiella pneumoniae</i> and <i>Klebsiella oxytoca</i>	<i>K. pneumoniae</i> , <i>K. oxytoca</i> , and other coliforms
8	Membrane Biofouling	Lytic phage against <i>Delftia tsuruhatensis</i>	<i>Delftia tsuruhatensis</i>
9	Reduction of Pathogenic load	Lytic coliphages against <i>E. coli</i> PTCC1399 and <i>E. coli</i> SBSWF27	<i>E. coli</i> PTCC1399, <i>E. coli</i> SBSWF27, and other coliforms
10	Controlling Foaming in Wastewater Treatment	Phages (NOC1, NOC2 and NOC3) against Nocardioforms	Nocardioforms
11	Reduction of Pathogenic load	Phages against <i>E. coli</i> , <i>P. aeruginosa</i> and <i>S. Typhi</i>	<i>E. coli</i> , <i>P. aeruginosa</i> and <i>S. Typhi</i>
12	Reduction of Pathogenic load	Phage (PA25) against <i>P. aeruginosa</i>	<i>P. aeruginosa</i>
13	Reduction of Pathogenic load	Phage against <i>Salmonella Typhi</i>	<i>Salmonella Typhi</i>

Amidst mounting challenges like shifting lifestyles, widespread water pollution, and deteriorating water conditions, the paramount significance of ensuring clean and safe water cannot be emphasized enough. Rapid industrialization, urban expansion, and intensified agriculture have led to a surge in toxic wastewater, posing serious threats to both human health

and the environment (Marutescu et al., 2023). The coliform group of bacteria, along with fecal streptococci, are more frequently utilized as indicators of fecal contamination in waters (Kromberg, 2018; US EPA, n.d.). Certain studies have proposed that *Salmonella* might be a better indicator of water quality than coliforms (McEgan et al., 2013; Moriñigo et al., 1990; Redouane et al., 2023). Additionally, some researchers suggest that *P. aeruginosa* should be considered as an index of fecal pollution, as it has been found in both drinking water and inadequately treated water supplies, even when fecal coliforms are absent (Highsmith and Abshire, 1975; Januário et al., 2020; Marutescu et al., 2023). Ganges River has been known for its non-putrefying and self-cleaning properties. However, the use of its bacteriophages for environmental applications has not been studied. There are only limited studies that have focused on the special properties of non-putrefying (self-cleansing) and rejuvenation (Dutta et al., 2020; Dwivedi et al., 2020; Nautiyal, 2009; Rachel Samson et al., 2019) of the Ganges River. Based on the available literature, we have attempted to summarize the bacteriophage diversity reported from the Ganges River through a classical (culture-based) approach (**Table 2**) and a metagenome-based approach (**Table 3**).

Table 2: A tabular comprehension of bacteriophage diversity identified from the Ganges River through a culture-based approach.

Phages against Pathogen	Location	Strength of study	Limitations	Reference
<i>Escherichia coli</i>	Gomukh	The first culture-based study showing the presence of bacteriophages from Gomukh	Lack of detailed characterization of phages, No <i>in-vitro</i> toxicity data	(Khairnar, 2016)
ESKAPE group and <i>S. Typhi</i>	Prayagraj, Mirzapur,	The first study to demonstrate the lytic activity of	Study restricted only to plaque assays, no	(Mishra and Nath, 2020)

	and Varanasi	phages against the ESKAPE group of pathogens	morphological and genomic characterization of phages	
<i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. aureus</i> , <i>K. pneumoniae</i> , <i>Proteus</i> sp., <i>Citrobacter freundii</i> , <i>Morganella morgani</i> , and <i>A. baumannii</i>	Varanasi	A first prospective study using customized bacteriophages in the treatment of chronic non-healing wounds with a success rate of 84% for non-diabetic patients and 74% for diabetic patients	Cocktail of phages from Ganges River together with ponds, sewer of municipal corporation, No detailed characterization of monophages for cocktail compatibility	(Patel et al., 2021)
MDR <i>K. pneumoniae</i>	Haridwar and Rishikesh	Phage titer 10^{10} PFU/ml, Combinatorial approach with streptomycin resulted in 6 log reduction of <i>K. pneumoniae</i> , no <i>in-vivo</i> toxicity in zebrafish model	No toxicity data with mammalian cells, Lack of detailed characterization of phage with respect to genome to ascertain therapeutic potentials	(Sundaramoorthy et al., 2021)

MDR <i>P. aeruginosa</i>	Ganga ghats from Varanasi	A novel phage from the <i>Autographiviridae</i> family, active on MDR <i>P. aeruginosa</i> with a truly lytic genome	Only <i>in-vitro</i> study, lack of cytotoxicity, endotoxin, and <i>in-vivo</i> data	(Rathor et al., 2022)
<i>K. pneumoniae</i>	Sundarlal hospital sewer (BHU), Durgakund pond, Varanasi	An <i>in-vivo</i> study demonstrating the success of phage therapy in eradicating MDR <i>K. pneumoniae</i> septicemia in a mouse model	Lack of detailed characterization of phages in terms of genomic aspects, No comparative account of phages with antibiotics	(Bashir et al., 2022)
<i>A. baumannii</i>	Kanpur	A proof of concept study that made use of bacteriophages as a disinfectant to lyse-specific MDR <i>A. baumannii</i> on surfaces	Lack of detailed characterization of phages in terms of genomic aspects	(Rathor et al., 2023)

Table 2: A tabular comprehension of bacteriophage diversity reported from the Ganges River through a metagenome-based approach.

Location	Sequencing technology	Strength of study	Limitations	Reference
Bijnor, Narora, Kannauj, Kanpur, Allahabad, Allahabad Sangam, Mirzapur and Varanasi	Illumina NextSeq and MinION Mk1b (Oxford Nanopore Technologies, UK)	The first metagenomic report portraying the phage profile from the sediment of the Ganges River	Only one sample combining sediments from all locations, Inconclusive for phage diversity for each location	(Kumar et al., 2021)
Kanpur, Farakka	Illumina Truseq	The first metagenomic report that documents bacteriophage and pathogenic bacteria from both sediment and water	Limited to only the Kanpur location, lack of culture-based proof of phages against pathogenic bacteria	(Behera et al., 2023)
Haridwar, Bijnor, Kanpur, Allahabad (<i>Prayagraj</i>), Varanasi, Patna, Bhagalpur	MinION Mk1b (Oxford Nanopore Technologies, UK)	First metagenomic report mapping bacteriophage diversity and functions together with	Lack of culture-based proof of phages against pathogenic bacteria	(Samson et al., 2023)

the resistome of
the microbiome
in the sediments
(for 2 years)
along a 1500 km
stretch

Based on the literature, specific research gaps were identified, leading to the formulation of key questions that this study aims to address. **Figure 9** illustrates the study's purpose by defining the current research gaps and outlining how they can be bridged.

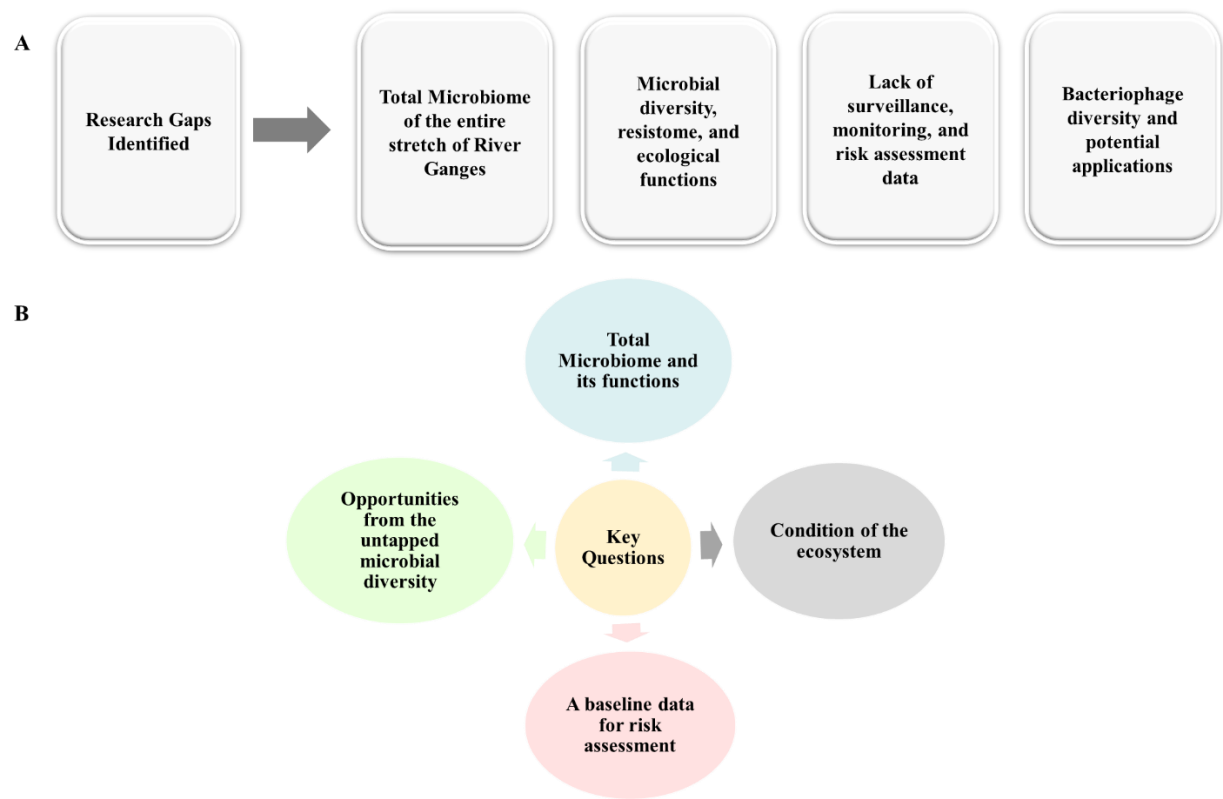


Fig. 9: Research gaps and key questions to bridge these gaps. (A) A pictorial representation highlighting the primary research gaps identified from the literature. **(B)** The key questions formulated to address these gaps show the pathway from identifying limitations in the existing literature to proposing targeted research strategies. This framework outlines

the study's objectives in bridging these gaps, ultimately contributing to a better understanding and management of antimicrobial resistance.

1.5 The Rationale of the Study

- Achieving a holistic understanding of the total microbial diversity and functions from the pristine and anthropogenically dynamic stretches to overcome the knowledge gap.
- Need for surveillance, monitoring, and risk assessment data defining the resistome and trophic status of the river over a specified period.
- To use metagenomic data as a roadmap to isolate phages with promising applications for One Health initiatives.

1.6 Objectives and Division of Chapters

- **Metagenomic investigation of microbiome and resistome across the anthropogenically impacted stretch of River Ganges (Chapter 2)**
- **Assessment of the microbiome and the resistome across the pristine stretch using a shotgun approach (Chapter 3)**
- **Isolation, characterization, and evaluation of the bacteriophages for potential applications in one health (Chapter 4)**

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Chapter 2

*Metagenomic analyses of the microbiome,
resistome, and antimicrobial potentials
along the anthropogenically impacted
stretch of the Ganges River*

2.1 Confluence Microbiome Along the Lower Stretch

Abstract

Background

River confluences are ecological hotspots for microbial Diversity and functions. The Ganges is India's national river and is amongst the most significant fluvial systems with religious and economic importance. The Yamuna River is the longest tributary of the Ganges by length and accounts for 16% of its water. It meets the Ganges at *Prayagraj*, India, to form a unique confluence known as *Triveni Sangam*.

Rationale

The influence of the Yamuna River on taxonomic and functional aspects of the microbiome in the Ganges along its confluence remains unexplored. Therefore, in the present study, we have used a MinION-based shotgun metagenomic sequencing to comprehend the microbial Diversity along the confluence zone.

Results

Grouped by the confluence, the Yamuna River (G15 Y) and immediate downstream of confluence (G15DS) showed a higher abundance of *Proteobacteria* and a lower abundance of *Bacteroidetes* and *Firmicutes*, as compared to the upstream (G14 DS, 15US) and farther downstream of the confluence (G16 US, G16 DS). Similarly, a higher abundance of *Euryarchaeota* was observed only in G15Y and G15DS, indicating the influence of the Yamuna River. Functional analysis revealed the influence of Yamuna River on xenobiotic degradation, resistance to toxic compounds, and antibiotic resistance interceded by the autochthonous microbes at the confluence and succeeding downstream locations.

Significance

Overall, our metagenomic investigation presented first insights into the microbiome of the Ganges River along its religiously significant confluence zone with the Yamuna River. The occurrence of similar taxonomic and functional profiles of microbial communities before the confluence and farther downstream of the confluence suggested a transient influence of the Yamuna River, followed by a possible restoration or rejuvenation, thereby revealing yet other special property of the Ganges River.

Outcome

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1. Introduction

In a fluvial system, confluences (*Sangam* in the Sanskrit language) stand as the nodal points of connectivity between the tributaries and their trunk stream. The juxtaposition of the tributary with the main stem creates a unique pattern of habitat heterogeneity at the confluence, rendering them as the hotspots of biological Diversity (Benda et al., 2004). Tributary confluences augment biodiversity due to the gathering of species from multiple catchments, thereby positively influencing the size of the meta-community from which local communities assemble (Besemer et al., 2013). The network dynamics hypothesis speculates habitat discontinuity, geomorphology alterations, and a shift in the aquatic ecology at the tributary confluence (Rice et al., 2008). However, there are limited studies on the changes in the microbial community pattern that happen at the confluence points, including the upstream and downstream locations (Payne et al., 2017; Scherwass et al., 2010; Widder et al., 2014).

The Ganges is the largest and most religiously significant riverine system in India, originating as Bhagirathi from Gomukh, the snout of the Gangotri glacier (Khairnar, 2016). However, the Ganges proper emerges at Devprayag in Uttarakhand, India, from the confluence of two of its source streams, namely the Bhagirathi River and the sediment-laden-Alaknanda River (Penn, 2013). Spanning 2,525 km, the Ganges drains one-fourth of Indian subcontinents and also functions as a lifeline for millions of people (Bhutiani et al., 2016). The Ganges basin is one of the most fertile and densely populated regions in the world. During its course of run from Gomukh to the Bay of Bengal, the Ganges encounters around fifteen tributaries, which account for 60% of its total water, making it the third largest river in the world in terms of the amount of water discharged (Zhang et al., 2018). The water of the Ganges has been known to possess unusual healing and non-putrefying properties since ancient times, yet there are limited experimental proofs to support it.

Among all the tributaries of the Ganges, Yamuna is the longest tributary by length. Originating from the Yamunotri glaciers in the lower Himalayas, the Yamuna River joins the Ganges at the "*Triveni Sangam*" in Prayagraj (Paul, 2017). Yamuna River is one of the most polluted rivers in India due to the domestic and industrial effluents, untreated sewage, wastewater, and other toxic substances (Misra, 2010; Rani et al., 2013). Of the total water of the Ganges, 16% is contributed by the Yamuna River. As a result of this, the pollution levels of the Ganges are further amplified. Previous studies on the Ganges are restricted to its water quality, increased water pollution due to human intervention, discharge of domestic and sewage waste, rapid industrialization, the influx of tourists, and religious and cultural practices on the banks of the river (Bhardwaj et al., 2010; Farooquee et al., 2008; Srivastava et al., 1996).

Recent advancements in sequencing technology have paved the way for a better understanding of the total microbial Diversity. The whole genome sequencing approach is utilized to study the taxonomic and functional spectrum of microbial communities. Lately, the microbial community of the Ganges with respect to human gut microbes and antibiotic resistance genes has been profiled (Zhang et al., 2018). It is a well-established fact that there is an interrelationship of microbes and their distinctive functions with the ecosystem (Barberán et al., 2016). Our focus is on unveiling another distinct property of the River Ganges with experimental evidence of a probable restoration of the microbial and functional Diversity per se after its confluence with the Yamuna River. Therefore, it becomes imperative to delve ahead of taxonomic Diversity and understand the functional potentials of the microbes inhabiting therein. To provide insights into the autochthonous and allochthonous microbial community structure and their functions, we investigated functional aspects of the microbial community with respect to xenobiotic degradation resistance to toxic compounds along with the antimicrobial resistome profile.

In this study, for the first time, we have comprehended the total Diversity of the Ganges River across its confluence with the Yamuna and the subsequent downstream locations using the MinION sequencing platform. Furthermore, on the basis of the convergence of Ganges and Yamuna, we hypothesized that the pattern of microbial Diversity observed upstream of confluences would be similar to that of the farther downstream of the confluence with certain significant changes at the confluence and the immediate downstream.

2. Methodology

2.1 Sampling and study site details

During December 2017, sediment samples were collected from the left bank, center, and right bank of the river from seven locations comprised of upstream of the confluence of Ganges (G14DS and G15US), Yamuna River (G15Y), the confluence of Yamuna and Ganges (G15S), immediate downstream of confluence (G15DS), and farther downstream of confluence (G16US, and G16DS) (**Fig. 1**). Details of the sampling locations and their grouping, is provided in the supporting information (**Table 1**). A total of 21 sediment samples were collected in sterile polypropylene containers, retained on ice, transported to the laboratory, and stored at 4°C for further processing.



Fig. 1. Map illustrating the sampling locations and their grouping.

Table 1: Details of the sampling locations and the samples investigated in this study.

Groupings	Sample code	Location	Latitude	Longitude
Upstream of confluence	G14DS	Kanpur Downstream	26°37.9005' N	80°49.1344' E
	G15US	Allahabad upstream	25°51.6161' N	81°68.5440' E
Sangam (Confluence)	G15(S)	Allahabad Sangam	25°42.3628' N	81°88.8116' E
Yamuna	G15(Y)	Allahabad Yamuna	25°32.0510' N	81°78.9160' E
Immediate Downstream	G15DS	Allahabad Downstream	25°39.3014' N	81°91.2330' E
Farther downstream	G16US	Varanasi upstream	25°13.778'N	83°01.513'E
	G16DS	Varanasi downstream	25°18.404'N	83°0.856'E

2.2 Whole metagenomic DNA extraction, sequencing, and analysis

For each location, the sediment samples collected in triplicates were pooled together, and the total DNA was extracted using RNeasy PowerSoil Total RNA Isolation Kit (Qiagen) and RNeasy PowerSoil DNA Elution Kit (Qiagen) with certain modifications (detailed protocol is in the supporting information). The DNA quality and concentrations were assessed by Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific) and Qubit fluorometer (Thermo Fisher Scientific) using the Qubit BR assay kit (Thermo Fisher Scientific, Q32853). The integrity of the DNA samples was checked on 1% agarose gel using a gel documentation system (Protein Simple). The extracted DNA samples were subjected to whole metagenome sequencing using a long read and MinION platform (Oxford Nanopore Technologies, Oxford, UK). Prior to this, the library preparation was carried out using 1D Native barcoding genomic DNA (EXP-NBD103) and Ligation Sequencing Kit 1D (SQK-LSK108) protocol as per the manufacturer's instructions.

2.3 Data processing and analysis

The reads were base-called using Albacore (v2.2.7) (Sanderson et al., 2018). Poretools (a toolkit for analyzing nanopore sequencing datasets) was used for further Quality Check (QC) (Loman and Quinlan, 2014). Error correction was performed using Canu, which has an MHAP-based error correction module that generates good-quality long reads (Koren et al., 2017). The fastq files were then uploaded to the MG-RAST server for taxonomic and functional analysis. The MG-RAST uses the SEED-subsystem database together with the KEGG (Kyoto Encyclopedia of Genes and Genomes) database to assign taxonomy and functional attributes to the microbial communities. The taxonomic Diversity of sequenced reads was interpreted using the M5RNA (multisource non-redundant ribosomal RNA) database on the MG-RAST server with an E-value cut-off = $1 \times e^{-5}$ and sequence identity of 60%. The Operational Taxonomic Units (OTU) table downloaded from the MG-RAST server was used for the taxonomic analysis.

2.4 Statistical analysis

Alpha diversity for bacteria and archaea was computed using Chao1 and Shannon indices in QIIME 1 (Quantitative Insights Into Microbial Ecology) (Caporaso et al., 2010). Singletons were removed, and the OTU table was rarefied to equal sampling depth based on the sample having the lowest sequencing reads. Principal Component Analysis (PCA) plot for showing beta diversity was constructed using Bray-Curtis matrices in MicrobiomeAnalyst (Dhariwal et

al., 2017). Cytoscape v3.2.7 (Vey and Moreno-Hagelsieb, 2012) was used to generate networking plots to understand the shared and unique functions possessed by the microbial communities across different sampling sites.

2.5 Data Availability and accession numbers

Raw metagenome reads were submitted under MGRAST IDs mgm4804931.3 (G14DS), mgm4804930.3 (G15US), mgm4804937.3 (G15Y), mgm4804936.3 (G15S), mgm4804939.3 (G15DS), mgm4804938.3 (G16US) and mgm4804941.3 (G16DS).

3. Results and Discussion

The confluence of the River Ganges with the Yamuna creates a unique pattern with added biological values. These rivers are among the most sacred ones in India, with immense spiritual beliefs of people, mainly through mass bathing events like *Kumbh*, which is also a global attraction (Sridhar et al., 2015). However, no studies have focused on the changes in microbial dynamics as a result of their confluence. As compared to water, sediment is more reliable in terms of its stability and less variability, serving as a natural recorder for several biogeochemical functions occurring in an ecosystem (Li et al., 2016). Therefore, we have collected the sediment samples to provide insights into the autochthonous (G14DS, G15US, G15S, G15DS, G16US, G16DS) and the allochthonous (G15Y) microbial community composition and their diverse functions using culture-independent analysis. MinION is the latest sequencing technology generating read lengths of 10-20 kb, with low capital expenditure, and is the most portable sequencing platform. This device produces data in real-time and has a wide range of applications in genome sequence assemblies (Jain et al., 2016).

3.1 Bacterial Diversity and Community Composition

MG-RAST derived 90-97% of the reads were assigned to bacteria, and 1- 4% accounted for archaea. In the upstream of the confluence and at the confluence points (G14DS, G15US, and G15S), *Proteobacteria* (59.3%; 58.4.3% and 59.6%) was predominant, followed by *Bacteroidetes* (7.6%; 7.4% and 6.6%) and *Firmicutes* (6.7%; 7%; 6.1%). Similar observations were noted in the Yamuna River (G15Y), with an increase in the relative abundance of *Proteobacteria* (64.2%) and a decrease in *Bacteroidetes* (4.6%) and *Firmicutes* (5.4%). Further, the immediate downstream of the confluence (G15DS) observed an upsurge of *Proteobacteria* to 74.8%, while the percentage of *Bacteroidetes* and *Firmicutes* declined to 3.4% and 3.5%, respectively. However, this change in the bacterial community structure was transient, as the

farther downstream samples (G16US; G16DS) showed the relative abundances of *Proteobacteria* (60.2%; 55.6%), *Bacteroidetes* (8%; 6.1%), and *Firmicutes* (6.2%; 6.6%) comparable to the upstream of the confluence (**Fig. 2A**). At the class level, *Beta-Proteobacteria* was found to be dominant in all the samples, with a relative abundance of 20%, 20.4%, 20.8%, and 17.72% corresponding to G14DS, G15US, G15S, and G16DS. However, in G15Y, G15DS, and G16US, the relative percentages of *Beta-Proteobacteria* increased to 27%, 28.2%, and 23.1%, respectively. A sudden rise in terms of the occurrence of *Gamma-Proteobacteria* (28%) and a decline in *Delta-Proteobacteria* (6.4%) was seen in G15DS as opposed to other sampling sites (**Fig. 2B**).

The major bacterial phylum at all sites was *Proteobacteria*, with a predominance of class *Beta-* and *Alpha-Proteobacteria*, which are essential for primary production in a freshwater biome. This is comparable with the studies of other freshwater systems (Lirós et al., 2014; Tsagaraki et al., 2018). Another dominant class observed in our study was *Delta-Proteobacteria*, which encompasses members with varied anaerobic physiological Diversity and capability for reduction of iron and sulfate, fermentation, and dehalogenation (Liu et al., 2003). *Gamma-proteobacteria* are facultative or obligate anaerobes with diverse metabolic plasticity and are significant contributors to nitrogen cycling (Franco et al., 2017). The predominance of members of *Delta* and *Gamma Proteobacteria* could be correlated with the intensive anthropogenic, domestic, and industrial activities that have set a platform favorable for the anaerobes to dwell in.

At the genus level, *Geobacter* (8-14%), *Burkholderia* (7-10%), *Pseudomonas* (6-9%), *Candidatus Solibacter* (6-7%), *Anaeromyxobacter* (5-6%) were dominant in all sites. However, in G15DS, an unusual shift in the relative percentage of *Geobacter* (3%), *Burkholderia* (7.2%), *Pseudomonas* (46.8%), *Candidatus Solibacter* (3.4%), *Anaeromyxobacter* (2.6%) was seen. Although *Beta-Proteobacteria* dominated at the class level, members of class *Delta-Proteobacteria*, *Acidobacteria*, and *Gamma-Proteobacteria* were the chief contributors at the genus level, governing the functioning of this ecosystem. Compared to the other freshwater rivers such as Chao Phraya and the Tha Chin in Thailand (Thoetkiattikul et al., 2017), the Tinto River in Spain (Sánchez-Andrea et al., 2012), the Amazon River in South America (Ghai et al., 2011), and the Godavari River in India (Jani et al., 2018), the Diversity of River Ganges differed at the generic level. A predominance of *Geobacter*, *Burkholderia*, *Pseudomonas*, *Candidatus Solibacter*, and *Anaeromyxobacter* was noted for all the samples (**Fig. 2C**).

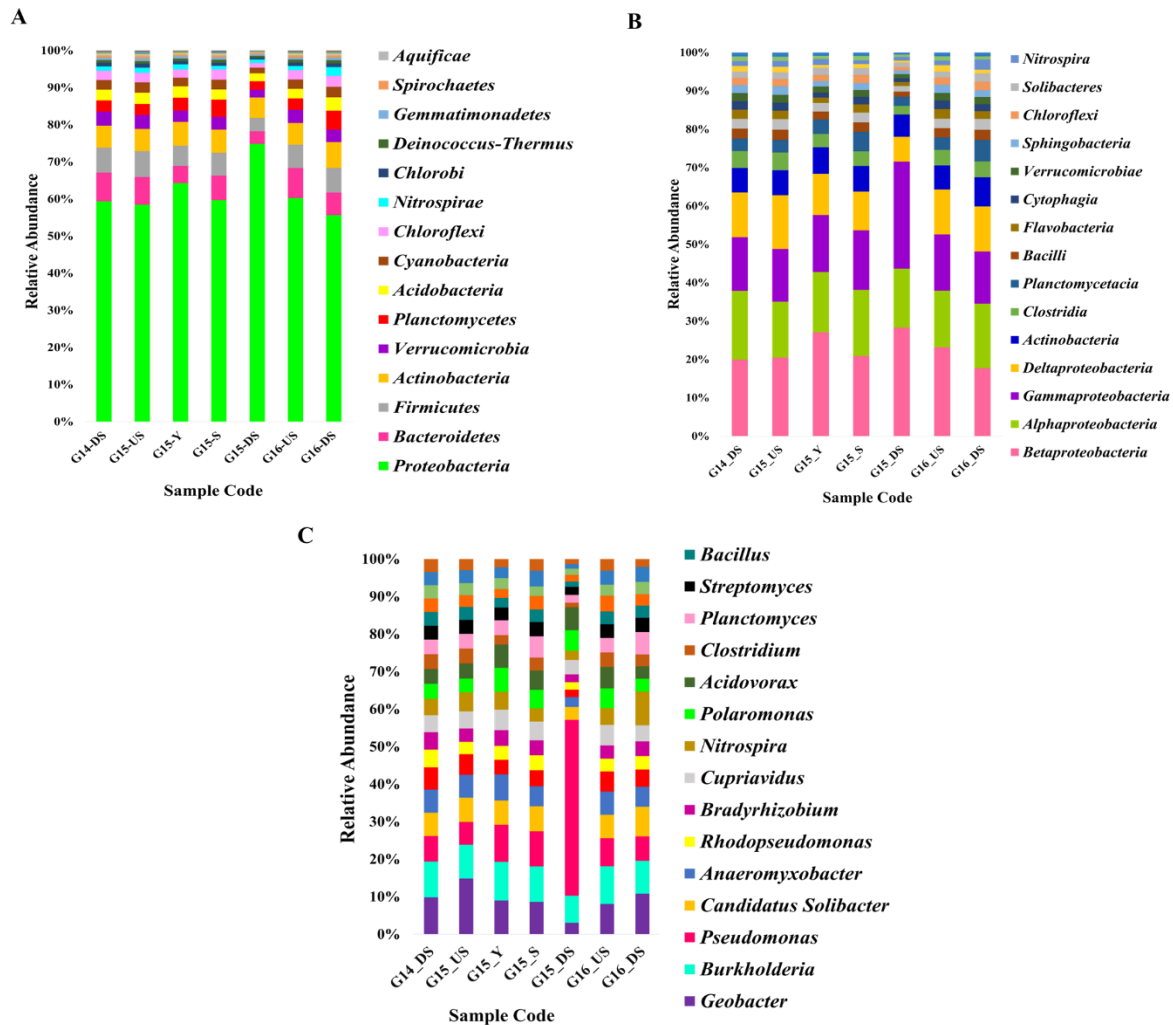


Fig. 2. Bacterial community composition (Top 15) along the confluence: A) Relative Phylum abundance, B) Relative Class abundance, C) Relative Genus abundance.

Studies have shown that species of *Geobacter* and *Anaeromyxobacter* are strictly anaerobic (Coates et al., 2001; Onley et al., 2017). They can readily be isolated from aquatic freshwater environments and are fundamentally Ferric iron Fe (III)-reducing bacteria. *Candidatus Solibacter* is an anammox-anaerobic ammonia-oxidizing bacterium (AOB) that is mainly associated with wetlands (Ma et al., 2017). The dominance of anaerobic genera in all the samples could be attributed to the increased level of organic load as a result of pollution in these rivers. Also, the dead carcasses of animals and humans, which are led into these rivers directly as a matter of religious beliefs, serve as a driving force towards the increase in the anaerobic bacteria. This can be explained in terms of the decomposition of the cadavers by putrefying bacteria, where the dominant ones are the anaerobes. A higher relative percentage of *Pseudomonas* in G15Y, G15S, and G15DS can be correlated with the allochthonous inputs of pollutants by Yamuna River along its confluence with the Ganges at *Triveni Sangam* (Singh

et al., 2015) and the anthropogenic activity like mass bathing, leading to an increase in *Pseudomonas* (Bhasin et al., 2015). Despite the variations in the bacterial communities' post-confluence, the diversity pattern of farther downstream samples was comparable to that of the upstream of the confluence.

3.2 Archaeal Community Composition

Archaea are fundamental to the functioning of various environments, yet very little is known about their ecological roles in freshwater sediments (von Schiller et al., 2017). This study is the first report that highlights the archaeal Diversity of the River Ganges and River Yamuna along their confluence. Archaeal communities showed a predominance of *Thaumarchaeota* in G14DS; G15US; G15S, G16US and G16DS with a relative abundance of 65.7%; 65.6%; 65.1%; 58.3%; and 68.3%, respectively. However, in G15Y and G15DS, the percentage of archaeal phyla varied. A higher relative abundance of phylum *Euryarchaeota* (44% and 40%) was observed in G15Y and G16US, respectively. For G15DS, a distinct archaeal diversity was observed with *Euryarchaeota* (61%), followed by *Thaumarchaeota* (33.4%) (**Fig. 3A**). *Thaumarchaeota* group is ammonia-oxidizing archaea (AOA) that possesses a chemolithoautotrophic mode of nutrition, thus contributing mainly to nitrification (conversion of ammonia to nitrite) and autotrophic carbon fixation. This can be correlated with the functioning of the nitrogen cycle occurring in the Ganges and Yamuna Rivers. The AOB and AOA convert ammonia to nitrite, and the nitrifying bacteria – *Nitrospira* (Daims and Wagner, 2018), detected in our study would oxidize nitrite to nitrate, thus aiding in nitrogen leaching.

At the genus level, *Nitrosopumilus* (61-62%), *Crenarchaeum* (6-9%), *Methanosarcina* (4-9%) reads dominated in all samples (**Fig. 3B**). A decrease in the relative abundance was observed for *Nitrosopumilus* in G15Y and G15DS (47.5 and 30.3%). Furthermore, the sample immediate to the confluence, i.e., G15DS, showed distinct differences in the distribution pattern of Archaeal genera like *Methanosarcina* (9.7%), *Thermococcus* (4.45%), *Archaeoglobus* (3.46%) and *Pyrococcus* (3.35%). *Crenarchaeota* includes archaea thriving in high-temperature environments like hot springs and hydrothermal vents, and it has been reported to be associated with nutrient- and particle-rich waters (Stoica and Herndl, 2007). The *Euryarchaeota* comprises methane-oxidizing archaea and halophiles that flourish well in highly polluted sediments (Saia et al., 2010). A similar trend was observed at the genus level, where the relative abundance of *Nitrosopumilus* (AOA) belonging to *Thaumarchaeota* was higher in all the samples except G15Y and G15DS.

As compared to the other locations, *Thermococcus*, *Pyrococcus*, *Methanosarcina* and *Methanococcoides*, *Archaeoglobus*, *Natronomonas*, *Haloarcula*, and *Haloterrigena* were more abundant in G15Y, G15S and G15DS and to some extent in G16US with respect to *Methanosarcina*. However, this difference was not significant. The pollution levels of Yamuna River seem to affect the confluence downstream (G15DS), which in turn results in a transient metabolic shift from ammonia oxidation to methane oxidation. Community transfer as a result of confluence was observed at the phylum level, and it was carried forward at the genus level. However, the persistence of a more unvarying pattern of bacterial and archaeal Diversity at phylum and genus level at pre-confluence and farther downstream of the confluence suggested a transient influence of Yamuna River on the confluence of the Ganges, extending its immediate downstream region only.

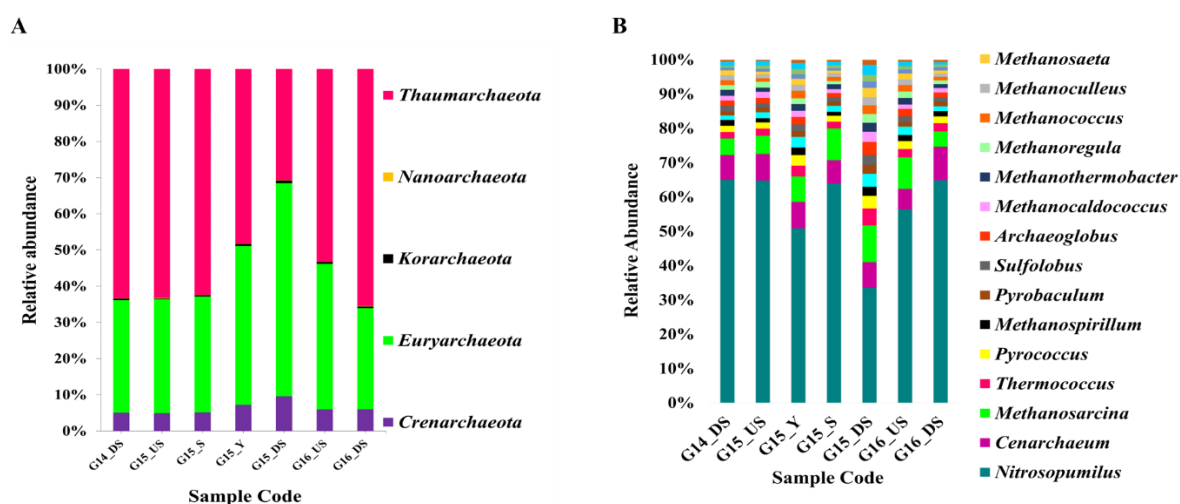


Fig. 3. Archaeal community composition along the confluence: A) Relative Phylum abundance, B) Relative Genus abundance.

3.3 Estimation of microbial diversity indices

The rarefaction curves were plotted as species richness (Y-axis) as a function of number of reads obtained per sample (X-axis). The rarefaction curves almost reached an asymptote, indicating that sufficient depth of sampling was achieved to encompass the overall taxonomic Diversity. Assessment of the diversity indices revealed the differences in the complexity of bacterial and archaeal communities, signifying a diverse community composition of the sediment samples of the Ganges River. The Chao1 richness estimator and Shannon diversity indices indicated a relative decrease in bacterial diversity and a relative increase in the archaeal Diversity for G15Y and G15DS. (**Fig. 4A**). Beta diversity, using a count-based Bray Curtis dissimilarity matrix, showed G15DS to be the most dissimilar on axis-1 (69%) with a

value of more than 0.2. (**Fig. 4B**). The Sangam and Yamuna samples are placed closely, indicating the influence of the Yamuna River on the microbiome of River Ganga during the confluence. However, the post-confluence locations merge back to the pre-confluence group, suggesting a transient influence of the Yamuna River on the microbiome of the Ganges.

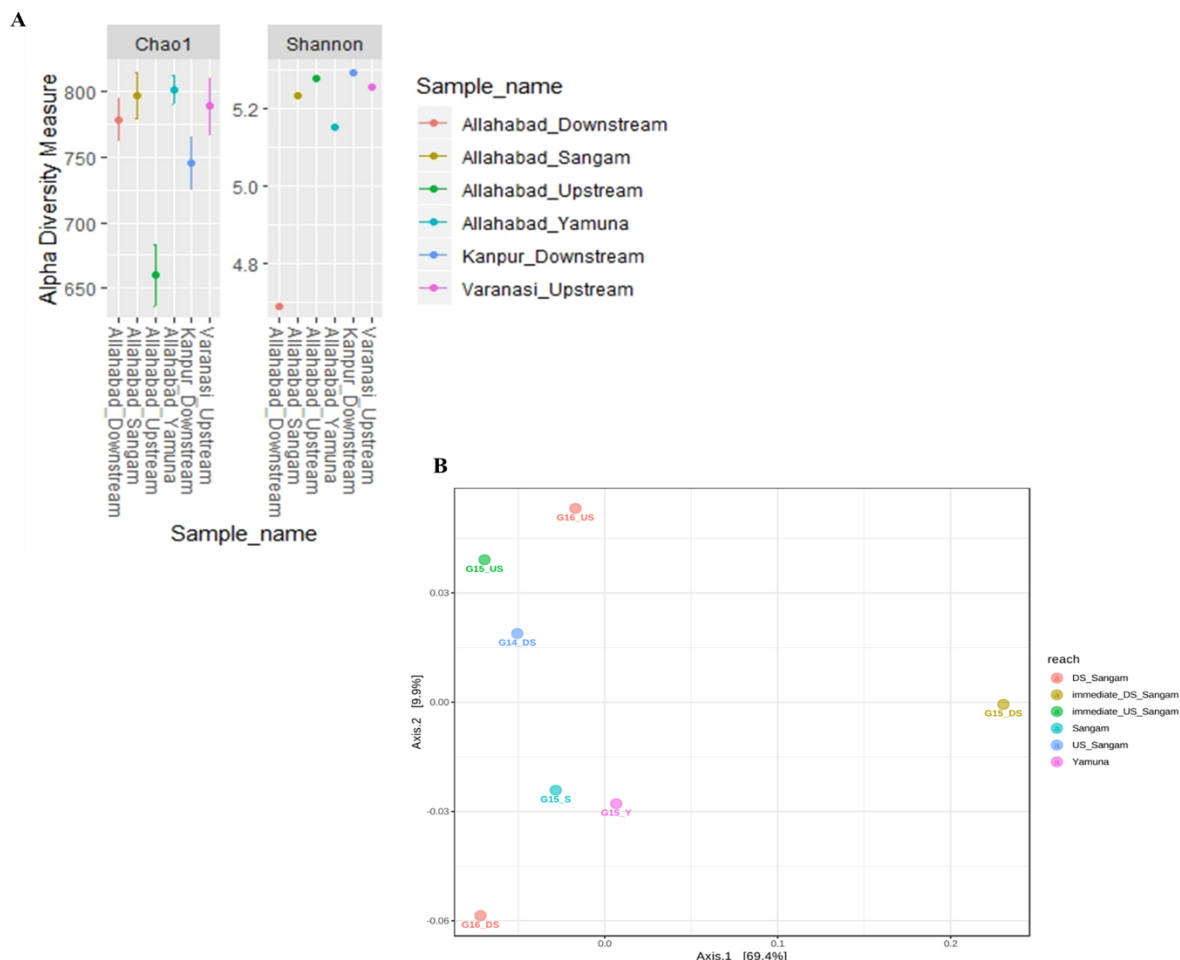


Fig. 4. Alpha and Beta diversity measures of the confluence microbiome: A) Observed alpha diversity indices (Chao1 and Shannon) for the taxonomic diversity. Each colored point corresponds to a sediment sample from a particular location. **B)** PCoA (Principal Component Analysis) plot using Bray-Curtis matrix showing Beta diversity of the samples. Each point represents samples from a single location, and the distance between the points signifies the compositional dissimilarity among the sample locations.

3.4 Predictive Functional Diversity

3.4.1 Diversity of xenobiotic biodegradation and metabolism

Metagenomic study of the diverse microbial communities in freshwater environments paves the way for exploring novel genes involved in metabolic functioning. Due to the alarming

levels of pollution of the Ganges and Yamuna, it becomes necessary to investigate the functional potentials of microbes. Targeting this facet, we focused primarily on the genes involved in the metabolism of xenobiotic compounds' resistance to antibiotics and toxic compounds. Further, we investigated whether the functional profile supports the restoration hypothesis. Amongst all the functions, 60% of the sequences were assigned to metabolism, which encompasses amino acid metabolism, biosynthesis of secondary metabolites, carbohydrate metabolism, cofactors and vitamins metabolism, energy metabolism, glycan synthesis and metabolism, lipid metabolism, metabolism of terpenoids and polyketides, nucleotide metabolism and xenobiotic biodegradation and metabolism (**Fig. 5A and 5B**). Annotated pathways for the metabolism of xenobiotic compounds such as Chlorocyclohexane and Chlorobenzene, Benzoate, Aminobenzoate, and Nitrotoluenes were present in all samples except G15US.

Additionally, the potential for degradation of steroids, drugs, and herbicides such as dioxin, caprolactum, and atrazine was observed in G15Y. These additional metabolic features, except for steroid and dioxin metabolism (exclusive of G15Y), were also present in the confluence and the immediate downstream, i.e., G15S and G15DS. However, the farther downstream locations G16US and G16DS showed xenobiotic degradation profiles comparable to that of the upstream of the confluence (**Fig. 5C**). The degradation of xenobiotics accounted for only 1-2% of the total metabolic functions, with G15US comprising only 0.6%. Classifying this aspect in detail unveiled the highest percentage of reads assigned to benzoate degradation, followed by degradation of chlorocyclohexane, chlorobenzene, nitrotoluene, and aminobenzoate in all the samples except G15US. Benzoate and chlorobenzene are aromatic compounds and significant contaminants of the dye and food processing industries, as well as chemical and degreased products (Xie et al., 2009). Chlorinated compounds (Cyclochlorohexane), nitrobenzene compounds (Nitrotoluenes), dioxins, and steroids are characterized as the principal xenobiotics and are known to be recalcitrant (Pal et al., 2018). Direct or indirect discharge of these compounds has led to water pollution in these rivers and an unforeseen effect on the microbial communities.

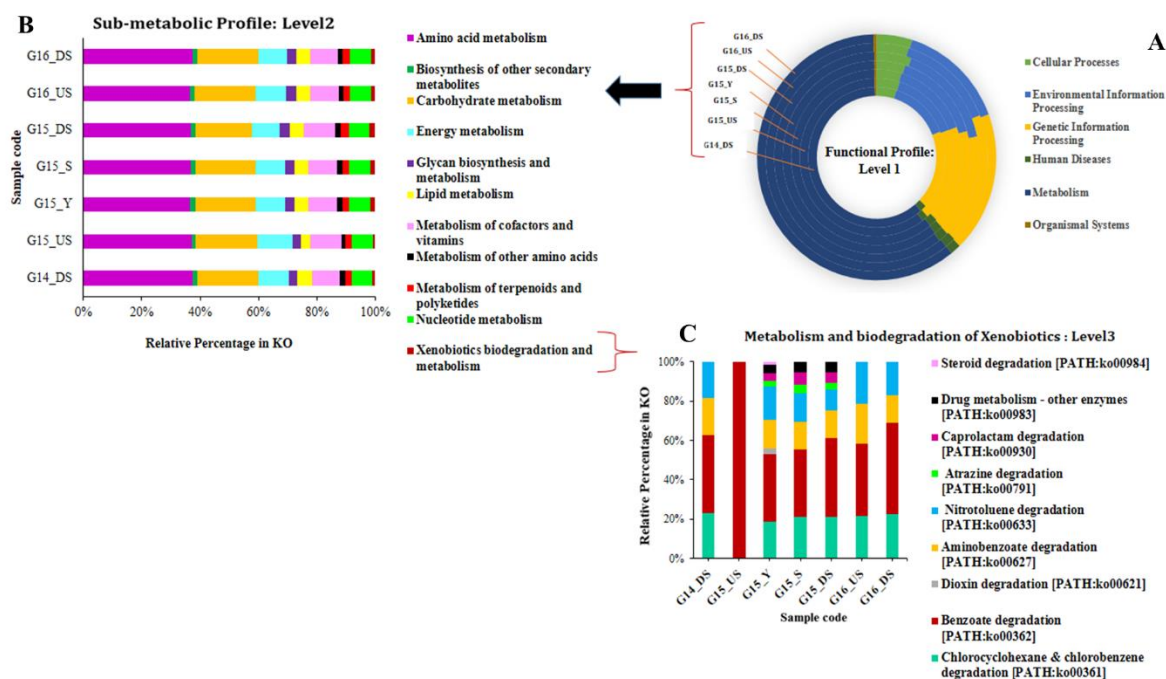


Fig. 5. Classification of reads assigned to metabolism according to KEGG database: A) Relative Percentage of reads assigned to all functions, **B)** Sub-metabolic profile, **C)** Biodegradation of xenobiotic compounds.

Degradation of these compounds from the microbial groups of River Ganges and Yamuna has not been studied. However, from this study, it can be inferred that these rivers house microbes with diverse bioremediation capabilities. There are several reports on anaerobic degradation of benzoate, cyclochlorohexane, and nitrotoluenes by bacteria and archaea such as *Geobacter*, *Pseudomonas*, *Nitrosopumilus*, and *Methanosarcina*, which were found to be dominant in this study, signifying their role in biological remediation under anaerobic conditions (Krzmarzick et al., 2018; Thelusmond et al., 2019). Interestingly, dioxin and steroid degradation were exclusive to G15Y. Dioxin is a polychlorinated biphenyl (PCB) that is resistant to physical, chemical, and biological degradation and has several adverse health implications. The presence of dioxin can be correlated with the poorly managed recycling practices of electronic waste (e-waste), which has gradually resulted in the discharge of these toxic effluents into the river. Furthermore, it was observed that the degradation potentials for caprolactam (a cyclic amide used in the manufacturing of synthetic fibers (Otzen et al., 2018) and atrazine (a primary representative of triazine ring-containing herbicides (Sene et al., 2010) were present in G15Y, G15S, and G15DS only. Although the changes affected the G15S and G15DS, none of these additional sets of genes were detected in distant sites, and a pattern very similar to that of the confluence upstream (G14DS) was observed.

3.4.2 Antimicrobial and heavy metal resistance

Further, the SEED database was used to decipher the genes conferring resistance to toxic compounds such as heavy metals and antibiotics. Resistance to heavy metals such as zinc, mercury, copper, cobalt, cadmium, and arsenic and antibiotics such as fluoroquinolones and beta-lactams were observed in all the samples. Apart from this, genes conferring Multiple Drug Resistance (MDR) due to the presence of efflux pumps were seen in all the samples. In addition to these genes, G15Y encompassed an additional set of genes supplementing diverse functionality. These genes include resistance to chromium compounds, copper homeostasis, presence of mercuric reductase, a separate MDR cluster, tolerance to Colicin E2, and Methicillin Resistance Staphylococci (MRSA).

Interestingly, the simultaneous presence of these genes was observed at the confluence (G15S) and the immediate downstream (G15DS) sample. Several studies have shown an increased load of heavy metals to be associated with amplified levels of river pollution (Jaishankar et al., 2014; Pal et al., 2018; Tchounwou et al., 2012). However, genes responsible for resistance against chromium compounds, copper homeostasis, and tolerance to colicin and multidrug efflux pumps were first seen in more polluted G15Y that is possibly getting carried forward to G15S and G15DS and disappearing farther downstream (**Fig 6A**). This suggests a transient influence of confluence followed by a probable restoration. Additionally, genes conferring resistance against antibiotics such as beta-lactams, multidrug resistance, and fluoroquinolones were noted in this study.

To delineate further and understand the antimicrobial resistance (AMR), MegaRES was used to annotate the metagenomic samples, classifying them on the basis of genes imparting resistance to a particular class of antibiotics and also revealing the mechanism of resistance lying underneath. From the categorization, a unique pattern of distribution of AMR genes with respect to resistance to different classes of antibiotics such as aminocoumarins, aminoglycosides, beta-lactams, elfamycins, lipopeptides, Macrolides-Lincosamide-Streptogramins (MLS), MDR, phenicol, rifampin, and sulfonamide was observed. The relative percentage of aminocoumarins and rifampin resistance was higher in G14DS (10.78%, 23.52%); G15US (11.27%, 41.81%); G16DS (8%, 36%) as compared to that of G15Y (0.47%, 0.47%); G15DS (0.14%, 0.84%); G16US (1.31%, 1.31%) (**Fig. 6B**). Resistance to aminoglycosides and sulfonamides was maximum at G15Y (24.88%, 14.92%), which appeared to be carried downwards at G15S (21.98%, 0.86%), G15DS (20.53%, 0.28%). Strikingly, confluence G15S also harbored specific distinct mechanisms, such as higher resistance to beta-

lactamases (50.86%) and lipopeptides (2.5%), respectively, that were possibly passed downstream to G15DS (33.6%, 0.421%), while started diminishing from G16US (1.31%) and were absent in G16DS (**Fig. 6C**). Further, to comprehend the shared and the unique mechanism of antibiotic resistance, a networking plot was constructed using Cytoscape v3.2.7 (**Fig. 6D**).

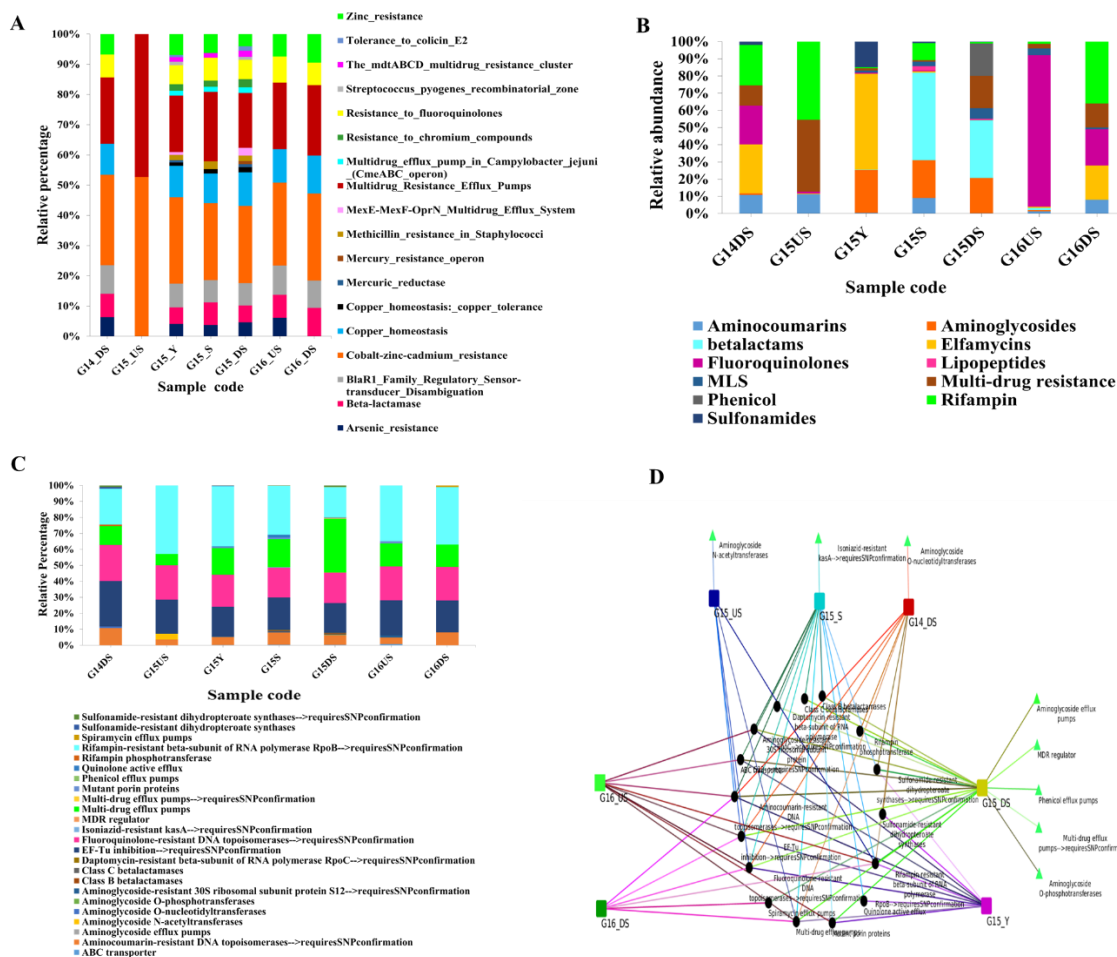


Fig. 6. Resistome profile of the confluence microbiome: A) Grouping of the reads mapping to toxic compounds (heavy metals and antibiotics) resistance according to KEGG database, **B)** Antibiotic resistance profile at the class level, **C)** Mechanism of resistance against different classes of antibiotics, **D)** Network plot showing resistance mechanisms unique to a particular location (arrows pointing outwards) and those shared in common by all the locations (Black dots).

Several studies have been carried out profiling the antibiotic resistance of the Ganges River (Reddy and Dubey, 2019; Zhang et al., 2018). However, to the best of our knowledge, there are no reports highlighting the restoration of Ganges regardless of the allochthonous inputs of AMR genes from River Yamuna into River Ganges at the confluence and the immediate downstream. Competence for resistance against aminoglycoside antibiotics is observed only

for G15Y, G15S, and G15DS, typifying the influence of the Yamuna River. Furthermore, an increase in the resistance against beta-lactamases is seen only at G15S and G15DS. This can be linked to the mass bathing of the pilgrims at the *Triveni Sangam* with a religious belief of being healed by the water of the River Ganges (Ahammad et al., 2014). This practice adversely affected the microflora over time by the addition of microbes with resistant genes, followed by the sharing of those with their counterparts through various gene transfer mechanisms (Jani et al., 2018).

Since this sampling was carried out before the mass bathing event (*kumbh*), additional studies need to be effectuated to unravel the underlying effect of mass bathing on the alteration of the microbial and functional profile of the confluence during and after the *Kumbh*. From the mechanisms of AMR, a higher relative abundance of multidrug efflux pumps was observed. Studies have shown an increase in AMR by *Pseudomonas* due to the alteration of multidrug efflux pumps under hypoxic conditions (Moradali et al., 2017). Increased hypoxic conditions at G15DS, with a dominance of *Pseudomonas* at the genus level, can be associated with the increase in the mechanism of resistance due to multidrug efflux pumps. Thus, a pattern where G15Y and G15S sway G15DS and, to some extent, G16US for AMR was observed. Nevertheless, G16DS shows an AMR profile comparable with G14DS, suggesting a probable restoration.

4. Conclusion

Overall, the present study underscores the importance of the transient effect of the Yamuna River on the taxonomic and functional diversity of the Ganges River at the confluence. Although the changes affected the immediate downstream, a restoration of microbial communities and their related functions was observed in the wake of the confluence. Therefore, this is a proof-of-concept study that reveals yet another unique property of apparent restoration/rejuvenation of the Ganges River post-confluence. However, an elaborative and in-depth approach for sampling would be needed over a spatiotemporal scale over the years to ascertain the changes in the confluence microbiome with similar results. Furthermore, studies on the entire lower (anthropogenically impacted) stretch of the River Ganges will also shed light on the possible role of the inherent microbiome in conferring the apparent restoration properties to the Ganges River.

5. References

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2.2 The Microbial Diversity and Resistome Along the Lower Stretch

Abstract

Background

The Ganges River is a unique riverine system in India with socio-cultural and economic significance. The lower stretch of the Ganges is marked by its debouchment from Himalayan regions to Gangetic plains, with dense civilization and massive anthropogenic influx along its catchment area contributing to most of its pollution.

Rationale

Only a few metagenomics-based studies have emphasized the presence of ARGs and MRGs in this riverine system. However, these studies are limited to specific locations of the river Ganges and lack a holistic understanding of the resistome encompassing its vast geographical area independently.

Results

This study employed a whole metagenome-based nanopore sequencing approach to delineate the microbial community structure and their associated antibiotic/metal resistance genes (A/MRGs) from the sediments of the Ganges along its 1500 km-long lower stretch. Comprehensive microbiome analyses revealed *Proteobacteria* (45.89 ± 4.16%), *Actinobacteria* (9.64 ± 1.46%), *Chloroflexi* (9.36 ± 2.19%), and *Acidobacteria* (8.54 ± 3.37%), as the predominant bacterial phyla. *Thaumarchaeota* (41.91 ± 15.97%) and *Euryarchaeota* (35.93 ± 13.54%) were the dominant archaeal phyla in all the samples across both seasons. Resistance genes against 23 different types of metals and 28 classes of antibiotics were observed. The key taxa associated with the dominant ARGs and MRGs were *Pseudomonas* and *Burkholderia*.

Significance

This study suggests the accumulation of antibiotics and metals as the driving force for the emergence of resistance genes and the affiliated bacteria trafficking them. The present metagenomic assessment provided evidence-based baseline data detailing the need for comprehensive, long-term biological and physicochemical monitoring and mitigation strategies towards the contaminants associated with ARGs and MRGs in this nationally important river.

Outcome

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1. Introduction

The occurrence of antibiotic and metal resistance genes (A/MRGs) is essentially ancient, but their propagation and dissemination are augmented through various anthropogenic actions (Yang et al., 2019). The World Health Organization (WHO) has stated that ARGs are a new pollutant. Excessive metals and antibiotics in any environment impose selection pressure on the microbial communities and eventually alter the native diversity (J. Chen et al., 2019). Human activities primarily influence freshwater ecosystems such as rivers and lakes and, therefore, serve as hotspots for the proliferation and evolution of ARGs and MRGs. These include several significant reservoirs that drain into the rivers, such as discharges from hospitals, sewage treatment plants, industries, and agricultural/ domestic/ urban runoff (Li et al., 2016). The excessive accumulation of heavy metals and antibiotics in the riverine system would gradually result in bacteria trafficking ARGs and MRGs being released into the food chain, consequently menacing public health (Muurinen et al., 2022). In recent studies, the water quality deterioration of urban rivers has become an important issue, as they are pivotal in the sustainable development of cities. However, significant changes in the water quality across urban settlements have been attributed to the copious release of anthropogenic pollutants and fecal contamination, which results in the emergence of ARGs, MRGs, and the associated resistant bacteria (H. Chen et al., 2019; Wang et al., 2019).

The Ganges is India's national river that serves as a lifeline for millions of people. It originates from the Gangotri glacier as River Bhagirathi and travels downward, joining the Alaknanda River at Devprayag, where its name commences as the Ganges (Khairnar, 2016). Being an epitome of spirituality and civilization, the water of this mighty riverine system meets the diverse needs of people regarding their subsistence. The lower stretch of the Ganges is marked by its debouchment from Himalayan regions to Gangetic plains, with dense civilization along its catchment area (Bhutiani et al., 2014). Several anthropogenic activities along the basin and at the shore of this river have contributed most to its pollution. Besides, the discharge of various untreated wastes, such as industrial and sewage, has further altered the water quality of this riverine system (Jani et al., 2018). Recently, Naqvi et al. reported dumping over 50 MLD (million liters per day) of hazardous waste and 140 MLD of domestic waste in Kanpur (Haider Naqvi SK, 2020). Another study by Srivastava et al. mentioned that Ganges' water was unfit for bathing and drinking at Haridwar and Rishikesh (Srinivas et al., 2019).

Additionally, Ganges water at other locations such as Bihar, Kanpur, Varanasi, *Triveni Sangam* in Allahabad, Ghazipur, Buxar, Patna, Bhagalpur, and Howrah-Shivpur in West Bengal was neither potable nor suitable for bathing (Dutta et al., 2020). Fecal pollution in rivers is another significant concern, as it increases the risk of harmful microbes with antibiotic resistance. Ganges River beyond Kanpur has fecal coliforms at alarming levels, rendering it unacceptable for bathing and drinking. These pollutants ultimately result in stagnant water conditions that have a colossal impact on the native microbial communities of the Ganges and public health (Srinivas et al., 2019).

Previous studies on the Ganges River have documented water pollution due to excessive amounts of heavy metals such as Mn, Zn, Cr, Cu, Ni, Cd, Pb, and Hg (Boral et al., 2020; Pal et al., 2018). Others have reported selective isolation of fecal coliforms and antibiotic-resistant bacteria (Ali et al., 2021; M Vortmann, 2015). Only a few metagenomics-based studies have emphasized the presence of ARGs and MRGs in this riverine system (Behera et al., 2020; Kumar et al., 2021; Reddy and Dubey, 2019; Zhang et al., 2018). However, these studies are limited to specific locations of the river Ganges and lack a holistic understanding of the resistome encompassing its vast geographical area independently. Recent developments in high-throughput sequencing technologies have empowered us with enhanced insights into the microbial community structure and its associated functions (Sanderson et al., 2018). Therefore, this is the first study to provide comprehensive insights into the total microbiome and the associated resistome (ARGs and MRGs) of the River Ganges about 1500 km of its anthropogenically impacted course from Haridwar to Bhagalpur along two seasons, using MinION nanopore sequencing technology.

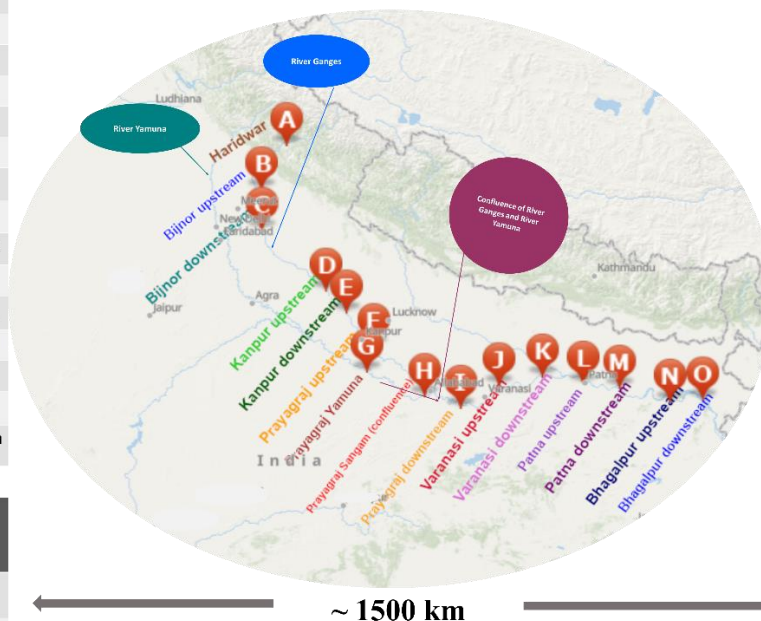
2. Methodology

2.1 Sample Collection and description of the study sites

In the present study, an approximate 1500 km long transect of the River Ganges was explored to map the reservoirs of antibiotic and heavy metal resistance genes. The sediment samples (depth ~20 cm) from fifteen locations, at two different seasons, were collected in triplicates (n=90) (n denotes the number of samples) from the left bank (LB), center (M), and right bank (RB) of the river, in December 2017 and June 2018 (**Fig 1**).

Sample code	Location
12DS	Haridwar
13US	Bijnor upstream
13DS	Bijnor downstream
14US	Kanpur upstream
14DS	Kanpur downstream
15US	Prayagraj upstream
15Y	Prayagraj Yamuna
15S	Prayagraj sangam
15DS	Prayagraj downstream
16US	Varanasi upstream
16DS	Varanasi downstream
17US	Patna upstream
17DS	Patna downstream
18US	Bhagalpur upstream
18DS	Bhagalpur downstream

Sample prefix	Season	Sampling Year
G	Post-monsoon	2017
JG	Pre-monsoon	2018



A total of 90; $n=45 \times 2$ sediment samples were collected from 15 locations spanning 1500 kms of River Ganges from Haridwar to Bhagalpur across both the seasons (2017,2018)

Fig. 1: Schematic illustration of the Ganges riverine system portraying the sampling locations across two seasons, pre-monsoon (2017) and post-monsoon (2018).

The physicochemical parameters such as temperature, pH, electrical conductivity (EC), and dissolved oxygen (DO) were measured *in-situ* using respective probes (**Table 1**). The samples were collected in 250 mL of sterile polypropylene solution bottles (Hi-Media), preserved on dry ice, and transported to the laboratory for further study. Further, each triplicate sample was pooled location-wise ($n=15 \times 2$) and sent for a detailed elemental analysis to an accredited laboratory (Bioresource Biotech Pvt. Ltd, Pune, India).

Table. 1: A tabulated summary of physicochemical parameters such as temperature, pH, electrical conductivity (EC), and dissolved oxygen (D.O.) for all the locations.

Station_ID	Season	Color	Odor	pH	Temperature °C	DO (mg/Liter)	Electrical conductivity(μs/cm)
G12DS	Post-Monsoon	Light green	No odour	6.9	17	10.8	181
JG12DS	Pre-Monsoon	Light green	No odour	7.96	20	9.1	156
G13US	Post-Monsoon	No color	No odour	8.5	19.4	10.5	225

Chapter 2.2

JG13US	Pre-Monsoon	No color	No odour	8.03	23.3	8.7	198
G13DS	Post-Monsoon	Light brown	No odour	8.32	17	10.9	233
JG13DS	Pre-Monsoon	No color	No odour	8.12	22.1	8.6	194
G14US	Post-Monsoon	Light brown	No odour	8.1	19	8.7	353
JG14US	Pre-Monsoon	No color	No odour	8.27	23.2	6.9	347
G14DS	Post-Monsoon	Light brown	Rotten egg	9.1	19.9	9.5	342
JG14DS	Pre-Monsoon	Dark brown	No odour	8.6	22.9	7.8	354
G15US	Post-Monsoon	Light green	Fishy	9.1	19.9	8.9	386
JG15US	Pre-Monsoon	Light green	No odour	8	22.7	7.6	371
G15Y	Post-Monsoon	Grey-black	foul	8.4	22	7.7	688
JG15Y	Pre-Monsoon	Grey-black	foul	7.9	23.5	7.2	665
G15S	Post-Monsoon	No color	No odour	8.8	21	9.5	760
JG15S	Pre-Monsoon	No color	No odour	8	24.3	8.7	739
G15DS	Post-Monsoon	No color	No odour	9	22	9.8	415
JG15DS	Pre-Monsoon	No color	No odour	8.3	24.8	7.7	389
G16US	Post-Monsoon	Light greenish and brownish	No odour	8.67	20	8.5	715
JG16US	Pre-Monsoon	Light greenish	No odour	7.8	24.1	7.1	722
G16DS	Post-Monsoon	Light greenish	No odour	8.65	21	7.8	728
JG16DS	Pre-Monsoon	Light greenish and brownish	No odour	7.6	24.9	7.3	725
G17US	Post-Monsoon	Light greenish and brownish	No odour	8.77	20	6.81	465
JG17US	Pre-Monsoon	Light greenish and brownish	No odour	8.2	23.9	6.9	434

G17DS	Post-Monsoon	Light greenish and brownish	No odour	8.89	20.5	6.65	480
JG17DS	Pre-Monsoon	Light greenish and brownish	No odour	8	22.5	6.2	396
G18US	Post-Monsoon	Light greenish and brownish	No odour	8.5	21.7	6.37	450
JG18US	Pre-Monsoon	Light greenish and brownish	No odour	7.9	24.2	6.9	458
G18DS	Post-Monsoon	Light greenish and brownish	No odour	8.4	23.4	6.05	465
JG18DS	Pre-Monsoon	Light greenish and brownish	No odour	8.1	24.6	6.4	448

2.2 Extraction of the metagenomic DNA and MinION-based shotgun sequencing

Water-saturated sediment samples were collected in triplicates. Subsequently, the samples (LB, M, and RB) were pooled together location-wise, and the total DNA was extracted using RNeasy PowerSoil Total RNA Isolation Kit (Qiagen) and RNeasy PowerSoil DNA Elution Kit (Qiagen) with certain modifications as mentioned in our previous study (Samson et al., 2019). The extracted DNA samples were multiplexed with 1D Native barcoding genomic DNA EXP-NBD103 and EXP-NBD 113, and the library preparation was carried out using Ligation Sequencing Kit 1D (SQK-LSK108) as per the manufacturer's instructions with certain modifications and loaded onto FLO-MIN-106D-R9 flow cells (Oxford Nanopore Technologies). The sequencing run was carried out for ~56 hours using the MinION sequencing platform.

2.3 Data processing and analysis

The raw reads (Fast5) were base-called using Albacore (v2.3.4). Demultiplexing of the fastq reads was done with qcat software v1.1.0 (ONT) (Vasiljevic et al., 2021). Porechop tool (v0.2.1) was employed to trim the adapters from the end of the reads and the chimeric

sequences from the middle of the reads. Additionally, Porechop was run along with barcode demultiplexing to minimize the risk of cross-barcode contamination. Variations in the library sizes of the datasets were normalized using the DESeq2 package (Zhu et al., 2019). Further, the rarefaction of the data was achieved using MG-RAST (Metagenomic Rapid Annotations using Subsystems Technology). The read statistics, quality score, and read length were assessed by Nanostats in NanoPlot (De Coster et al., 2018). Further, to eliminate the biases, the resulting data distribution was evaluated using the Shapiro-Wilk normality test (p-value threshold of 0.05), followed by the confirmation using a Q-Q plot (San et al., 2020). These rarified datasets were used then used for downstream taxonomic and functional analysis.

2.4 Microbial diversity and Statistical analysis

Annotation of the metagenomic datasets for microbial taxonomy and functional composition was achieved using MG-RAST. An E value cut off 1×10^{-5} and 60% of sequence identity were the default criteria used by the MG-RAST web server for the downstream analysis. Additionally, another metagenome classifier, Kaiju v1.7.3, was used to ascertain a higher sensitivity and precision for the taxonomic reads (Menzel et al., 2016). Singletons were removed from the (Operational Taxonomic Unit) OTU file. Further, the OTU table was rarefied to equal sampling depth. Alpha diversity was computed using Observed OTUs (number of different OTUs in the sample), Shannon diversity index (evenness of the sample), and Simpson's index (richness and evenness of the sample) was estimated using the phyloseq package in R (McMurdie and Holmes, 2013). Principal Component Analysis (PCoA) plot for beta diversity estimation was constructed using the Bray-Curtis dissimilarity matrix. Significance in the dissimilarity measure was evaluated using ANOISM and PERMANOVA (Adonis test p 0.05).

Additionally, to estimate the compositional homogeneity between the groups, the Betadisper test (p 0.05) was applied to the datasets. To control the errors arising from multiple comparisons, Benjamini-Hochberg (BH.) FDR procedure and Bonferroni correction were used. Furthermore, to understand the influence of physicochemical and heavy metals on microbial diversity, a non-metric multidimensional scaling (NMDS) plot based on the Bray-Curtis dissimilarity matrix was constructed in R using the vegan package. The envfit function in R was used to fit vectors of the physicochemical variables onto the NMDS ordination. For taxonomic analysis, ANOVA with BH correction was used to ascertain significant differences ($p < 0.05$).

2.5 Resistome analysis

The fastq files were uploaded to NanoARG to gain deeper insights into the detection and quantification of Antibiotic resistance genes (ARGs), and metal resistance genes (MRG). The default parameter cutoff by NanoARG is (--iden 25 --cov 40 --evaluate 1e-5 --prob 0.5). MRG annotation (--evaluate 1e-5 --id 25 --coverage 1e-5) was carried out using the Bacmet database by NanoARG. Kruskal-Wallis (KW) test ($p = 0.05$) was applied to the samples grouped by pre- and post-monsoon to ascertain significant ARGs and subtypes. The centrifuge database in NanoARG was used to assign the taxa to a particular ARG/MRG. Briefly, for each sampling location, their respective data table, post-analysis was downloaded from NanoARG. The data were sorted based on the gene name, category (ARG/MRG), and the cognate taxonomic annotation. The relative percentage of host for the respective gene category of the resistome was then plotted using GraphPad Prism v9.4.

2.6 Data Availability

The metagenomic datasets used in this study are publicly available in MG-RAST under the project name 'Ganga_Lower Stretchdata_NCL2019'. This data has also been submitted to the Sequence Read Archive (SRA) portal under the bio project **PRJNA902095**.

3. Results and discussion

3.1 Microbiome of the Ganges

3.1 A: Bacterial community composition

A total of 152 bacterial phyla and 3256 bacterial genera were observed at all the locations in both seasons. *Proteobacteria* ($45.89 \pm 4.16\%$) was the most prevalent phylum, followed by *Actinobacteria* ($9.64 \pm 1.46\%$), *Chloroflexi* ($9.36 \pm 2.19\%$), *Acidobacteria* ($8.54 \pm 3.37\%$), and *Bacteroidetes* ($7.35 \pm 3.39\%$) in all the samples across both the seasons (**Fig. 2A**). Certain metagenomic studies with River Ganges have also reported *Proteobacteria* and *Actinobacteria* as the two most dominant phyla. However, these studies are limited to determining microbial diversity in the Ganges River with 16S rDNA sequences (Zhang et al., 2018) and bacterial diversity along with ARGs and MRGs in the River Ganges at Varanasi

(Reddy and Dubey, 2019). Additionally, several members of *Proteobacteria* and *Bacteroidetes* phyla comprise clinically significant pathogens with multidrug resistance. Their abundance indicates contamination of water and sediments with human-associated clinical/ infectious wastes.

A seasonal comparison between all the samples was made to understand compositional changes in the bacterial taxonomy. The overall bacterial taxonomy remains similar at the phylum level across both seasons ($p > 0.99$; ANOVA with BH correction) (**Fig. 2B**). However, *Candidatus Handelsmanbacteria* ($p = 0.0041$), *Candidatus Kryptonia* ($p = 0.0173$), and *Candidatus Kaiserbacteria* ($p = 0.0356$) were among the significant taxa at phylum level that varied with the seasonal change. Besides, *Bacteroidetes* ($p = 0.0016$), *Acidobacteria* ($p = 0.0017$), *Candidatus Rokubacteria* ($p = 0.0019$), *Armatimonadetes* ($p = 0.0042$), and *Firmicutes* ($p = 0.0043$) were among the significant taxa enriched at all the sampling locations in this study. *Candidatus Handelsmanbacteria* and *Candidatus Kaiserbacteria* were significantly enriched in the post-monsoon samples. At the same time, *Candidatus Kryptonia* was more prevalent in pre-monsoon samples (**Fig. 2C**). This is the first study to report these candidate phyla in the River Ganges. Besides, for other dominant phyla, although not significant yet, an increase in the relative abundance of *Acidobacteria* (6-10%) was observed in the post-monsoon samples. In contrast, *Chloroflexi* (10%) and *Bacteroidetes* (8%) showed an increase in the pre-monsoon samples (dry season). This corroborates with the findings of Maestre et al., wherein the aridity affects the composition of *Chloroflexi* and *Acidobacteria*, increasing the abundance of the former and decreasing the latter (Maestre et al., 2015). A study on seasonal variations in the bacterial profile of Dongjiang River showed a similar pattern where phylum *Bacteroidetes* was in higher abundance during the dry season than in the wet season (Sun et al., 2017).

At the genus level, *Streptomyces* (17-32%) dominated at all the locations except for G13US, JG14 US, J/G14DS, G15Y, JG16US, J/G16DS, where a predominance of *Nitrospira* (19-32%). There are limited studies on freshwater systems, where *Streptomyces* has been reported as the most abundant genera (Hamm et al., 2017; Passari et al., 2018). *Streptomyces* are considered excellent elaborators of pharmaceutical products such as antibiotics and industrial enzymes and are well known as a prominent source of novel secondary metabolites (Ma et al., 2020; Passari et al., 2018). This whole metagenome sequencing-based information could be crucial in exploring sediments of the river Ganges for the isolation of promising strains of *Streptomyces* and its potential bioprospection. Additionally, the dominance of *Nitrospira* often occurs in close association with ammonia-oxidizing archaea/bacteria that

convert ammonia to nitrite, which is further oxidized to nitrate by *Nitrospira* (Mehrani et al., 2020). Other prominent genera include *Pseudomonas* ($16.08 \pm 12.65\%$), *Bradyrhizobium* ($4.55 \pm 1.55\%$), and *Sphingomonas* ($4.75 \pm 1.43\%$) (**Fig. 2D**). Seasonal variation did not have a significant impact on the bacterial diversity at the genus level ($p > 0.99$; ANOVA with BH correction) (**Fig. 2E**).

Pseudomonas was predominant in JG15Y, and J/G15DS ($n = 28-67\%$). However, a significant increase (Wilcoxon test p -value = 0.0156) in the genus *Pseudomonas* was observed at G15DS (67.24%) and JG15DS (45.97%). This could be correlated with the substantial increase in the relative abundance of *Proteobacteria* at J/G15Y ($50.4 \pm 2\%$) and J/G15DS ($55.2 \pm 5.1\%$). This result corroborates our previous study where the confluence of Yamuna River with the Ganges at *Triveni Sangam, Prayagraj*, results in a transient taxonomic shift at the immediate downstream location G15DS. The *Pseudomonas* genus spikes in the Yamuna River at *Prayagraj* and the immediate downstream sample of the Ganges River (G15DS). River Yamuna is well-known for its polluted trophic status. A sharp increase in the *Pseudomonas* could be an attribute of this confluence, with excessive human interference alongside the banks of the River Ganges (Samson et al., 2019). Additionally, the presence of *Pseudomonas* as the dominant genus at Bijnor could be due to the discharge of around 24 Million Liter Per Day (MLD) of wastewater (WW) (17%) from the four-point sources of drains, namely, Hemraj Drain, Bijnor Sewage Drain, Chhoiyad drain and Malan River (carrying sewage of Nazibabad town and later merging with river Ganges at Bijnor) (CPCB (Central Pollution Control Board), 2013).

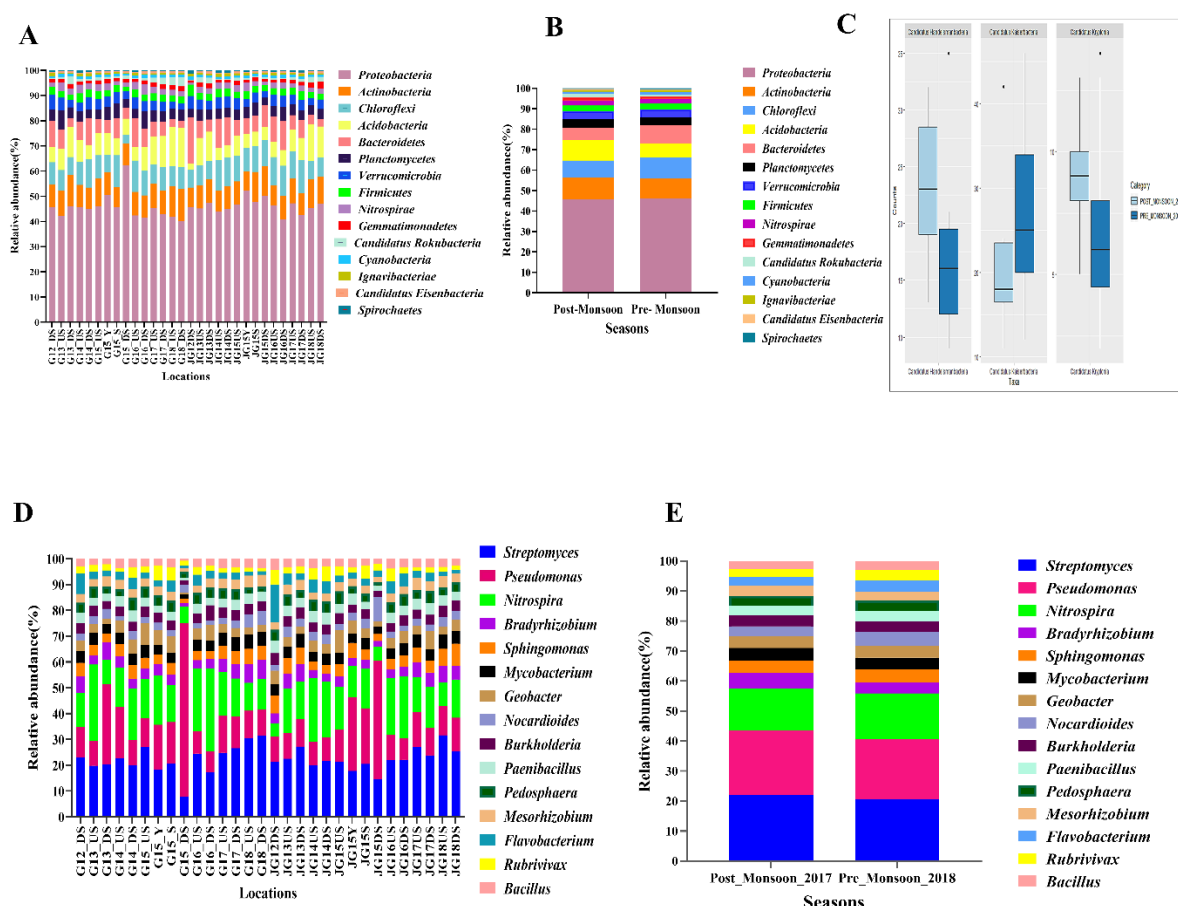


Fig. 2: Bacterial community composition across the lower stretch of River Ganges. A: Relative abundance of top 15 bacterial phyla. **B:** A seasonal comparison of the top 15 bacterial phyla showing compositional homogeneity. **C:** Graph showing the significant taxa at the phylum level that varied with the seasonal change. **D:** Relative abundance of top 15 bacterial genera. **E:** A seasonal comparison of the top 15 bacterial genera showing compositional homogeneity.

3.1 B: Archaeal community composition

River Ganges has been underexplored for archaeal diversity. Aiming for this, we analyzed our metagenomic datasets for dissecting the archaeal diversity. Analysis revealed the presence of a total of 22 archaeal phyla and 234 archaeal genera. A higher prevalence of *Euryarchaeota* was observed in all the samples with a mean relative percentage of ($38.93 \pm 13.54\%$), followed by *Thaumarchaeota* ($37.91 \pm 19.97\%$); *Candidatus Bathyarchaeota* ($6.23 \pm 3.91\%$); *Candidatus Woesearchaeota* ($3.14 \pm 2.46\%$); and *Crenarchaeota* ($2 \pm 0.45\%$) (**Fig. 3A**). Members of phylum *Euryarchaeota* comprise of methanogenic archaea, anaerobic

methanotrophs, and halophilic archaea that thrive in sediments with a higher pollution load (Saia et al., 2010; Stoica and Herndl, 2007). The *Thaumarchaeota* group consists of ammonia-oxidizing archaea (AOA), which are essential for nitrification processes in the aquatic biomes (Wang et al., 2020a, 2020b). Although a predominance of *Euryarchaeota* was observed at the phylum level, members of the phylum *Thaumarchaeota* were more enriched at the genus level. A significant fraction was contributed by *Candidatus Nitrosotenuis* ($19.75 \pm 12.76\%$), *Nitrosarchaeum* ($18.70 \pm 13\%$), *Candidatus Methanoperedens* ($8.52 \pm 9.43\%$), and *Methanosarcina* ($7.29 \pm 5.95\%$). Several studies have suggested a prevalence of AOA and methanogens in various wastewater (WW), WW treatment plants, and activated sludge (Ibarbalz et al., 2016; Płaza et al., 2021; Sharma et al., 2012; Vítězová et al., 2020). The presence of these genera in the River Ganges indicates water contamination through anthropogenic interference, which might act as a driving force toward the formation of the archaeal community structure (**Fig. 3B**). Further, a seasonal comparison of the samples for scoring differences in the archaeal diversity at phylum and genus level, revealed no significant differences (ANOVA with BH correction) (**Fig. 3C, D**). To our knowledge, this is the first study summarizing archaeal community composition along ~1500 km transects of River Ganges for two seasons.

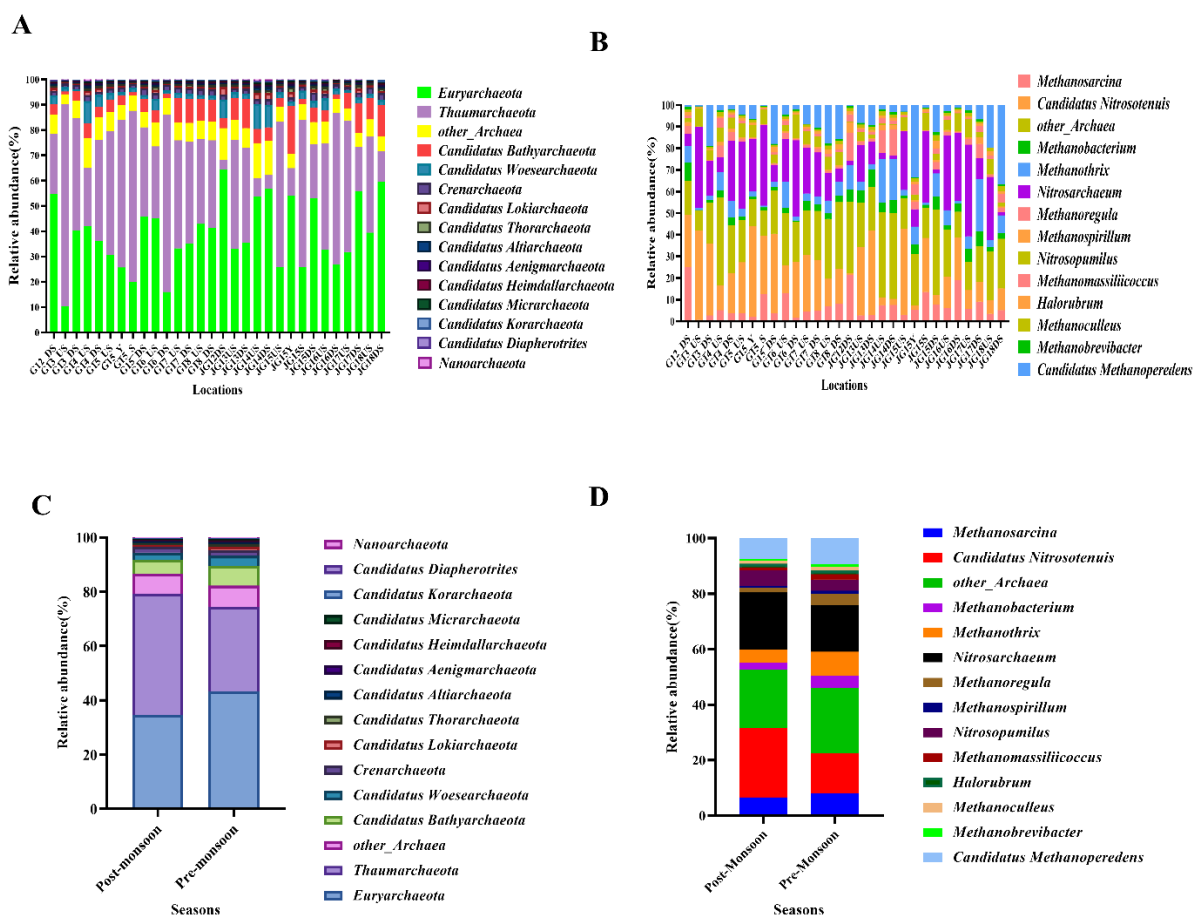


Fig. 3: Archaeal community composition across the lower stretch of River Ganges. A: Relative abundance of top 15 Archaeal phyla. **B:** Relative abundance of top 15 Archaeal genera. **C:** A seasonal comparison of the top 15 Archaeal phyla **D:** A seasonal comparison of the top 15 Archaeal genera.

3.1 C: Viral community composition

River Ganges harbor different microbial life yet remain unexplored for its viral diversity. In this study, we have attempted to delve into the diversity of dsDNA viruses in the River Ganges from Haridwar to Bhagalpur over two seasons. At the order level, a significant fraction was assigned to unclassified viruses ($62.55 \pm 9.79\%$), followed by *Caudovirales* ($33.55 \pm 8.49\%$) and *Herpesvirales* ($3.90 \pm 8.49\%$) (**Fig. 4A**). As compared to the prokaryotic and eukaryotic sequences in the metagenomic datasets, the viral sequences are underrepresented in the reference databases. This, in turn, inevitably impacts the reported viral diversity in the environmental metagenomes and results in a large proportion of unclassified viral sequences (Palermo et al., 2021). The presence of several unclassified viruses in the River Ganges opens the door for exploring its untapped viral diversity for various applications. Another identifiable

fraction includes the order of bacteriophages *Caudovirales*. This observation is consistent with the viral diversity of other freshwater systems, where *Caudovirales* accounted for numerical superiority (Cai et al., 2016; Ge et al., 2013; Lu et al., 2022). Further classifying the viral reads at the family level revealed a predominance of the family of giant viruses- *Mimiviridae* (31.68 $\pm$ 11.16%), followed by *Siphoviridae* (19.09 $\pm$ 5.06%), *Myoviridae* (11.23 $\pm$ 4.44%) and *Phycodnaviridae* (7.38 $\pm$ 2.83%) (**Fig. 4B**). Certain metagenomics-based studies on the viral diversity of eutrophic rivers and lakes have revealed the dominance of *Mimiviridae* and bacteriophage (*Siphoviridae*, *Myoviridae* and *Podoviridae*) families (Malki et al., 2020; Nogueira et al., 2022; Palermo et al., 2019; Runa et al., 2021). Similarly, for the River Ganges, a significant pollution load from various anthropogenic activities commences downstream of Haridwar (CPCB (Central Pollution Control Board), 2017). A higher prevalence of these viral families could be attributed to the resulting magnitude of water contamination, which leads to deterioration in the water quality of the river Ganges, thereby modulating the microbial community structure. Additionally, seasonal changes dismally affected the viral fraction (ANOVA $p > 0.05$ with BH correction) (**Fig. 4C, D**).

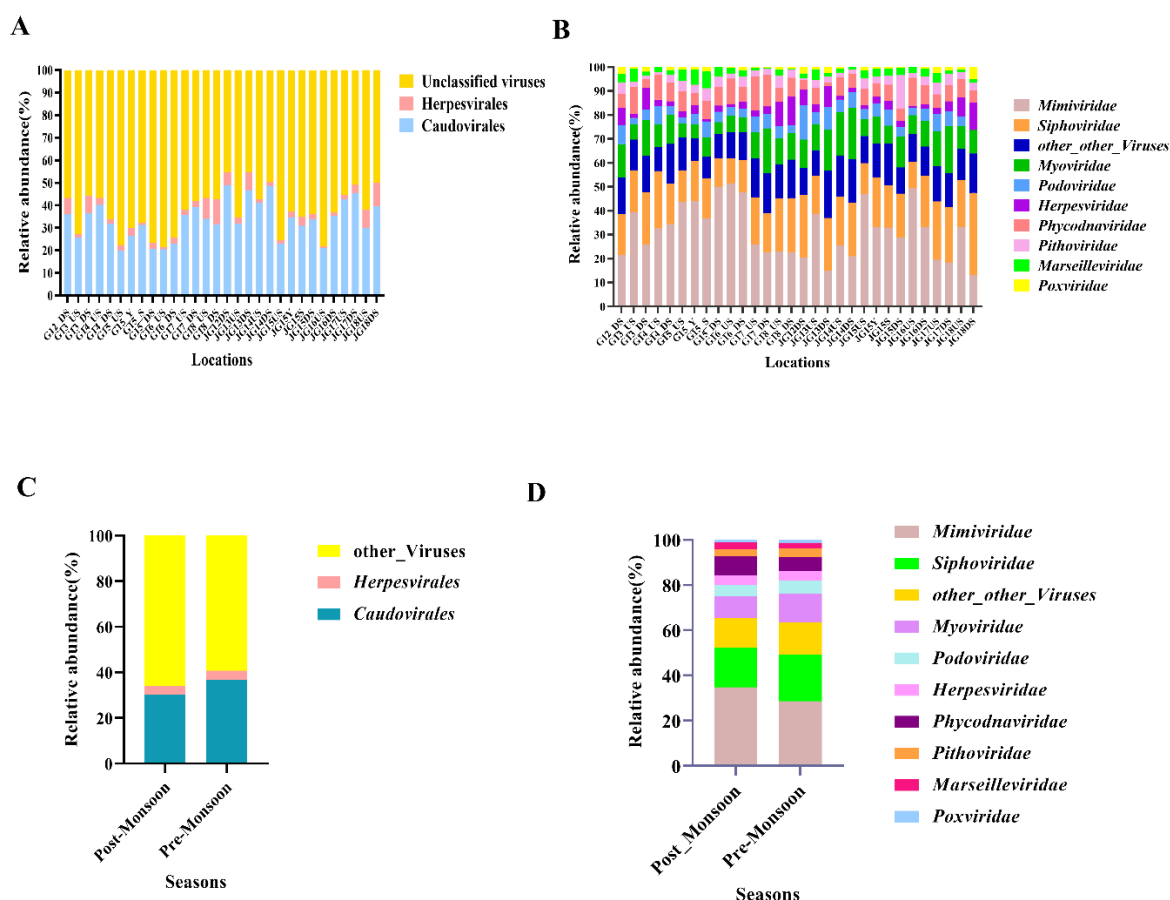
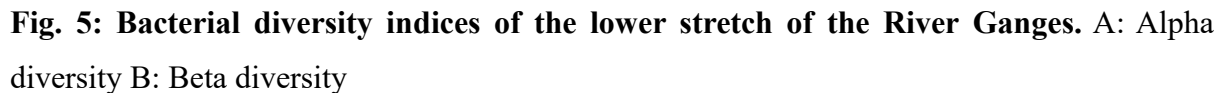


Fig. 4: Viral community composition across the lower stretch of the River Ganges. A: Relative abundance of viral order. **B:** Relative abundance of top 10 viral order. **C:** A seasonal comparison of viral order. **D** A seasonal comparison of viral order.

3.2 Estimation of Microbial Diversity Indices

Alpha diversity of the microbial community across two seasons was assessed using observed Shannon and Simpson's diversity indices. Although not significant, a higher number of different OTUs were observed in the post-monsoon season. However, a relative increase in the Shannon diversity (5.42) and Simpson's index (0.98) was observed for the microbial community, indicating a higher richness and evenness for the pre-monsoon samples (**Fig. 5A**). Beta diversity was computed to understand the microbial compositional changes, including seasonal differences, using the Bray-Curtis dissimilarity matrix. A lower dissimilarity was observed between the samples across both seasons on axis 1 with 39% variation (Adonis test $p > 0.05$), indicating no significant influence of seasonal variation on the microbial diversity (**Fig. 5B**).



3.3 A: Abundance, occurrence, and diversity of MRGs

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metals may increase in the aquatic environments. They can contrive into the human population, livestock, and crops in due course of time (Komijani et al., 2022). Therefore, monitoring the abundance and diversity of heavy metals in freshwater aquatic systems is necessary to assess their ecological risks. Targeting this facet, we investigated the prevalence of metal resistance genes and the associated taxa in the metagenomic datasets using the BacMet database in NanoARG. Analysis revealed the presence of MRGs against 23 various metals across all the sampling locations. The predominant MRGs conferred resistance against copper ($22.38 \pm 5.97\%$), zinc ($21.15 \pm 7.43\%$), silver ($11.79 \pm 3.90\%$), arsenic ($10.09 \pm 4.51\%$), and iron ($7.93 \pm 4.13\%$) (**Fig. 6A**). However, upon seasonal comparison, no significant differences in the compositional heterogeneity of the metal resistance genes across all the locations were observed ($p > 0.05$ ANOVA with BH correction).

Similar observations were reported by Reddy et al., wherein the most prevalent MRGs at the banks of the River Ganges at Varanasi were Cu, Zn, Ag, Fe, Cr, and As (Reddy and Dubey, 2019). Studies with other riverine systems, such as the Savannah River (USA) (Thomas et al., 2020), Xiangjiang River (China) (Jie et al., 2016), and Yangtze River (China) (J. Chen et al., 2019), have shown comparable results with Cu, Zn, and Pb/Cd/As being the most abundant MRGs. A substantial increase of MRGs against Cu and Zn could be correlated with the contamination of river water with fecal discharges from animal farms since these trace elements are used as feed supplements, clinically used antimicrobials, and multivitamins (Chakraborty et al., 2020). Several reports have highlighted Cu/Zn metals as the drivers for the development of antibiotic resistance in bacteria exposed to these metals. Also, wastewater influxes from various point and non-point sources and e-wastes could result in the abundance of Cu, Zn, and As (Reddy and Dubey, 2019; Samson et al., 2019). Besides, the river Ganges is revered as an epitome of spirituality. It receives various metals in the form of offerings from the people, which may affect the diversity of MRGs per se. The other prominent MRGs were against cobalt, nickel, and molybdenum. Additionally, resistance genes against toxic heavy metals such as mercury ($3.5 \pm 0.78\%$), lead ($3.3 \pm 0.76\%$), and cadmium ($1.92 \pm 0.12\%$) were also detected at a lower abundance in all the samples.

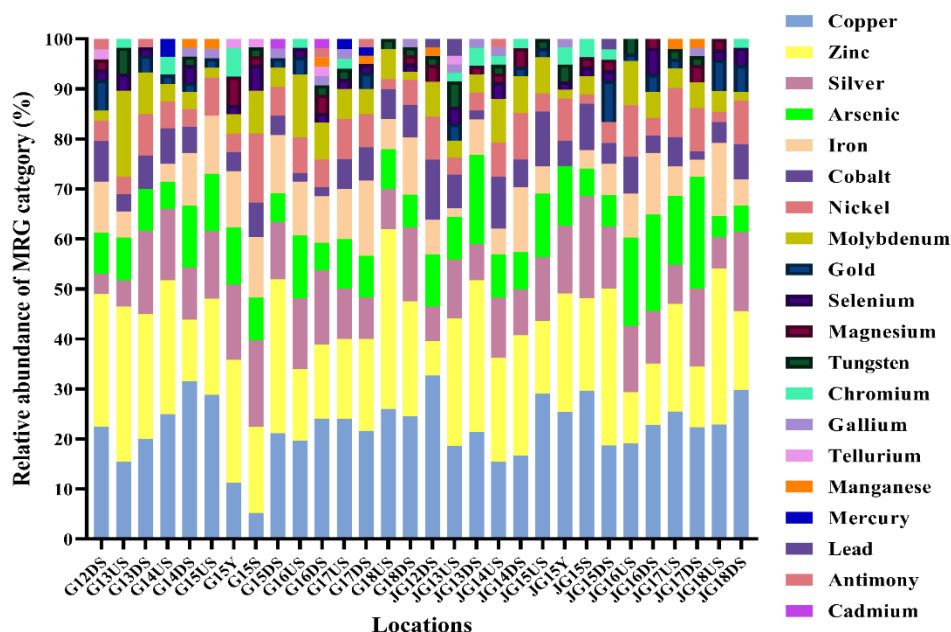


Fig. 6A: Abundance and diversity of metal resistance genes (MRGs) across the lower stretch of the River Ganges

3.3 B Influence of physicochemical factors and heavy metals on microbial diversity

Microbial communities in a riverine system are greatly influenced by their physicochemical parameters and the extent of heavy metal contamination (C. C. Huang et al., 2021). In the current study, we have assessed the effect of physicochemical properties such as pH, temperature, DO, and heavy metals (As, Cd, Cr, Hg, Ni, Pb, and Zn) on the microbial composition for both seasons. A higher concentration (mg/kg) of Zn (32-57) followed by Cr (8-50), Ni (4-14), and Pb (1-12) was observed at all the locations. However, the concentration of Hg was found to be enriched only in a few of the downstream sites of G12DS, JG13US, JG15US, and G17US. Seasonal changes dismally affected the prevalence of metals in the sediments of the river Ganges ($p > 0.05$ ANOVA with BH correction) (**Fig. 6B**).

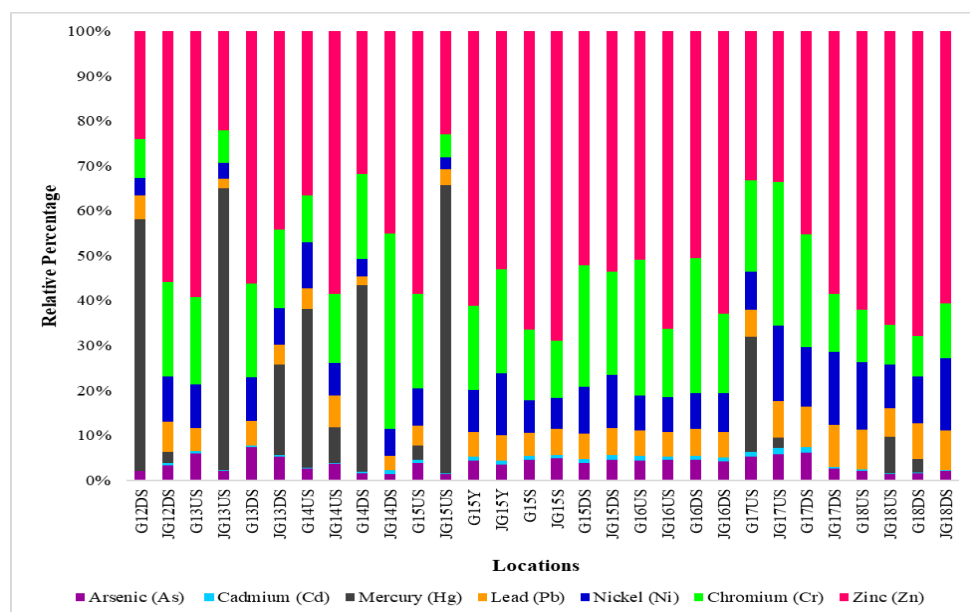


Fig. 6B: Abundance plot of elemental analysis along two seasons (For mercury $p=0.03$ Welsch ANOVA with BH correction)

Our results are consistent with other studies on the river Ganges in which a higher abundance of Cr, Co, Ni, Cu, Cd, Zn, Mn, and Pb metals through elemental analyses have been reported in its downstream (anthropogenically impacted) regions (Boral et al., 2020; Pandey and Singh, 2017). Further, we explored the association between the physicochemical parameters, elemental variables, and microbial community. Several studies have made known that long-term exposure to heavy metals (Cu, Cr, Ni, and Zn) alters the microbial community structure of the soil to varying degrees (Bååth et al., 1998; C. C. Huang et al., 2021; Zorz et al., 2019). The NMDS plot showed that temperature and heavy metals such as Hg, Cr, Cl, Zn, Pb, and Ni were the key drivers positively influencing the microbial diversity for all the sites across both seasons. In contrast, DO, pH, and arsenic showed a higher negative correlation with the axis than cadmium (**Fig. 6C**).

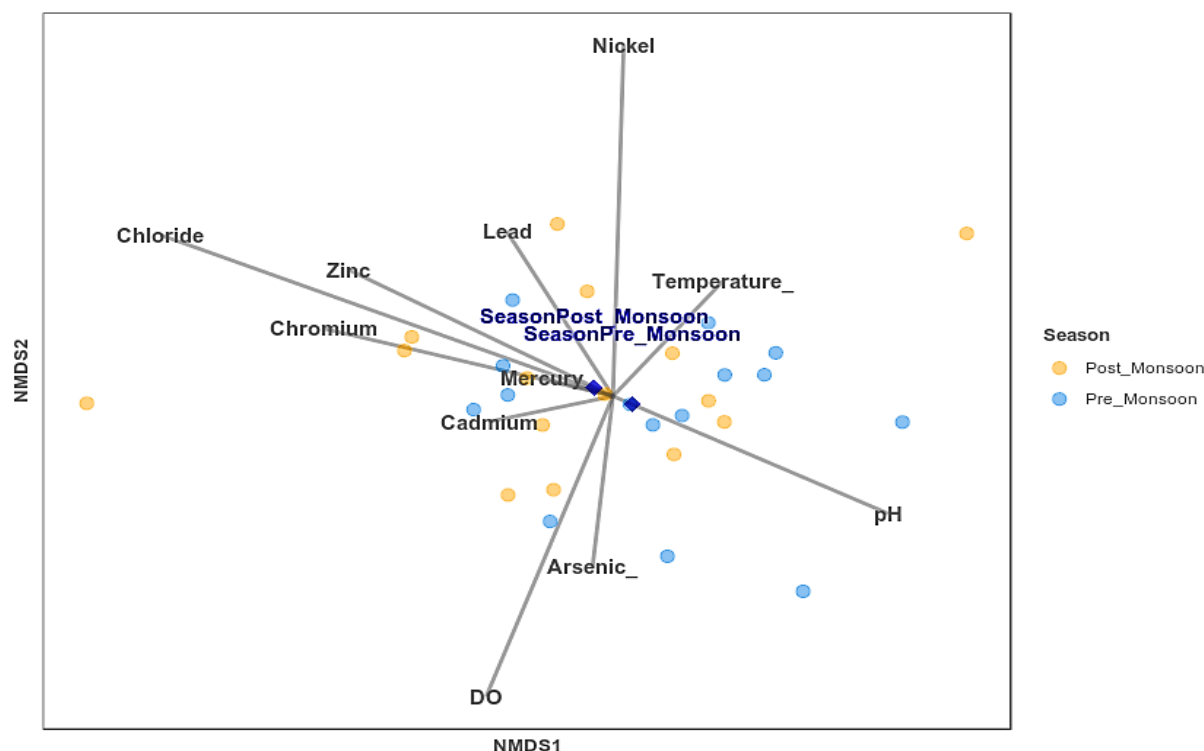


Fig. 6C: NMDS plot showing association among the physicochemical parameters, elemental variables, and microbial community along two seasons.

3.3 C: Abundance and dissemination of ARGs

Metal contaminants represent one of the chronic co-selection forces driving the antibiotic resistome in rivers. Aquatic biomes imperiled to extensive anthropogenic/urban activities are potential hotspots for the emergence of antibiotic-resistant bacteria (ARBs) (Reddy and Dubey, 2019). NanoARG-based analysis, together with MG-RAST functional profiling of the metagenomic datasets, was carried out to delineate a comprehensive resistome profile of the river Ganges. The analysis revealed the prevalence of genes extending resistance to a total of 28 classes of antibiotics. The most abundant ARGs across all the locations were those that offer resistance to multidrug/ multiple antibiotics (MDR) ($39.67 \pm 3.68\%$), followed by bacitracin ($7.70 \pm 1.37\%$); peptide ($7.65 \pm 0.95\%$); tetracycline ($6.86 \pm 1.23\%$) and glycopeptide ($5.3 \pm 1.14\%$) (**Fig. 7A**). This is consistent with the studies describing a higher prevalence of MDR genes through amplicon sequencing, antibiotic susceptibility testing and isolation of MDR bacteria from the River Ganges (Ali et al., 2021; Biswas et al., 2015; Reddy and Dubey, 2019; Zhang et al., 2018). However, these studies were confined to specific locations of the Ganges, and there was a dearth of holistic insight into its resistome along the

pollution ascent. To the best of our knowledge, this is the first study to comprehend a complete resistome profile of the River Ganges from Haridwar to Bhagalpur across both seasons.

Besides, the presence of bacitracin, glycopeptide, and tetracycline resistance in the River Ganges suggests contamination of the water and the underlying sediment with anthropogenic inputs from various sources such as sewage and hospital/industrial/domestic waste. Conversely, these observations vary when compared to the compositional profile of ARGs diversity and dissemination of ARGs with respect to other larger rivers across the world. Studies with the Yangtze River and River Wenyu, China, showed a predominance of bacitracin resistance genes and sulfonamide resistance genes, respectively (Liu et al., 2019; Wang et al., 2019). While in the Tiber River and Aniene River, Italy, a higher prevalence of Sulfamethoxazole resistance genes was observed (Grenni et al., 2019; Patrolecco et al., 2018). Additionally, for Fales River, Canada, and Sowe River, UK, the occurrence of resistance genes toward ciprofloxacin and ampicillin was observed, respectively (Amos et al., 2014; McConnell et al., 2018). However, in the River Ganges, a higher abundance of MDR genes has been observed, which is consistent with the other studies, as mentioned above. In consideration of the expanding nature of the AMR threat, the occurrence of MDR genes and their cognate hosts in the Ganges could pose severe threats to human health. Therefore, this study highlights the importance of surveillance and integrated management measures to prevent the dissemination of ARGs and potentially resistant bacteria.

Further, to understand significant ARG categories location-wise, the upstream and downstream sampling sites were grouped into a single location (e.g., G/JG13US, DS as Bijnor). ANOVA test with a $p=0.05$ and Bonferroni FDR corrections was applied to the grouping above. Notably, aminocoumarin ($p=0.041$), diaminopyrimidine ($p=0.000076$), mupirocin ($p=0.048$), and rifamycin ($p=0.0015$) were significantly enriched ARG categories (**Fig. 7B**). Aminocoumarins and rifamycin are classes containing potent antimicrobial compounds produced naturally by *Streptomyces* sp. (Cheenpracha et al., 2010). In addition, mupirocin- a blend of pseudomonic acid, is produced by *Pseudomonas fluorescens* (Sutherland et al., 1985). A predominance of *Streptomyces* and *Pseudomonas* at all locations highlights that members from these genera could drive antibiotic resistance in the other native microbial communities of the River Ganges. The selective pressure exerted by their antimicrobial compounds could act as a substantial driving force in shaping the microbial community structure of the River Ganges.

Further, to understand the detailed antibiotic resistance profile of the microbes in the River Ganges, categories of ARGs were investigated for their gene name (subtypes). A higher prevalence of genes *rpoB2* ($13.32 \pm 2.86\%$), *ompR* ($9.98 \pm 1.83\%$), *bcrA* ($9.08 \pm 2.05\%$), *tetA* (48) ($7.86 \pm 1.89\%$), multidrug ABC transporter ($6.08 \pm 1.61\%$), *patA* ($5.95 \pm 1.33\%$), *vanR* ($4.98 \pm 1.43\%$) and *bacA* ($4.57 \pm 1.33\%$) were abundant in all the samples as illustrated in **Fig. 7C**. In a signature-based-ARG source tracking study, Li et al. have linked the abundance of specific indicator ARGs to their source in each ecotype (Li et al., 2018). The occurrence of *mexE*, *mexF*, and *mexT* was linked to anthropogenic stressors impacting the Kalamas and Cam Rivers. Another noteworthy observation was the presence of *tetA* and *bacA* genes in the sediment samples of the Ganges. The *bacA* gene is characteristic of the human gut and human waste, while the *tetA* gene is an important candidate for hospital waste and wastewater-influent samples (Hu et al., 2013). Additionally, a higher incidence of *ompR*, *penA*, and *mexF* genes indicates sewage water contamination (Gupta et al., 2019). Additionally, the presence of *bcrA*, *vanR*, *tetA*, *bacA*, *arcB*, and *kdpE*, *macB* genes signify contamination of the riverine system with human and animal feces, respectively. These observations are indicative of anthropogenic stressors, and the associated genes signify contamination of the Ganges riverine system with human and animal feces, respectively.

Likewise, analysis of significant ARG groups for spatio-temporal variation revealed fosmidomycin to be more enriched in the pre-monsoon samples. At the same time, genes that confer tetracycline resistance were more prevalent in the post-monsoon season at all the locations (ANOVA, $p < 0.05$, with Bonferroni FDR corrections) (**Fig. 7D**).

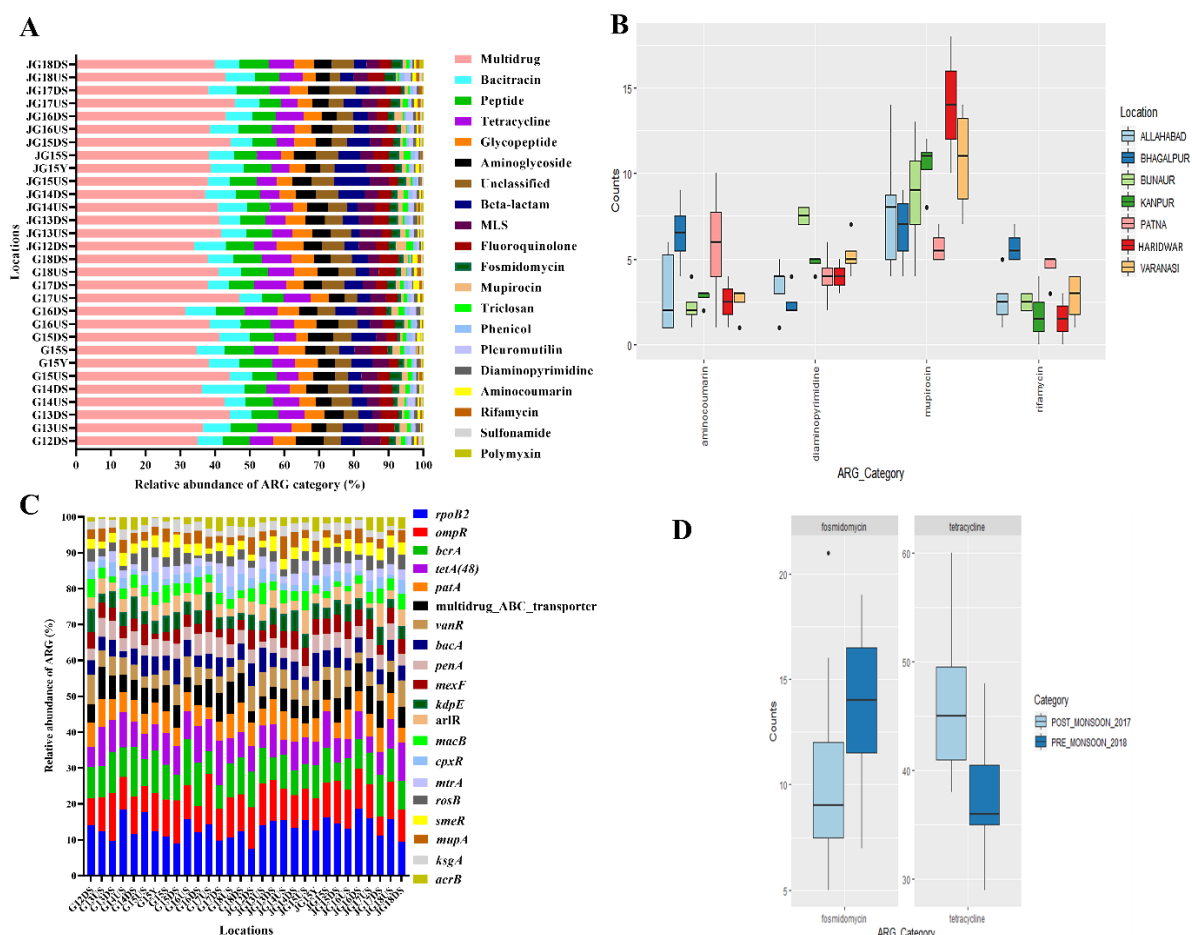


Fig. 7: Abundance and diversity of antibiotic resistome across the lower stretch of River Ganges. **A:** Graph illustrating categories of antibiotic resistance genes. **B:** Significantly enriched categories of antibiotic resistance genes. **C:** Relative abundance depicting the top 20 genes encompassing the antibiotic resistome. **D:** A seasonal comparison of the significant ARG categories.

3.3 D: Microbial hosts and associated functions for MRGs

In addition, we analyzed our data to understand the association between the microbial communities and the MRG categories. *Proteobacteria* comprised a significant fraction (91.37%) of members contributing to the metal resistome repertoire of the river Ganges, followed by *Actinobacteria* (4.31%), *Nitrospira* (3.44%), *Bacteroidetes* (0.86%), and *Firmicutes* (0.86%) (**Fig. 8A**). Subsequently, at the genus level, the taxonomic annotation revealed a total of 116 Bacterial species carrying MRGs conferring resistance to mainly Cu, Zn, As, Ag, Mg, and Hg. The entities were clustered into higher taxonomic rankings at the genus level, and the relative abundance of genera was plotted. A predominance of

Pseudomonas (28.44%), *Burkholderia* (4.31%), *Geobacter* (4.3%), *Xanthomonas* (3.44%), and *Pandoraea* (3.44%) was observed for a majority of the MRG categories (**Fig. 8B**).

Transition metals like iron, cobalt, nickel, copper, and zinc are prerequisites for several biological functions and virulence mechanisms in most microbes (Palmer and Skaar, 2016). To unveil the functions associated with each MRG type, we investigated our metagenomic datasets in MG-RAST (KEGG database). The analysis assigned maximum functions related to copper and zinc homeostasis. A higher relative abundance of Copper-translocating P-type ATPase (CopA) ($26.77 \pm 1.97\%$) followed by Response regulator of zinc sigma-54-dependent two-component system (ZraR) ($17.76 \pm 3.32\%$) and multicopper oxidase (CueO) ($12.13 \pm 1.91\%$) was observed in all the samples (**Fig. 8C**). CopA and CueO are crucial for maintaining copper homeostasis amid environmental fluctuations (Rensing and Grass, 2003). Since MRGs conferring resistance against copper are more enriched in all the samples, the occurrence of the corresponding functional components is evident.

Besides, certain studies have shown that resistance to copper and zinc offers advantages to members of the genus *Pseudomonas* and *Burkholderia* virulence (Schaefer, 2020; Virieux-Petit et al., 2022). These observations were consistent with the above discussion on MRGs-related hosts. The other prominent proteins include arsenical-resistance protein Acr3 ($8.94 \pm 2.70\%$), Co-Zn-Cd resistance protein Czc ($6.51 \pm 1.39\%$), and Mg/Co efflux protein CorC ($2.95 \pm 0.67\%$). Members of the Acr3 family are involved in the biotransformation of arsenic in different microbes, thereby offering them a survival advantage in metal-contaminated sites (Desoeuvre et al., 2016). A maximum abundance of functions associated with Cu, Zn, As, and multiple metal resistance suggests persistent exposure of the microbial community to the metal-contaminated urban/industrial discharges. Besides, most gram-negative and pathogenic bacteria possess multiple metal resistance genes, such as proteins from CorC and Czc families (Y. Huang et al., 2021; Nies, 1992). The taxa assigned to MRGs comprise gram-negative bacteria that are either pathogens or opportunistic, causing various human-associated infections. This is the first study on the river Ganges to comprehend metal resistome and the associated microbial diversity from Haridwar to Bhagalpur (~1500 km) across two seasons.

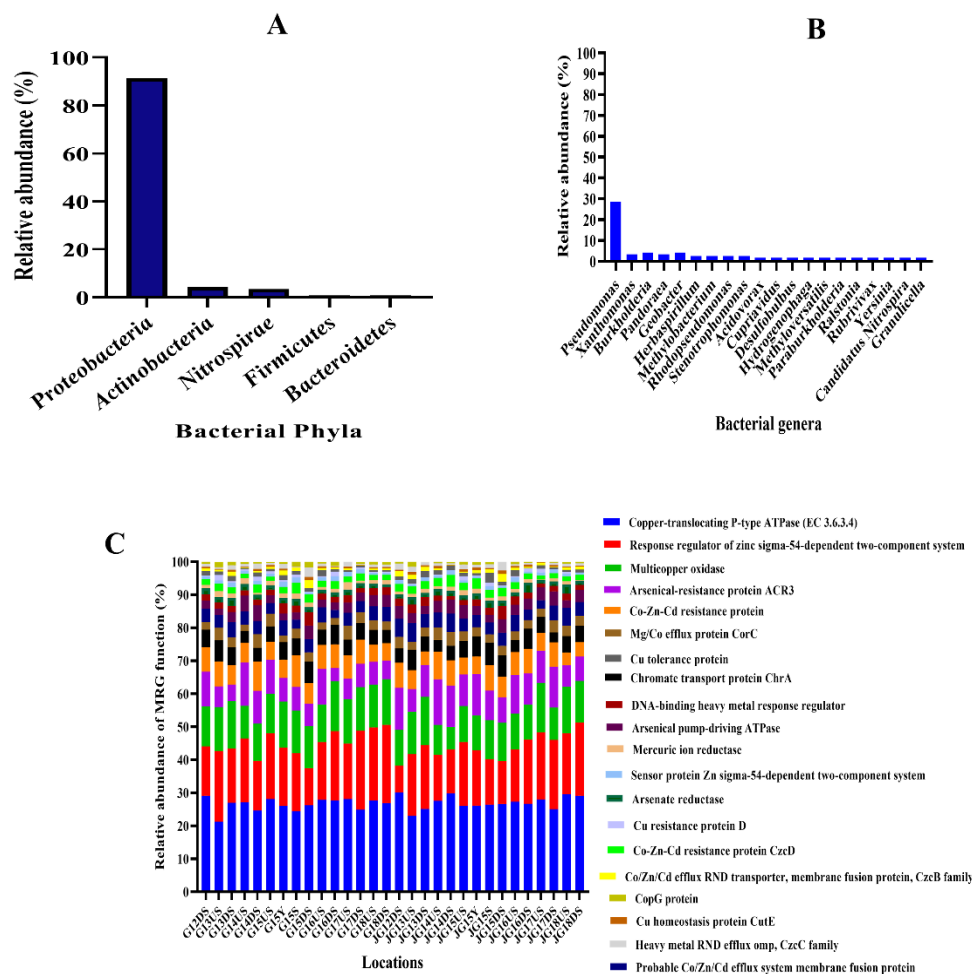


Fig. 8: Microbial diversity of taxa with metal resistance genes and the associated functions across the lower stretch of the River Ganges. A: Relative abundance of bacterial phyla conferring MRGs. **B:** Bacterial genera responsible for the MRG repertoire. **C:** Spatial composition of *in-silico* predictive functions associated with each MRG type.

3.3 E: Microbial hosts and associated functions for ARGs

The taxonomic annotation of the datasets was carried out to analyze the association between ARGs and microbial taxa. The Archaeal domain (99.5%) comprised a significant fraction of the ARGs. The major constituents among them include *Proteobacteria* (85.62%), *Actinobacteria* (6.98%), *Bacteroidetes* (1.2%), and *Chloroflexi* (1.12%) (**Fig. 9A**). At the genus level, *Pseudomonas* (35.6%), *Burkholderia* (11.8%), *Geobacter* (8.8%), *Streptomyces* (5.5%), *Stenotrophomonas* (5%), *Xanthomonas* (4.5%), *Ramlibacter* (4%), *Pandoraea* (4%), *Cupriavidus* (3.4%), and *Ralstonia* (3.1%) were the top 10 most abundant genera in all the locations (**Fig. 9B**). A similar observation was reported by Reddy et al., wherein *Pseudomonas* was found to be the dominant genus contributing to the ARG repertoire of the Ganges river at

Varanasi (Reddy and Dubey, 2019). Besides, most of the *Burkholderia* and *Stenotrophomonas* species are important nosocomial pathogens and are frequently encountered with *Cupriavidus*, *Pandoraea*, and *Ralstonia* species from the respiratory specimens of cystic fibrosis patients (Lipuma et al., 2015). Various anthropogenic influences in the forms of hospital-associated discharges, faecal contamination, and mass bathing could be linked to the prevalence of these taxa. Therefore, these findings value consideration toward water quality monitoring and management of the River Ganges across the urban settlements.

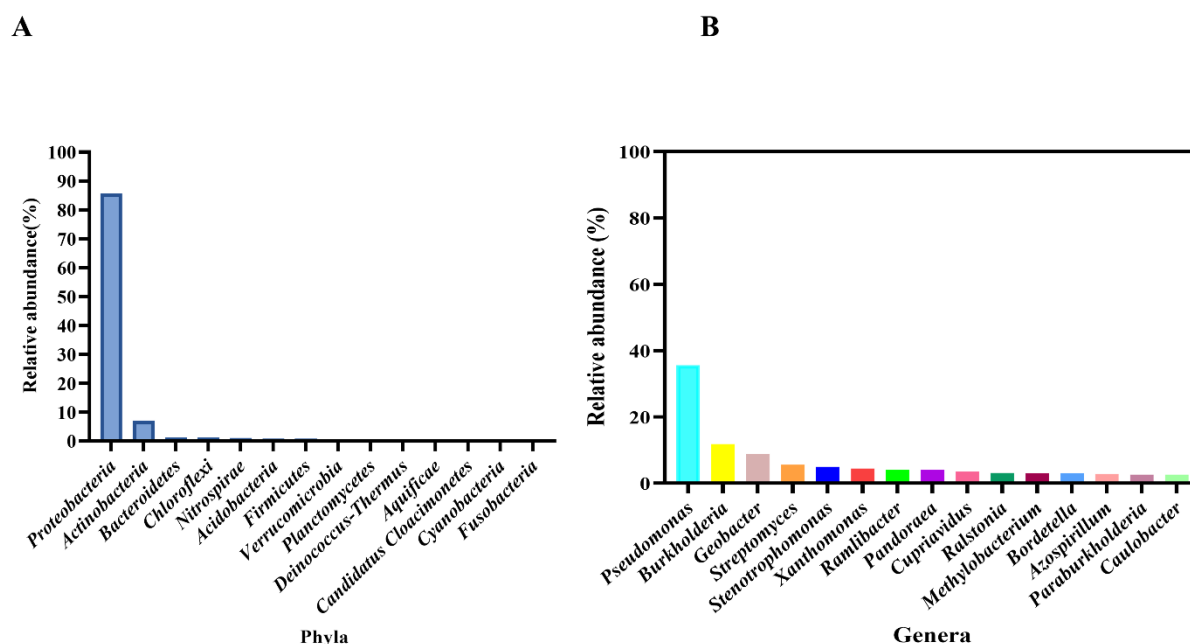


Fig. 9: Taxonomic annotation of the datasets showing ARGs bearing microbial taxa. A: Relative abundance at phylum level. **B:** Relative abundance at the genus level.

4. Conclusion

Overall, this is the first study to provide comprehensive insights into the total microbiome and the associated resistome (ARGs and MRGs) of the River Ganges about 1500 km of its anthropogenically impacted course from Haridwar to Bhagalpur along two seasons, using MinION nanopore sequencing technology. The results of the present study suggested the accumulation of antibiotics and metals as the driving force for the emergence of resistance genes and the affiliated bacteria trafficking them. A higher prevalence of ARGs and MRGs suggested prolonged exposure of the microbial communities to anthropogenic pollutants, posing a severe threat to the riverine ecosystem. Important nosocomial relevant pathogens, like

Pseudomonas, *Burkholderia*, and *Stenotrophomonas*, were the main potential taxa of ARGs and MRGs repertoire. The current study will significantly aid in the surveillance/ risk-based assessment of the River Ganges relating to long-term exposure to ARGs and multi-metal pollution. This data could be used to mitigate water pollution and improve the downstream trophic status of the River Ganges.

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2.3 Antimicrobial Potentials Along the Lower Stretch

Abstract

Background

The Ganges River in India has been well known for its centuries of non-putrefying (self-cleansing) and exceptional healing properties. The experimental foundation of the antibacterial property of the water from the Ganges River against *V. cholerae* was demonstrated by Ernest Hankin in 1896. The factor conferring this special property to the Ganges was later termed bacteriophages by D'Herelle.

Rationale

Lack of studies that provide a scientific explanation for the antimicrobial potential of the Ganges River in terms of its bacteriophage diversity and the diversity of secondary metabolite biosynthesis.

Results

Comprehensive microbiome analyses highlighted that despite the tremendous pollution load from various anthropogenic sources, the microbiome acts as a repository harboring functional potentials for the synthesis of secondary metabolite compounds with antimicrobial potentials. Additionally, a rich bacteriophage diversity with lytic potentials toward putative putrefying and pathogenic bacteria opportune their promising applications under the one-health concept.

Significance

Our study lays a foundational basis for understanding how the functional potentials of the autochthonous bacteria and bacteriophages could be involved in conferring special properties to the Ganges River. This data will be helpful in enhancing and expanding our understanding and unraveling the non-putrefying and rejuvenating special properties of this unique ecosystem.

Outcome

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1. Introduction

The post-antibiotic era marks an urgent need for new antimicrobial substances due to the increasing challenge of antimicrobial resistance (AMR). Any approach aimed at addressing the challenge of antimicrobial resistance must be comprehensive, involving multiple dimensions, disciplines, and global cooperation (Gordillo Altamirano and Barr, 2019). Therefore, to tackle AMR, the search for newer antimicrobials and emerging alternatives has been the current focus area of research. Microbial secondary metabolites are typically small molecules produced during secondary metabolism, occurring separately from the primary growth phase of microbes. These compounds encompass antibiotics, enzyme inhibitors, immunomodulatory agents, pigments, and toxins. These metabolites have a potential influence on the microbial community composition (Geers et al., 2022). There are limited studies that have investigated the potential of microbiomes from different ecosystems, such as soil, rhizosphere, endophytic plants, marine, and freshwater, to produce natural products (Chevrette et al., 2022; Fouillaud and Dufossé, 2022; Geers et al., 2022; Mendes et al., 2013; Sharrar et al., 2020). Given the vast array of microbial biosynthesis genes found in nature, even the most frequently sampled microbiomes offer the potential for discovering new classes of natural products (Fouillaud and Dufossé, 2022).

Additionally, bacteriophages, commonly referred to as phages, have also surfaced as promising options for tackling bacterial infections in the post-antibiotic era (Gordillo Altamirano and Barr, 2019). Besides, various phage-derived proteins or enzymes, such as endolysins, peptidoglycan hydrolases, and virion-associated depolymerases, have emerged as potential alternatives for addressing bacterial infections either independently or in conjunction with traditional antibiotics (Maciejewska et al., 2018; Sharma et al., 2018). Several studies have documented the isolation of bacteriophages from various ecological niches and their use in tackling pathogenic bacteria in humans, animals, poultry, aquaculture, food, and agriculture (Samson et al., 2024; Sieiro et al., 2020; Svircev et al., 2018). As traditional bioprospecting has only scratched the surface of the biosynthetic potential of various biomes, next-generation sequencing offers a new dimension to explore the untapped microbiome and their antimicrobial potentials.

River Ganges is the national river of India and has been an epicenter of civilization and culture. Since the beginning, this river has been perceived to possess certain special and unique properties of 'self-cleansing (non-putrefying) and special healing' (Kedzior, 2014; Khairnar,

2016a). The waters of the River Ganges have a rich history of demonstrating antibacterial properties, first studied by Ernst Hankin against *Vibrio cholerae* in 1896 (Hankin, 1896). However, a detailed insight into these antibacterial aspects was provided by Félix d'Hérelle, who discovered and termed the antagonist microbe of *V. cholerae* as a 'bacteriophage' (D'Herelle, 1917). It is well-known that despite tremendous anthropogenic activities and their related pollution load, the Ganges can rejuvenate itself rapidly (Paul, 2017; Reddy and Dubey, 2019; R. Samson et al., 2019; S.-Y. Zhang et al., 2018). This fact has also been witnessed amidst the COVID-19 lockdown, which provided a window period for the restoration of the Ganges River in the absence of any human intervention. The result, with a span of 2-3 months, was evident in the remarkable clean water, even at the most polluted sites (Dutta et al., 2020). The reason behind this is still unknown. Consequently, our knowledge of the special properties possessed by this riverine system remains fragmentary. Therefore, there is a need to fill these lacunae and delve deep into the scientific nature of the antimicrobial potentials possessed by the Ganges River.

The fact that aquatic ecosystems are a reservoir of a plethora of unique microbial diversity has led to their exploration of natural waters. Several studies have shown the biodiversity of viruses in fresh and marine waters in terms of their composition, seasonal variation, trophic status of the water system, and anthropogenic activities (Angly et al., 2006; Hewson et al., 2012; Palermo et al., 2019; Rohwer and Thurber, 2009). However, to date, there are no reports on the antimicrobial potentials of the Ganges River in terms of its bacteriophage diversity, its functions, the secondary metabolite diversity, and the biosynthetic pathways along the entire lower stretch of the Ganges River. Therefore, this study provides the first whole metagenome-based insights into the secondary metabolite potentials and bacteriophage diversity along the anthropogenically impacted stretch of the Ganges River. Additionally, we have also comprehended the spatiotemporal heterogeneity of the biosynthetic gene clusters, phage diversity, and their functions. Based on this information, the microbiome of the Ganges River can be explored for its antimicrobial potential to tackle AMR pathogens.

2. Methodology

2.1 Metagenomic data processing and analysis

The analysis pipeline utilized Albacore (v2.3.4) for base-calling FAST5 reads, followed by demultiplexing using qcat (v1.0.1) and adapter trimming with Porechop (v0.2.4). Prediction

of genes related to secondary metabolite biosynthesis and phage/host-phage functions, MG-RAST with an E-value cut-off of 1×10^{-5} and sequence identity of 60%, was employed using the KEGG database (Wilke et al., 2016). Taxonomy-based filtering using the RefSeq database in MG-RAST was focused mainly on bacteria to analyze genes associated with the biosynthesis of secondary metabolites. Additionally, for bacteriophages and their associated functions, viral reads were taken into consideration, followed by mapping their functions in Subsystems and KEGG databases of MG-RAST. The R packages phyloseq were used for alpha diversity and beta diversity analysis (McMurdie and Holmes, 2013).

2.2 Statistical Analysis

The samples were grouped on the basis of the season, i.e., the pre and post-monsoon, to estimate the alpha and beta diversity at the functional level. For alpha diversity, the richness and evenness were estimated using Shannon and Simpson's index. Beta diversity was estimated using the Bray-Curtis dissimilarity measure. The significance was evaluated using the Permanova (Adonis test, p-value threshold of 0.05) and betadisper test (p-value cut-off of 0.05). Data distribution was examined using the Shapiro-Wilk normality test (p-value cut-off of 0.05) and Q-Q plot. STAMP (Statistical Analysis of Metagenomic Profiles) (Parks et al., 2014) was employed to compare significant features grouped by seasons. Statistical significance assessment was ascertained with Welch's t-test (p-value threshold of 0.05) coupled with Benjamini-Hochberg FDR for multiple test corrections.

3. Results and Discussion

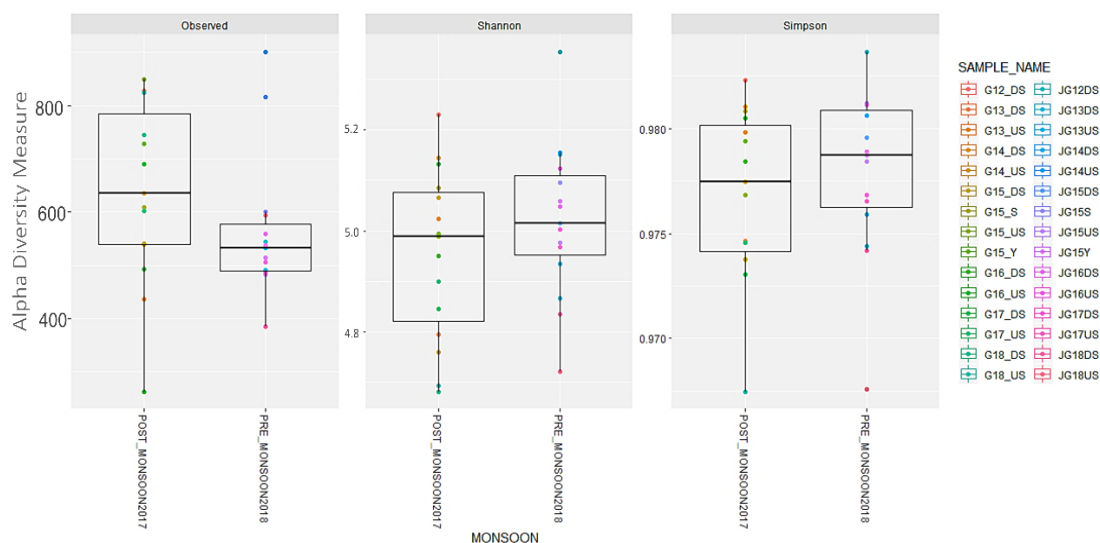
3.1 Bacteriophage diversity and functions

Recent advancements in metagenomics have significantly contributed to expanding our understanding of the virosphere in natural waters. Studies on aquatic virome have uncovered substantial genetic complexity and diversity of phages and their applications as therapeutics (Dolja and Koonin, 2018; Roux et al., 2012; Y. Z. Zhang et al., 2018; Zhang et al., 2019). River Ganges has been long known for its self-cleansing and non-putrefying properties, attributed primarily to its bacteriophages (phages) (Khairnar, 2016b). However, this river remains under-explored for the phage diversity across its anthropogenically impacted stretch. Targeting this facet, we annotated our data using MG-RAST to unveil the abundance and associated functions of the bacteriophage diversity. Analysis of the phage diversity was done with respect to the

season as the explanatory variable and phage diversity as a response variable, using observed indices, the Shannon function, and the Simpson index. Although pre-monsoon showed a lower phage diversity, as evidenced by the observed metric, it was not significant ($p>0.05$). In terms of richness and evenness of the dominant and rare types of bacteriophage, a relatively higher bacteriophage diversity was observed during the pre-monsoon season (**Fig. 1A**). Previous studies on species diversity, richness and evenness have suggested a relatively higher viral diversity in pre-monsoon due to the increased prevalence of available hosts, reduced run-off of river water, and increased nutrient availability (Jasna et al., 2018; Kristensen et al., 2010; López-Bueno et al., 2009).

Further analysis revealed the presence of bacteriophages from *Siphoviridae*, *Myoviridae*, and *Podoviridae* families against antibiotic, metal-resistant bacteria such as *Pseudomonas*; *Burkholderia*, *Stenotrophomonas*, *Cupriavidus*, *Pandoraea*, and *Ralstonia*. Besides, the presence of phages against several potential pathogens belonging to the ESKAPE group was also noted (**Fig. 1B**).

A



B

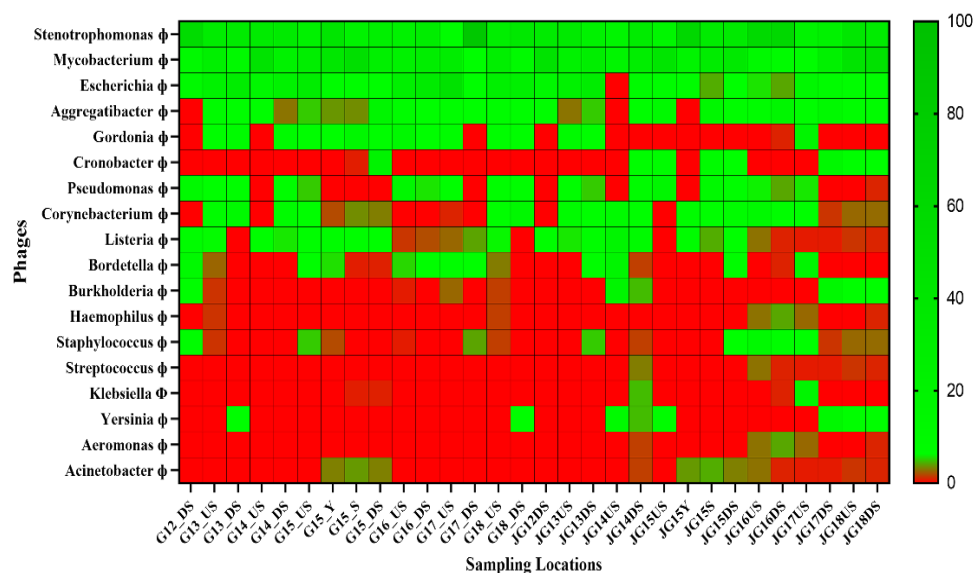


Fig. 1: Bacteriophage diversity along the lower stretch of the River Ganges. A: Elucidation of alpha diversity of bacteriophages with richness and diversity estimators. **B:** Bacteriophage diversity against potentially pathogenic bacteria at the human-animal-environmental interface.

Additionally, we grouped the host-phage data based on the occurrence of phages against putrefying bacteria, human pathogens, and plant pathogens (**Table 1**).

Table 1: A comprehensive table showing the presence of phages and their cognate host across the lower stretch of the River Ganges.

HOST	PHAGE
Putrefying pathogenic bacteria	
<i>Vibrio cholerae</i>	Vibrio phage
<i>Shigella</i> sp.	Shigella phage
<i>Enterobacter cloacae</i>	Enterobacteria phage
<i>Klebsiella pneumoniae</i> , <i>K. oxytoca</i>	Klebsiella phage
<i>Salmonella</i> sp, <i>S. enterica</i> , <i>S. Typhimurium</i>	Salmonella phage
<i>Clostridium</i> sp.	Clostridium phage
<i>Bacillus</i> sp.	Bacillus phage
<i>Campylobacter jejuni</i> , <i>Campylobacter</i> sp.	Campylobacter phage
Human Pathogens	
<i>Escherichia coli</i> , <i>Escherichia</i> sp.	Escherichia phage
<i>Aeromonas</i> sp.	Aeromonas phage
<i>Acinetobacter baumannii</i> , <i>Acinetobacter</i> sp.	Acinetobacter phage
<i>Pseudomonas aerogenes</i> , <i>P. putida</i> , <i>Pseudomonas</i> sp.	Pseudomonas phage
<i>Staphylococcus aureus</i>	Staphylococcus phage
<i>Streptococcus</i> sp.	Streptococcus phage
<i>Corynebacterium</i> sp.	Corynebacterium phage
<i>Listeria</i> sp.	Listeria phage
<i>Bordetella</i> sp.	Bordetella phage
<i>Burkholderia</i> sp.	Burkholderia phage
<i>Stenotrophomonas maltophilia</i>	Stenotrophomonas phage
<i>Mycobacterium</i> sp.	Mycobacterium phage
<i>Haemophilus</i> sp.	Haemophilus phage
<i>Aggregatibacter</i> sp.	Aggregatibacter phage
<i>Gordonia</i> sp.	Gordonia phage
<i>Cronobacter</i> sp.	Cronobacter phage
<i>Yersinia</i> sp.	Yersinia phage

Plant pathogens

<i>Xanthomonas oryzae</i> , <i>X. campestris</i>	Xanthomonas phage
<i>Xylella</i> sp.	Xylella phage
<i>Pseudomonas syringae</i>	Pseudomonas phage
<i>Ralstonia solanacearum</i>	Ralstonia phage
<i>Erwinia</i> sp.	Erwinia phage
<i>Clavibacter</i> sp.	Clavibacter phage

Based on this information, the River Ganges can be explored to isolate bacteriophages against various human and plant pathogens.

Furthermore, to comprehend phage-related and host-phage-linked functions in this riverine system, predictive *in-silico* functional annotation of the datasets was conducted using Subsystems and KEGG databases in MG-RAST. Analysis revealed the presence of phage-associated functions pertaining to their lifecycle, biological process, molecular function, and structural assembly (**Fig. 2A**). A predominance of integrases, excisionases, and prophage sequences was observed, suggesting the presence of phages with a temperate lifestyle (Howard-Varona et al., 2017). The prevalence of hits against phage-related proteins indicates a strong presence of a host-phage relationship. Interestingly, besides lysogenic lifestyle, our study also found hits for sequences related to phage-associated lytic activities (lysozyme, endolysin, N-acetylmuramoyl-L-alanine amidase, endopeptidases), replication and assembly-related proteins and enzymes (**Fig. 2B**).

These enzymes can be further used for bioprospection in therapy, diagnostics, and biotechnology-related applications. Also, phages and their enzymes obtained from this river can be used for the environmental remediation of other polluted rivers. Therefore, these findings are essential for mitigating various water-management and conservation policies to restore the Ganges River and other polluted rivers. As this is a preliminary study portraying phage diversity and its associated functions, further research is warranted to investigate and characterize the biological and genetic potentials of the phages for diverse applications.

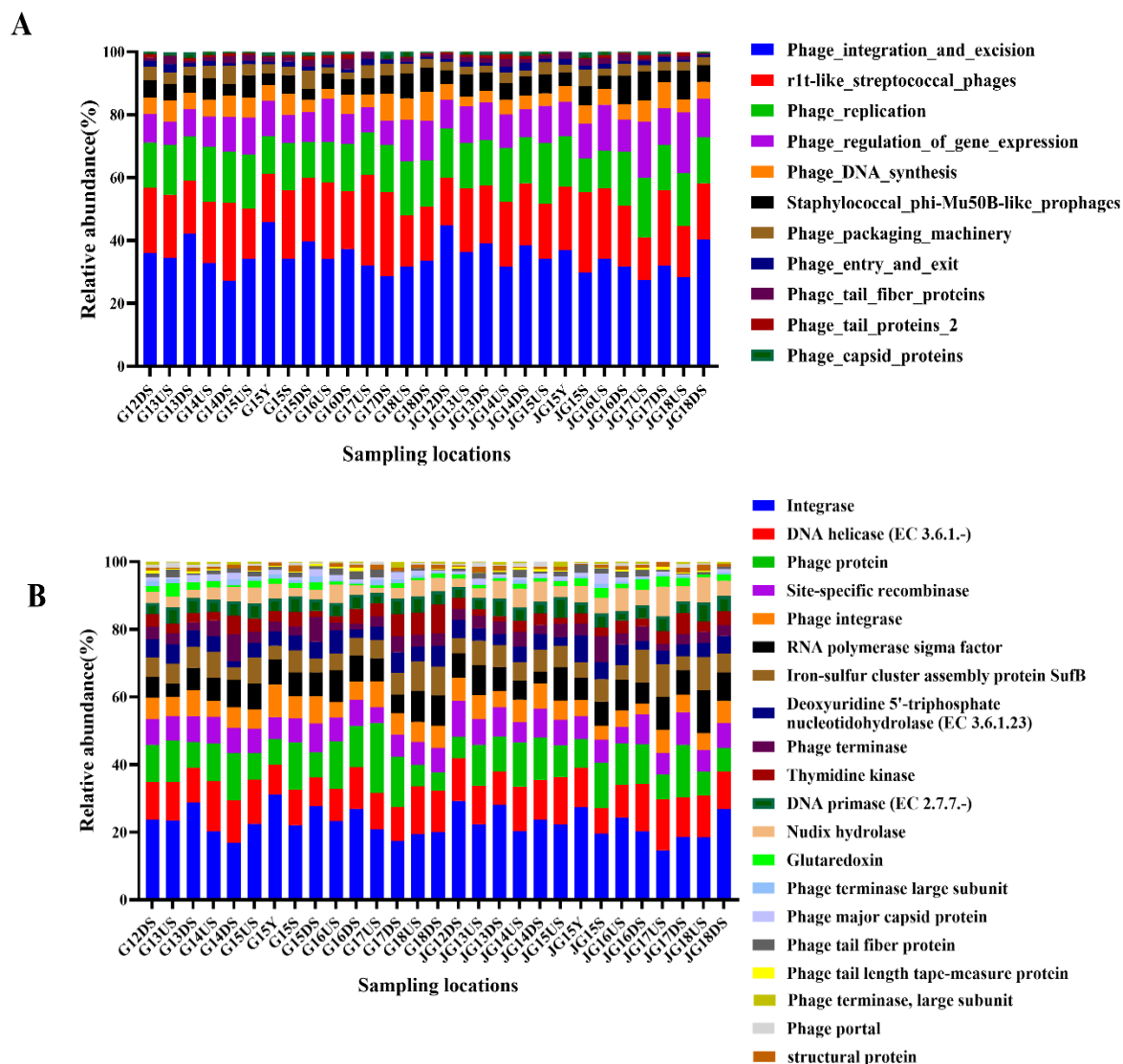


Fig. 2: Functional profile of the bacteriophage diversity across the lower stretch of the river Ganges. A: Predictive *in-silico* functional annotation of hits against host-phage-related proteins using MG-RAST. **B:** Predictive functions of phage-lifecycle-associated proteins and enzymes.

3.2 Taxonomic Diversity and Functions for Secondary Metabolite Biosynthesis

The taxonomic entities possessing genes for biosynthesis of secondary metabolite production were mapped to fourteen bacterial phyla. The major phyla contributing to the antimicrobial-linked biosynthetic gene cluster were *Proteobacteria* (55.6%), followed by *Actinobacteria* (36%), *Firmicutes* (11.8%), and *Bacteroidetes* (2.7%) (**Fig. 3**). Amongst the diverse phyla examined to date in aquatic and rhizospheric biomes, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, *Planctomycetes*, and *Cyanobacteria* have emerged as promising sources of antimicrobial compounds (Barka et al., 2016; Mendes et al., 2013; Mullis et al., 2019; Srinivasan et al., 2021). This observation suggests the substantial presence and involvement of these phyla in the biosynthesis of antimicrobial compounds within the riverine environment.

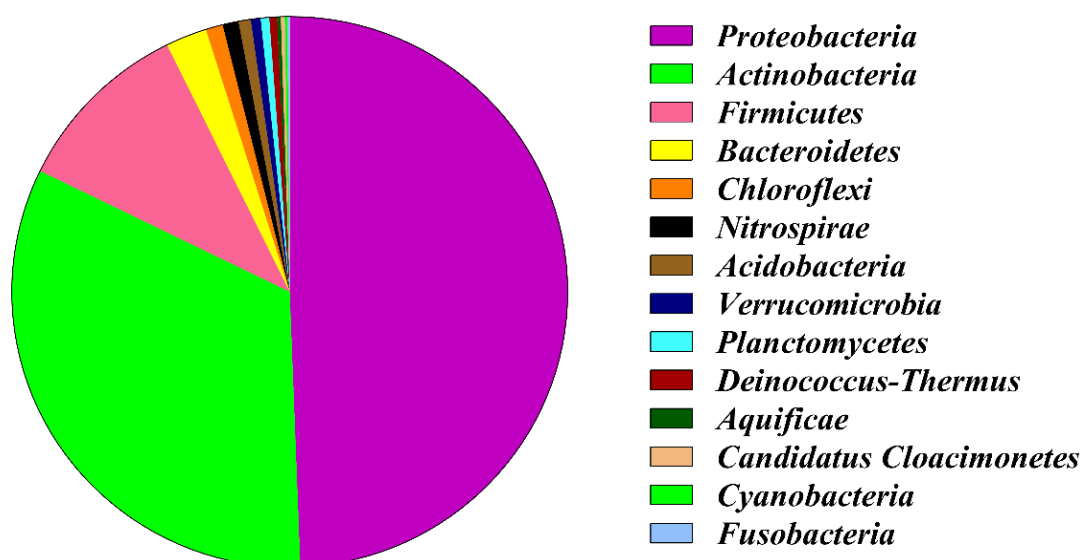


Fig. 3: Abundance of secondary metabolite biosynthesis-associated taxa.

The metagenomic analysis of diverse microbial communities in freshwater environments provides a platform for uncovering novel metabolic genes (Besemer et al., 2013). Analysis revealed several metabolic functions like metabolism of amino acids, carbohydrates, energy, glycan synthesis, lipids, cofactors and vitamins, biosynthesis of secondary metabolites, terpenoids, and polyketides, nucleotide and xenobiotic biodegradation and metabolism (**Fig. 4A**). However, to gain deeper insights and provide scientific reasoning toward the remarkable healing properties of the Ganges River, we focused our analysis primarily on the genes involved in the biosynthesis of secondary metabolite compounds. The analysis of secondary metabolites'

biosynthesis aspect annotated pathways for the biosynthesis of compounds that exhibit antimicrobial activity. There are only limited studies that have focused on the functional potentials of the microbiome (Behera et al., 2020; Rajput et al., 2022; Rout et al., 2024) and the special properties of non-putrefying (self-cleansing) and rejuvenation (Dutta et al., 2020; Dwivedi et al., 2020; Nautiyal, 2009; Rachel Samson et al., 2019) of the Ganges River. However, to the best of our knowledge, there are no reports on the Ganges River detailing the antimicrobial potentials of its microbiome in terms of secondary metabolite biosynthesis. Therefore, this data is crucial and would be helpful in the isolation of promising strains and the bioprospection of their antimicrobial potentials. Additionally, analysis of the differentially abundant metabolic features revealed biosynthesis of secondary metabolite function to be more prevalent in the pre-monsoon season (Fig. 4B). Certain studies have shown that an increase in the temperature influences the production of secondary metabolites, leading to an increase in the synthesis of antibiotics (Kuvarina et al., 2022; Pan et al., 2019). Therefore, we further analyzed our data with a specific focus on the gene clusters involved in the biosynthesis of secondary metabolites to ascertain the effect of temporal variation on its diversity.

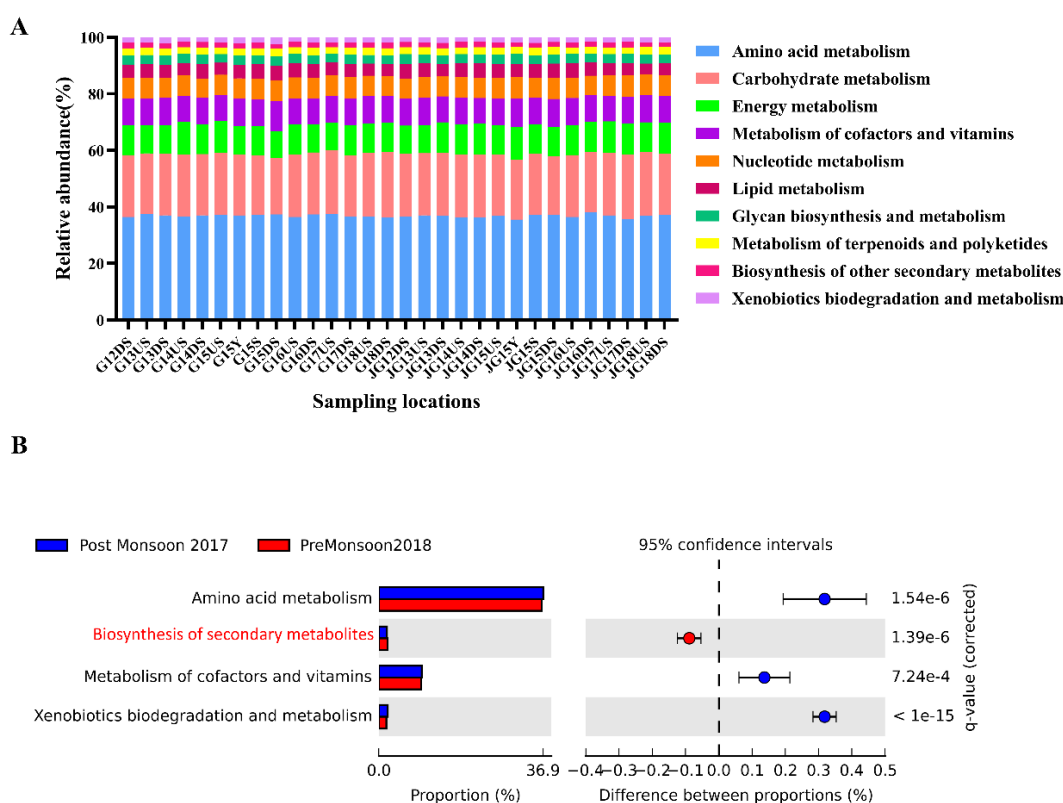


Fig. 4: Metabolic diversity along the lower stretch of the Ganges River. A: Predictive *in-silico* functional annotation of hits for overall metabolic diversity using MG-RAST. **B:** Differential abundance of significant metabolic function with respect to seasonal variation. The

extended error bar plot indicates the significant (Welsch's t-test, $p < 0.05$, Benjamini-Hochberg FDR corrections) group of metabolic features that vary with temporal fluctuations.

The alpha diversity of the genes involved in the biosynthesis of secondary metabolites using richness and diversity estimators like Observed metric, Shannon function, and Simpson index (**Fig. 5A**) revealed a considerably higher diversity in the pre-monsoon season. Additionally, the PCoA plot using NMDS matrices suggested a heterogeneous dispersion among the groups with a majority of variation at axis 1 (51.6%). Significant differences (Permanova, Adonis test $p < 0.05$) between the diversity of biosynthetic gene clusters were observed, suggesting a substantial effect of the seasonal variation (**Fig 5B**). These observations corroborate with previous findings, wherein higher temperatures above 15°C favor the synthesis of antimicrobial compounds to tackle the increased microbial competition (Bérđy, 2005; Kuvarina et al., 2022; Pan et al., 2019). This implies that the pre-monsoon season could be an ideal time for the bioprospection of microbes with a wide range of antimicrobial properties from the lower stretch of the Ganges River.

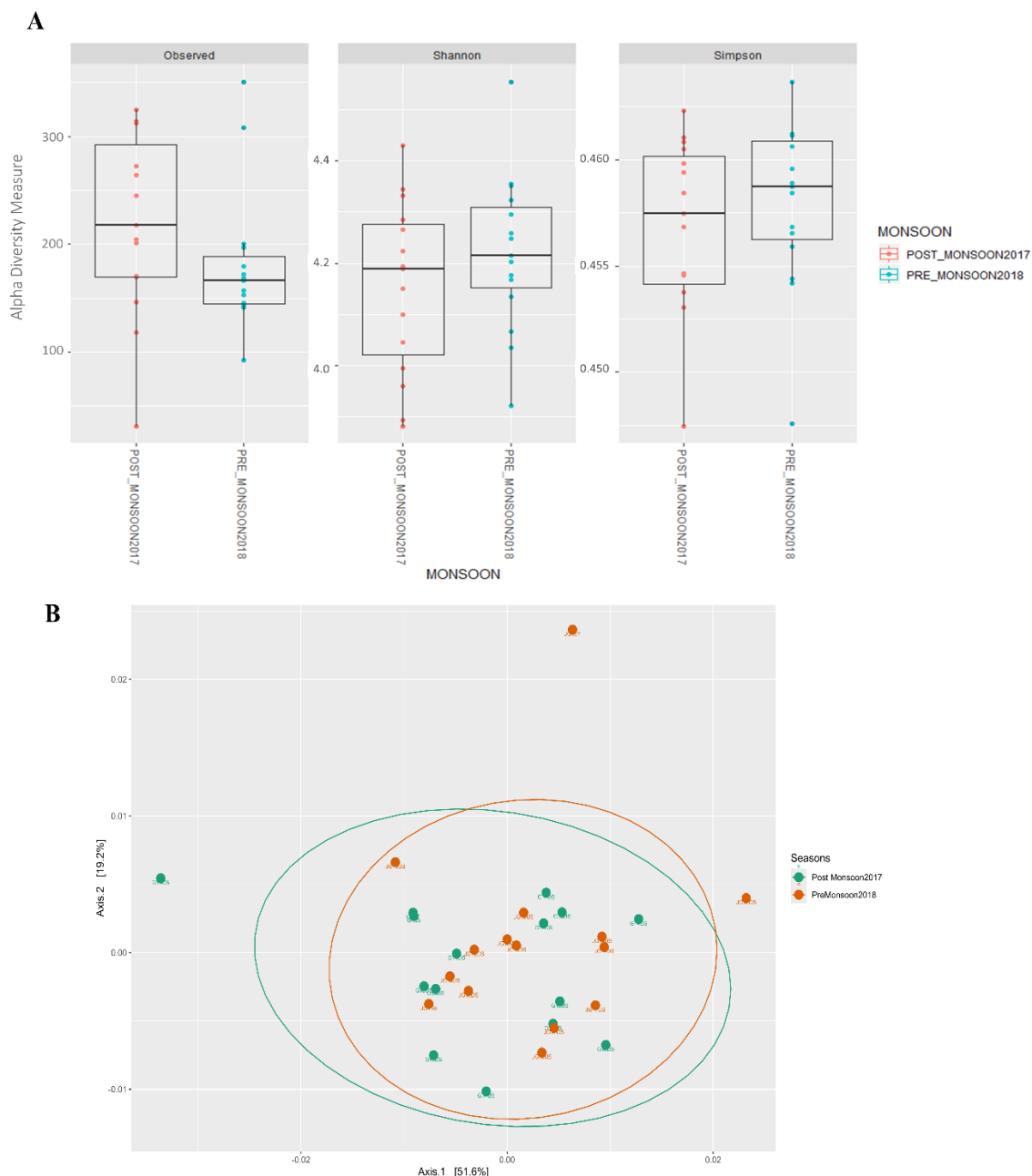


Fig. 5: Spatio-temporal diversity of biosynthetic genes (BSGs) involved in secondary metabolism. **A:** Box-plot illustrating alpha diversity of BSGs through diversity and richness estimators. **B:** PCoA plot with Bray-Curtis dissimilarity matrix depicting beta diversity. (Permanova, Adonis test, $p=0.03$, Beta-disper test- $p > 0.05$).

A significant fraction was assigned to Streptomycin biosynthesis ($65.41 \pm 10.11\%$), followed by phenylpropanoid biosynthesis (12.28 ± 5.93), and biosynthesis of penicillin and cephalosporins ($6.79 \pm 2.34\%$) (**Fig. 6**). The use of antibiotics like streptomycin, penicillin, and cephalosporins has been reported in the treatment of broad-spectrum bacterial infections in wounds, skin, and tissue. To date, there are limited studies that provide experimental validation

to substantiate the healing touch of the River Ganges (Khairnar, 2016c; Samson et al., 2023; Tyagi and Dubey, 2020). However, to the best of our knowledge, there are no reports that provide insights into the microbiome and the associated biosynthetic gene clusters in the River Ganges. Therefore, the presence of microbes with the potential to produce such antimicrobial secondary metabolites could attributed to one of the special properties of the River Ganges that could contribute to its healing touch.

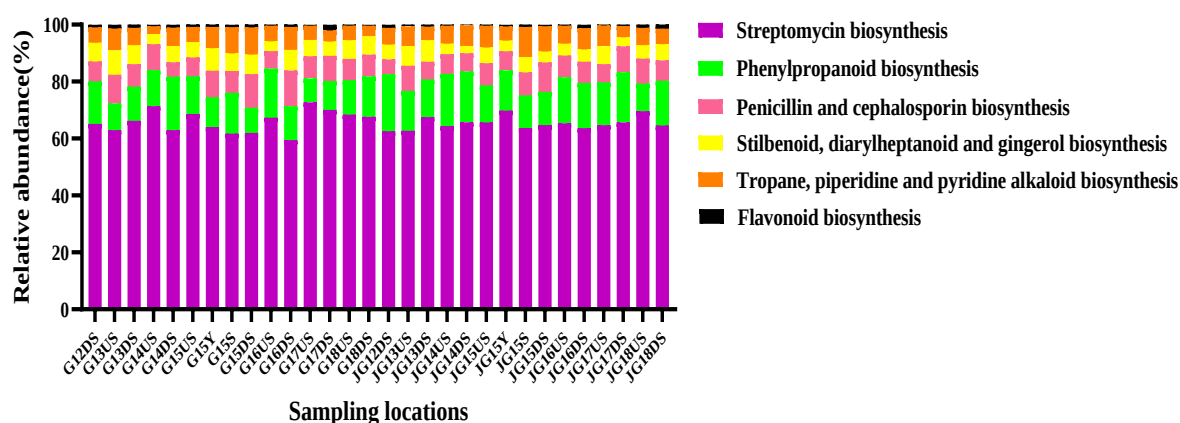


Fig. 5: Diversity of biosynthetic gene clusters with antimicrobial potentials along the lower stretch of the river Ganges.

4. Conclusion

The results underscore the microbial diversity within the Ganges River, showcasing the functional capacity of its bacterial diversity for synthesizing secondary metabolites. This data holds promise as a guide for bioprospecting and isolating microbes with antibacterial properties. Additionally, the diversity of bacteriophages displaying lytic activity against various potentially pathogenic bacteria was observed. These findings indicate the potential use of phages as emerging alternative therapeutics, aligning with the principles of one-health. This dataset contributes to our understanding of the unique properties of this ecosystem, suggesting its non-putrefying and rejuvenating characteristics. However, further investigations are necessary to fully elucidate the bioactive potential of the microbiota, including phages, to comprehensively grasp the promising capabilities of its microbial diversity and their role in shaping the unique properties of the Ganges.

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Chapter 3

*Metagenomic comprehension of the
microbiome and functions along the
pristine stretch of the glacier-fed Ganges
River*

3.1 Optimization of Total DNA Extraction and Amplification Methods

Abstract

Background

Glacier-fed-rivers (GFR) are unique environments with diverse microbial populations and ecological functions that are not fully understood. To gain deeper insights into the microbial processes and communities shaping GFR ecosystems, leveraging high-throughput sequencing is imperative. The Ganges River in India is a glacier-fed river; however, it remains largely unexplored for its microbiome along its pristine stretch encompassing its glacier origin.

Rationale

Only a few studies have focused on the microbiome and its functional potentials in the glacier-fed streams. However, there are no reports on the efficient method for extraction of DNA from the glacier-fed Ganges and its taxonomic profiling.

Results

In this study, fourteen different DNA extraction methods were evaluated to determine their effectiveness. Our results consistently demonstrated the inadequacy of established protocols designed for similar sample types in yielding sufficient and high-quality DNA, highlighting the challenging nature of glacier-fed rivers (GFRs). Notably, only one modified protocol from the DNeasy PowerMax soil kit (Qiagen) produced high-quality DNA, as indicated by the A260/A280 ratio. For increased DNA yields, the targeted amplification method employing the Long Amp enzyme proved to be optimal. Additionally, when assessing the amplification methods' influence on the taxonomic resolution of the lower-stretch Ganges sample, we found that only the Long Amp method showed a similar taxonomic profile with that of the native (non-amplified) DNA sample of lower stretch.

Significance

An efficient method for metagenomic DNA extraction and amplification method was developed to sequence the microbiome in samples with less DNA concentration, which could be helpful in the shotgun-based whole metagenomic analysis of other glacier-fed streams and forelands.

1. Introduction

The emergence of high-throughput sequencing technologies has revolutionized our ability to investigate microbial ecosystems in both extensively researched (Jansson and Hofmockel, 2018; Ji and Nielsen, 2015) and less explored environments (Hotaling et al., 2017; Jacobsen et al., 2012). Notably, less explored environments include high-altitude ecological niches, particularly glacier-fed streams. Among the latter are high-altitude ecosystems, notably glacier-fed streams that are undergoing rapid transformations due to climate change and shrinking glaciers, yet our understanding of their microbial diversity remains limited (Wilhelm et al., 2013). Sediments in glacier-fed streams and rivers pose extreme conditions with minimal microbial presence but significant mineral loads (Chanudet and Filella, 2008; Peter and Sommaruga, 2017; Wilhelm et al., 2013). River Ganges is the national River of India and is among the crucial glacier-fed rivers of the Himalayan ranges. The Ganges originates as River Bhagirathi (the source stream of the Ganges) from the Gomukh, the snout of the Gangotri glacier. It traverses ~2500 km and drains into the Bay of Bengal in the Sunderban Delta (Khairnar, 2016). However, the pristine stretch of the Ganges River remains unexplored for its total microbial diversity. High-resolution molecular techniques play a crucial role in delineating the extensive microbial diversity and associated functions, thereby enhancing our understanding of microbial ecology. However, to achieve this, it is imperative to extract nucleic acids of sufficient quantity and quality from such complex environmental samples (Roume et al., 2013).

Several studies have reported various protocols for extracting nucleic acids from different environments, providing valuable tools for researchers (Busi et al., 2020; Miller et al., 1999; Porebski et al., 1997; Xin and Chen, 2012; Zhou et al., 1996). However, only a few focus on sedimentary samples with very low microbial abundance typical of GFS/Rs (Busi et al., 2020; Wilhelm et al., 2013). Although nucleic acid extraction from glacial environments has been studied (Dancer et al., 1997), only one study has demonstrated their applicability to GFS sediments (Busi et al., 2020). A study by Lever et al. (2015) suggested that alongside low cell abundance, the complex mineral composition of GFS may influence extraction efficiency (Lever et al., 2015). Many studies have shown kit-based methods for DNA extraction and amplicon sequencing of GFS samples (Dancer et al., 1997; Peter and Sommaruga, 2017; Wilhelm et al., 2013). However, whole-genome shotgun sequencing requires a considerable amount of DNA input.

Amplifying less amount of DNA presents a fundamental hurdle in low-microbial load-related metagenomic or microbiome investigations due to potential biases of the sequencing coverage, guanine-cytosine (GC) composition, and alterations in species abundances. Certain rare specimens, such as challenging culturable microorganisms, genes from a single bacterium, and lesser DNA from fossil or museum specimens or polar environments, hold substantial scientific intrigue. However, current technologies are unable to sequence them without prior amplification (Pinard et al., 2006). One of the most widely used methods to overcome the bottleneck of minute quantities of DNA is whole genome amplification (WGA). The three primary types of WGA methods are degenerate oligonucleotide primed-polymerase chain reaction (DOP-PCR), the multiple displacement amplification (MDA) method, and multiple annealing and looping-based amplification cycles (MALBAC) (Liu et al., 2018). Prior investigations have reported that amplification approaches like MDA (Dean et al., 2002), primer extension pre-amplification PCR (Deleye et al., 2015), or DOP-PCR (Pinard et al., 2006) all introduce "considerable" levels of bias in comparison to unamplified controls (Liu et al., 2018). Recently, 1D Low input genomic DNA with PCR by Oxford Nanopore Technologies (ONT) was introduced for sequencing samples with less DNA concentration (Sanderson et al., 2018). However, this particular method has not been explored for sediment samples of GFRs.

In view of this, we have evaluated the efficacy of fourteen different DNA extraction methods to determine the most suitable approach for obtaining high-quality DNA from the sediments of the glacier-fed Ganges. Additionally, we have compared three different amplification methods with the unamplified control to understand the potential sequencing bias resulting from them in terms of taxonomic profiling. Therefore, the present study has attempted to deliver the most suitable method that yields anticipated diversity and taxonomic profiles, aiding in metagenome reconstruction. This study provides insights into nucleic acid extraction from sediments of the glacier-fed river, addressing methodological variability and laying the groundwork for future research.

2. Methodology

2.1 Metagenomic DNA Extraction and shotgun sequencing

Prior to the DNA extraction, the sediment samples from the left, center, and right banks of the River were pooled together according to the locations. Subsequently, the pooled samples from each location were subjected to DNA extraction. Efficient extraction of DNA from

sediments of glacier-fed aquatic ecosystems with low microbial load is challenging (Armbrecht et al., 2022; Guo et al., 2024). Therefore, we have evaluated fourteen different methods for the extraction of total metagenomic DNA from the sediments of the pristine stretch of the River Ganges. The details of the samples evaluated for DNA extraction are summarized in Table 1.

Table. 1: Details of the upper stretch samples processed and evaluated for total metagenomic DNA extraction with fourteen different classical and kit-based extraction methods.

Sample code	Location	Co-ordinates (Latitude (° N), Longitude (° E))
J18G1	Gomukh	30.73, 78.52
J18G2	Gangotri	30.92, 79.08
J18G3	Harshil	30.99, 78.94
J18G4	Maneri	31.03, 78.73
J18G5	Tehri	31.01, 78.71
J18G6	Rishikesh	30.75, 78.56

2.1.1 CTAB-based isolation of total metagenomic DNA

Cetyl–trimethyl–ammonium bromide (CTAB)-based modified protocol by Kachiprath et al., 2018 was evaluated for the extraction of metagenomic DNA from the sediments. The protocol is based on cell lysis through a CTAB-based buffer, followed by DNA purification with chloroform: isoamyl alcohol mixture, ensuring high-quality extraction.

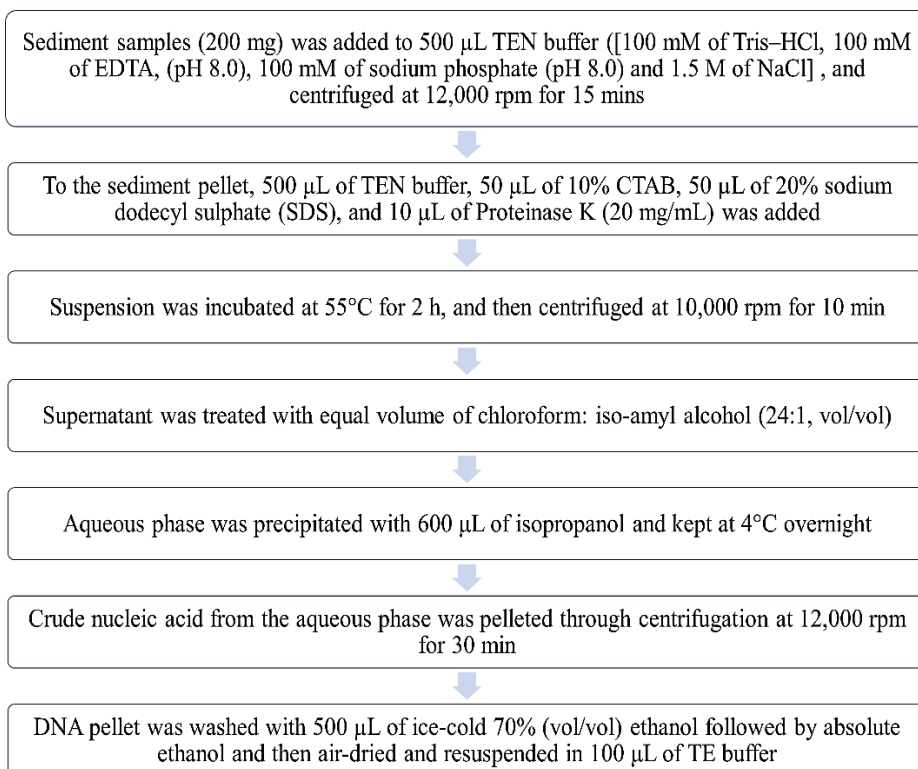


Fig. 1: A schematic flowchart illustrating the methodology of the CTAB-based isolation of the total metagenomic DNA.

2.1.2 CTAB-based isolation of total metagenomic DNA (Modified Protocol from Kachiprath et al., 2018). (CTAB_M)

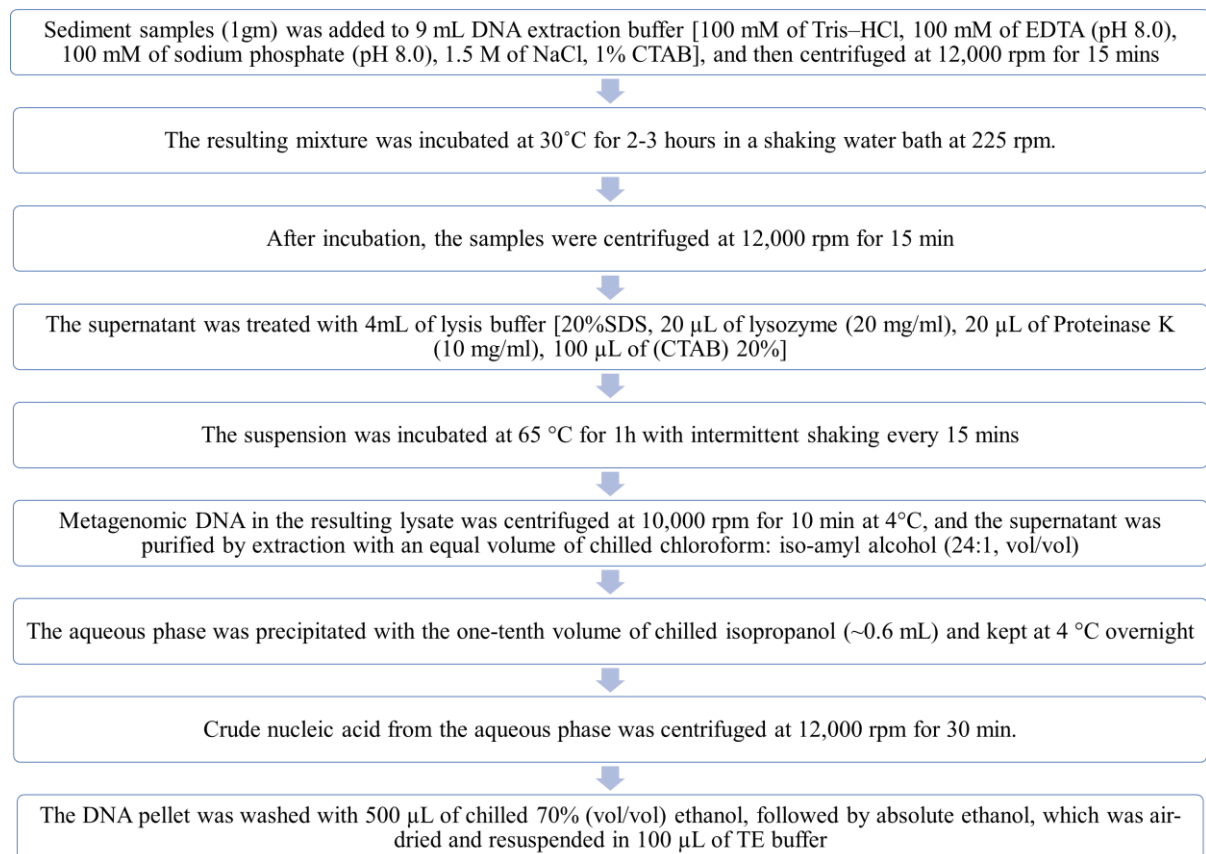


Fig. 2: A schematic flowchart illustrating the modified CTAB-based methodology for the isolation of the total metagenomic DNA.

2.1.3 MO BIO Powersoil Total RNA/DNA isolation kit (MoBio) (MSDP)

Total DNA was extracted from the samples using PowerSoil Total RNA Isolation Kit (MoBio) and PowerSoil DNA Elution Kit (MoBio) as per the manufacturer's instructions with certain modifications, which are illustrated in **Fig. 3**.

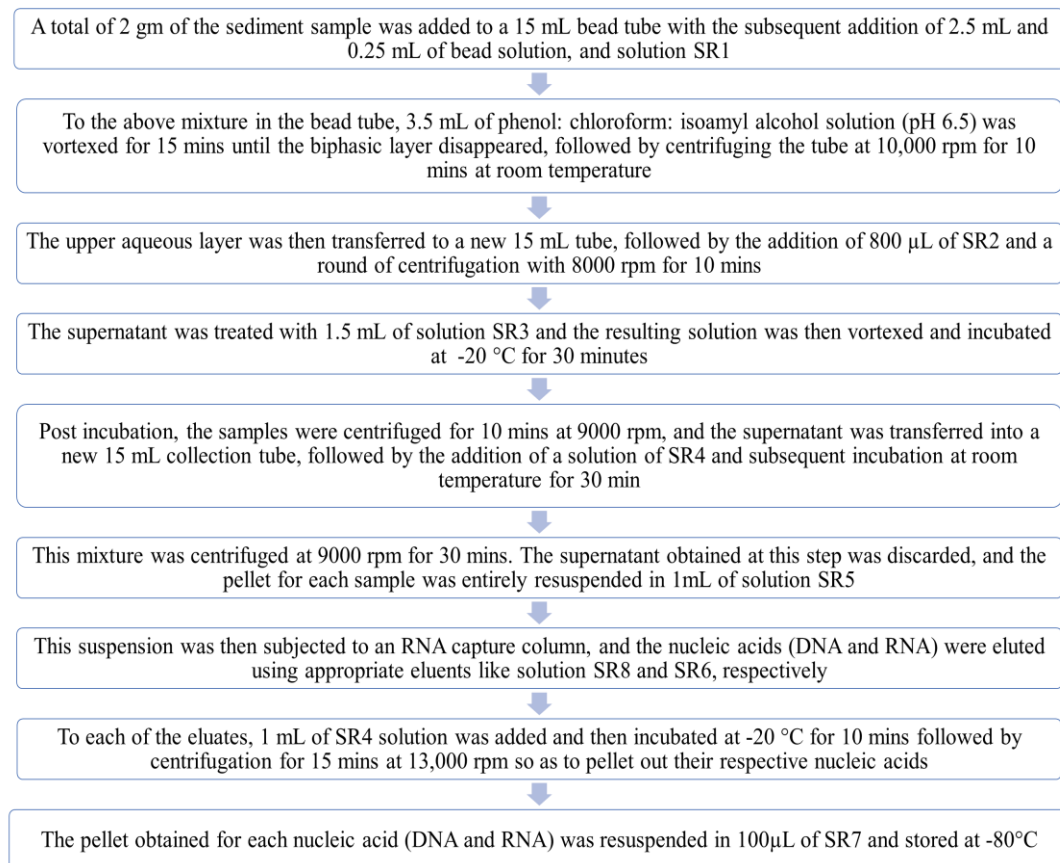


Fig. 3: A schematic flowchart depicting the methodology for isolating total RNA/DNA using the PowerSoil Total RNA Isolation Kit (MoBio) and the PowerSoil DNA Elution Kit (MoBio).

2.1.4 Direct Sediment with HipurA Bacterial Genomic DNA Purification Kit (HiMedia) (BGDP)

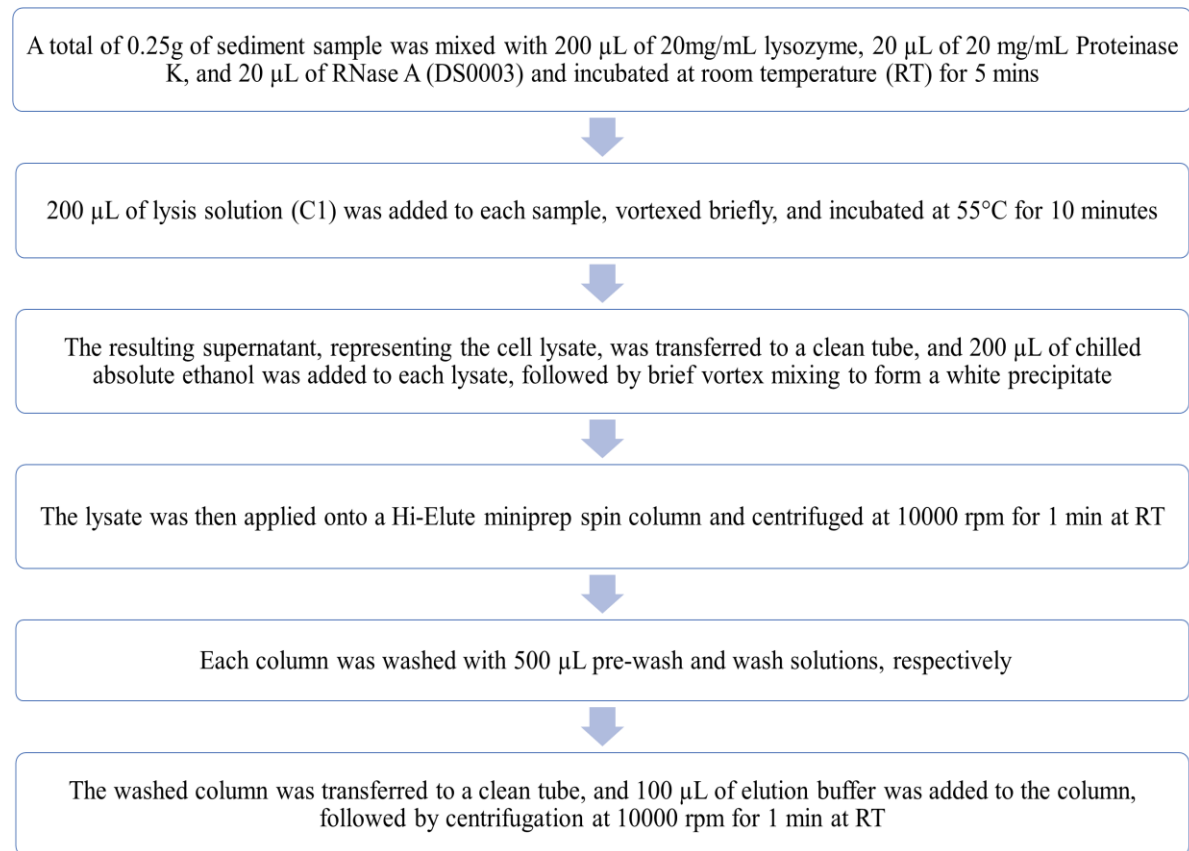


Fig. 4: A schematic flowchart depicting the methodology for isolating bacterial DNA using HipurA Bacterial Genomic DNA Purification Kit (HiMedia).

2.1.5 Sediment Suspension with HipurA Bacterial Genomic DNA Purification Kit (HiMedia) (Modified Protocol, BGDP_M)

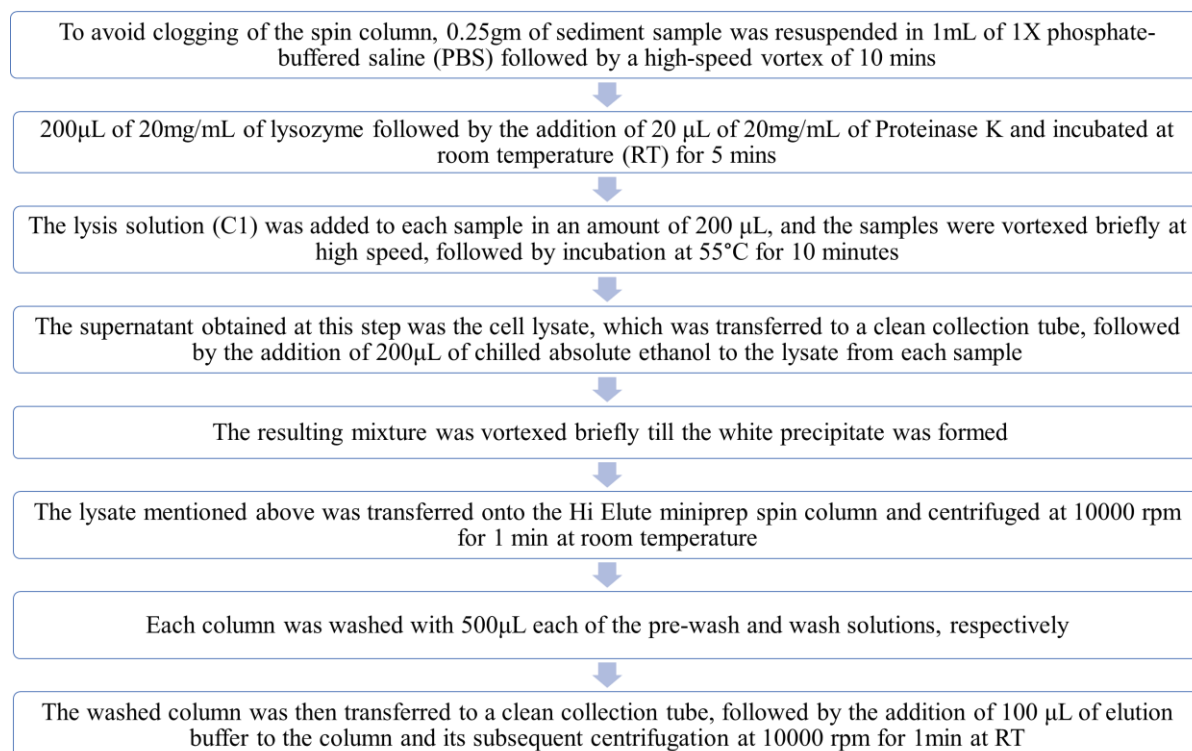


Fig. 5: A schematic flowchart depicting the modified methodology for isolating bacterial DNA using HipurA Bacterial Genomic DNA Purification Kit (HiMedia).

2.1.6 Direct Sediment Samples with DNA Xpress Reagent (Engineered Protocol) (DNA_XR)

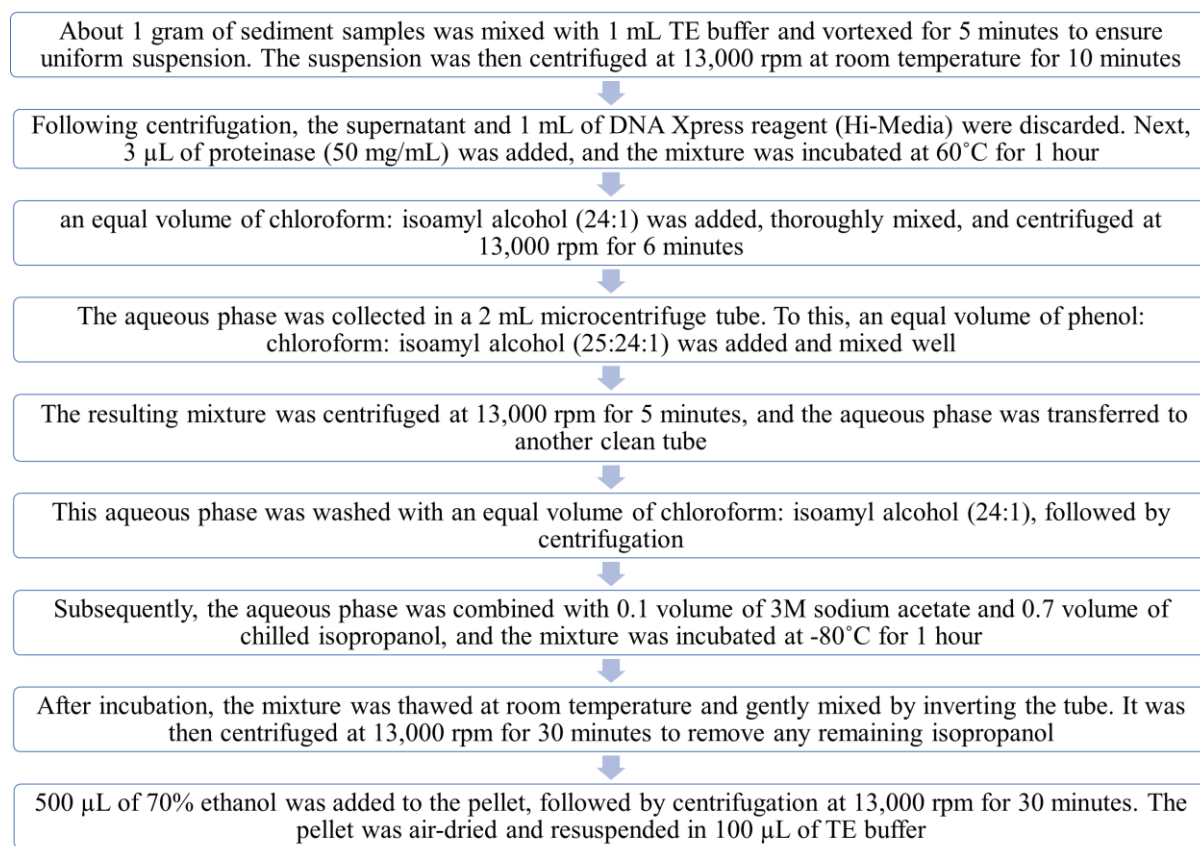


Fig. 6: A schematic flowchart portraying the engineered methodology for isolating metagenomic DNA using DNA Xpress Reagent (HiMedia).

2.1.7 DNA Xpress Reagent + SPECTL Buffer (Engineered Protocol) (DNA_XR+ SPECTL)

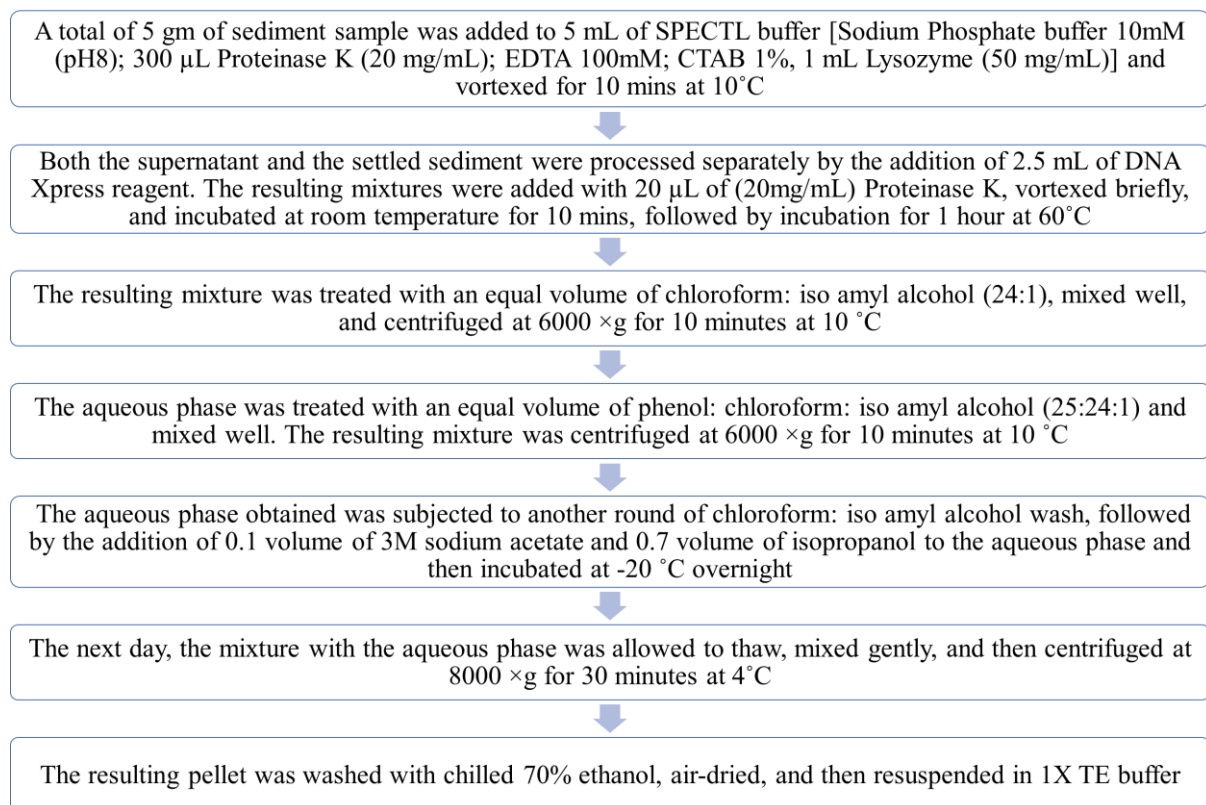


Fig. 7: A schematic flowchart portraying the engineered methodology for isolating metagenomic DNA using DNA Xpress Reagent (HiMedia) together with SPECTL buffer.

2.1.8 RNeasy Powersoil Total RNA Isolation Kit, RNeasy PowerSoil DNA Elution Kit (Qiagen), Rneasy PS

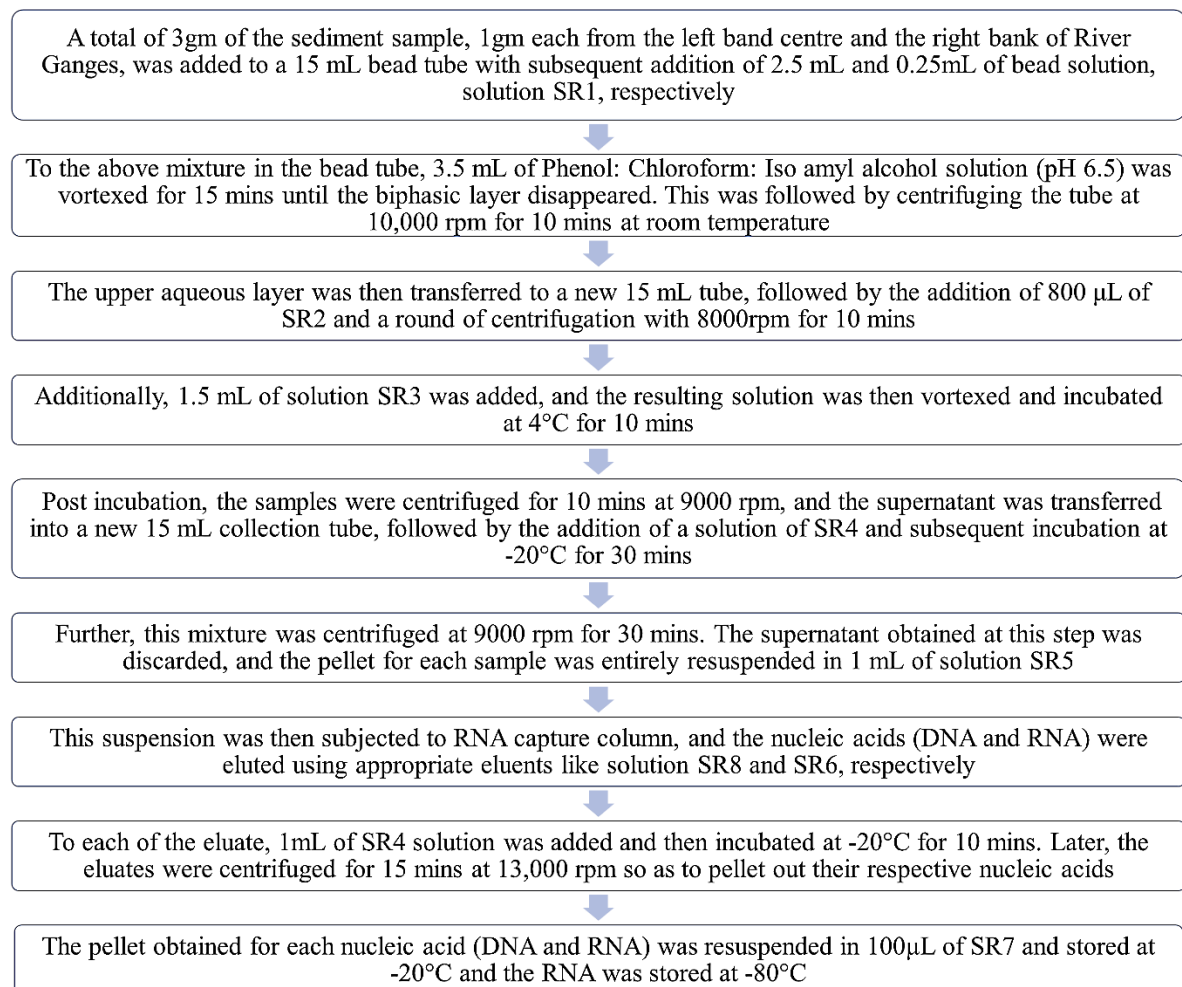


Fig. 8: A schematic flowchart detailing the methodology for isolating total metagenomic nucleic acids (DNA+RNA) using RNeasy Powersoil Total RNA Isolation Kit, RNeasy PowerSoil DNA Elution Kit (Qiagen).

2.1.9 Metagenomic DNA extraction with All Prep Viral DNA/RNA kit (Qiagen) (APV)

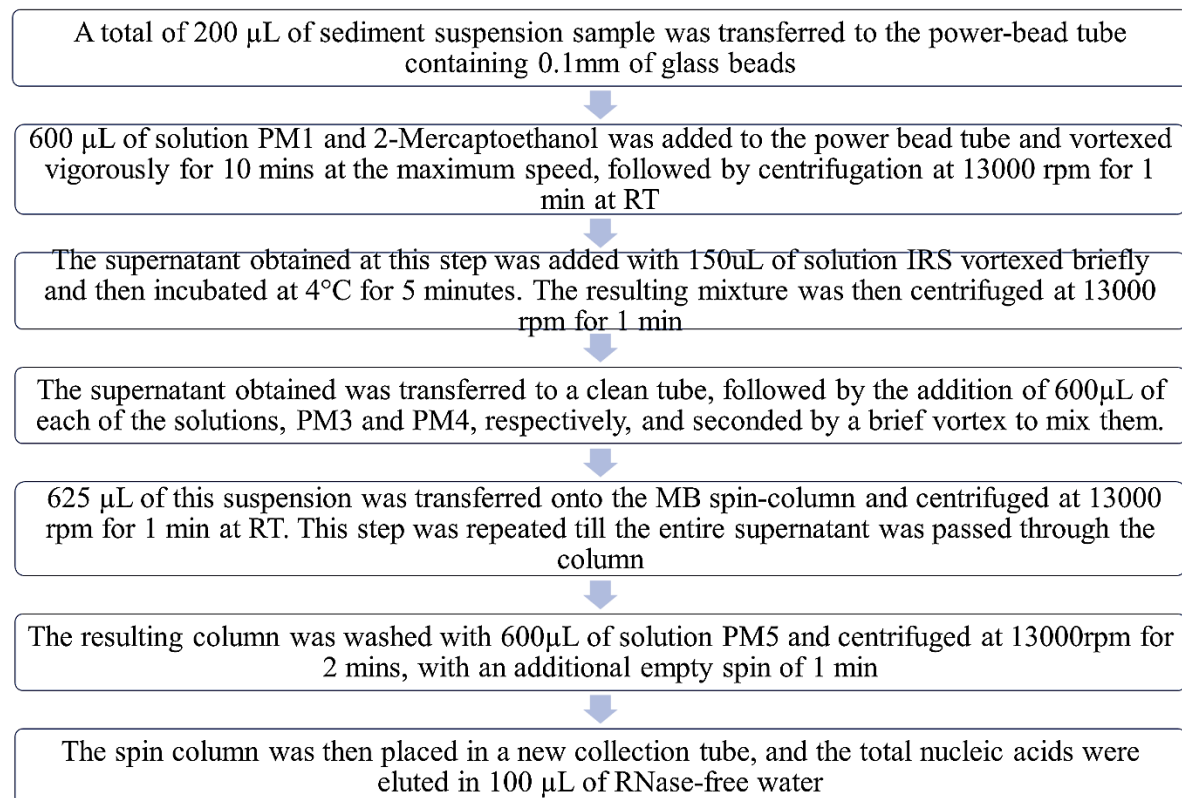


Fig. 9: A schematic flowchart detailing the methodology for isolating total metagenomic nucleic acids (DNA+RNA) using All Prep Viral DNA/RNA kit (Qiagen).

2.1.10 Metagenomic DNA extraction with All Prep Viral DNA/RNA kit (Qiagen) (Modified Protocol, APV_M)

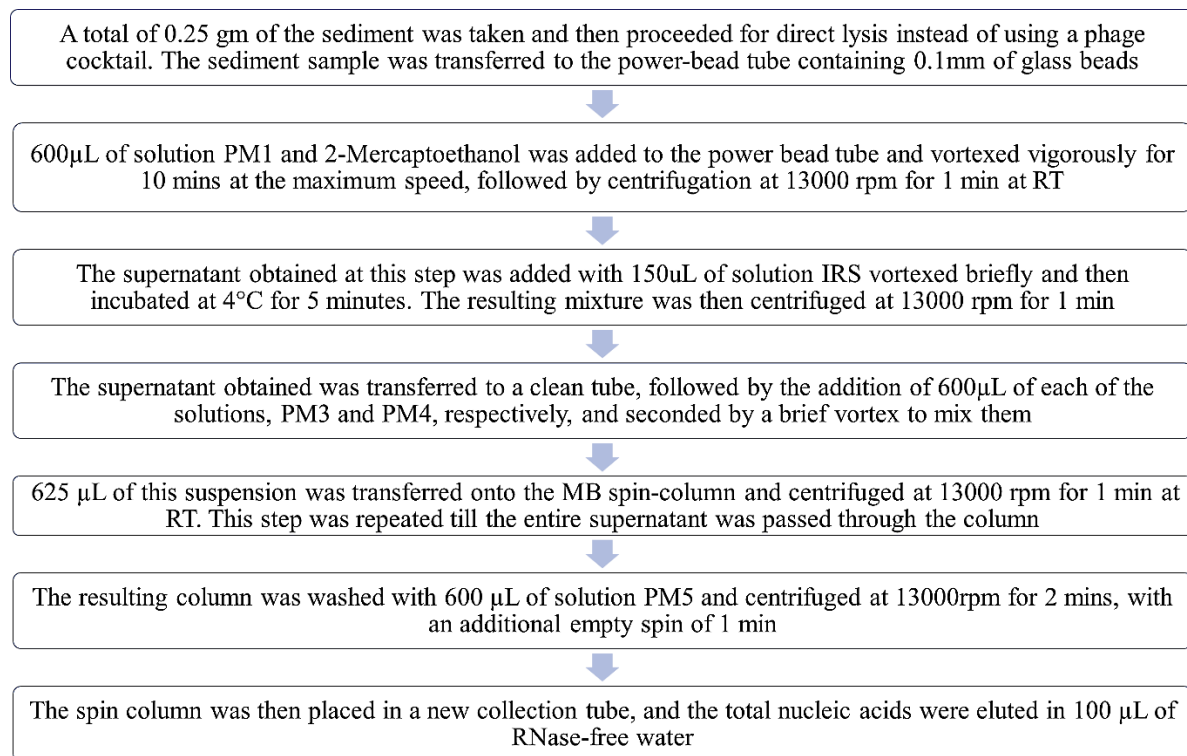


Fig. 10: A schematic flowchart detailing the modified methodology for isolating total metagenomic nucleic acids (DNA+RNA) using All Prep Viral DNA/RNA kit (Qiagen).

2.1.11 DNA extraction with PureLink™ Viral RNA/DNA Mini Kit (Thermo Fisher Scientific) (PL)

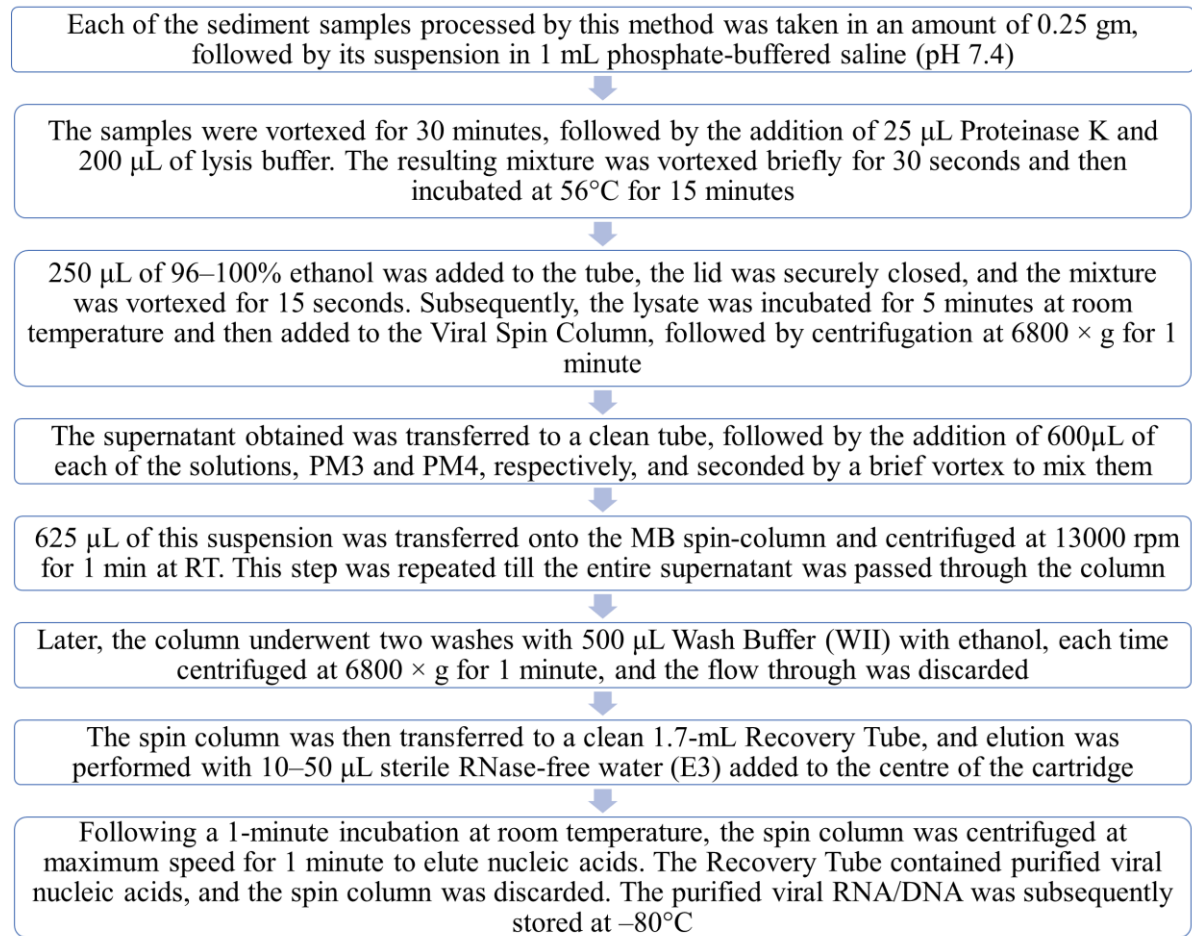


Fig. 11: A schematic flowchart describing the methodology for isolating total metagenomic nucleic acids (DNA+RNA) using PureLink™ Viral RNA/DNA Mini Kit (Thermo Fisher Scientific).

2.1.12 DNA extraction with PureLink™ Viral RNA/DNA Mini Kit (Thermo Fisher Scientific) (Modified Protocol, PL_M)

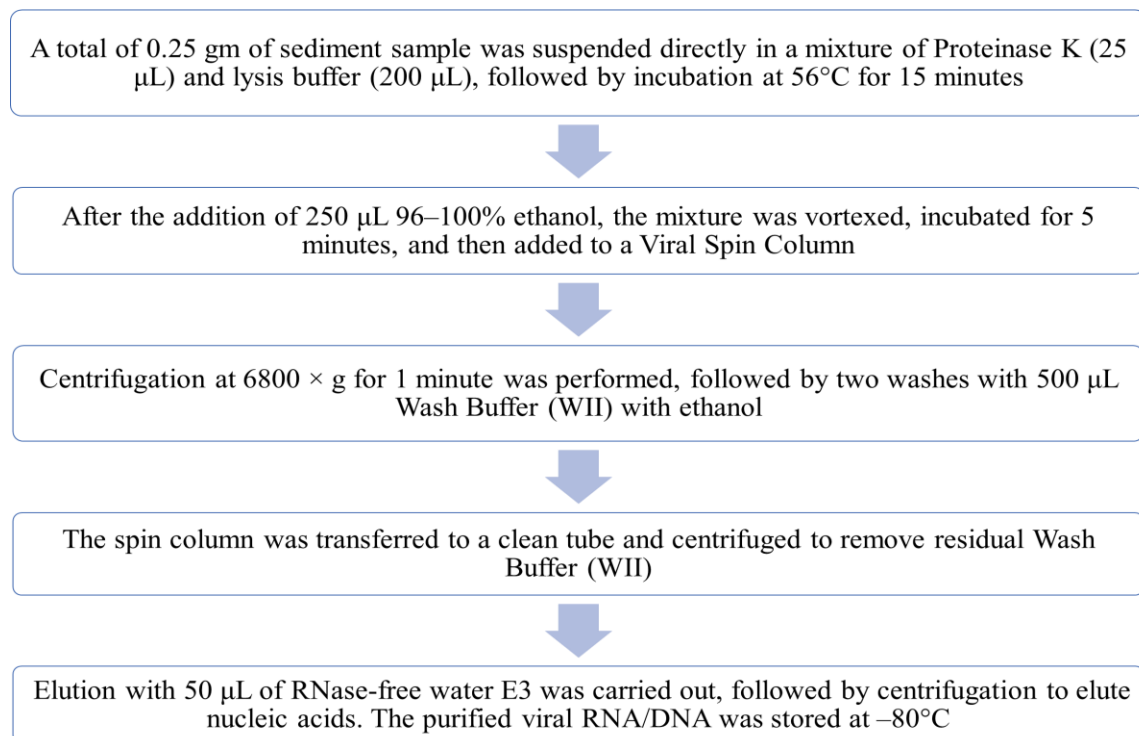


Fig. 12: A schematic flowchart describing the modified methodology for isolating total metagenomic nucleic acids (DNA+RNA) using PureLink™ Viral RNA/DNA Mini Kit (Thermo Fisher Scientific).

2.1.13 DNeasy PowerMax Soil Kit (Qiagen) (Pmax)

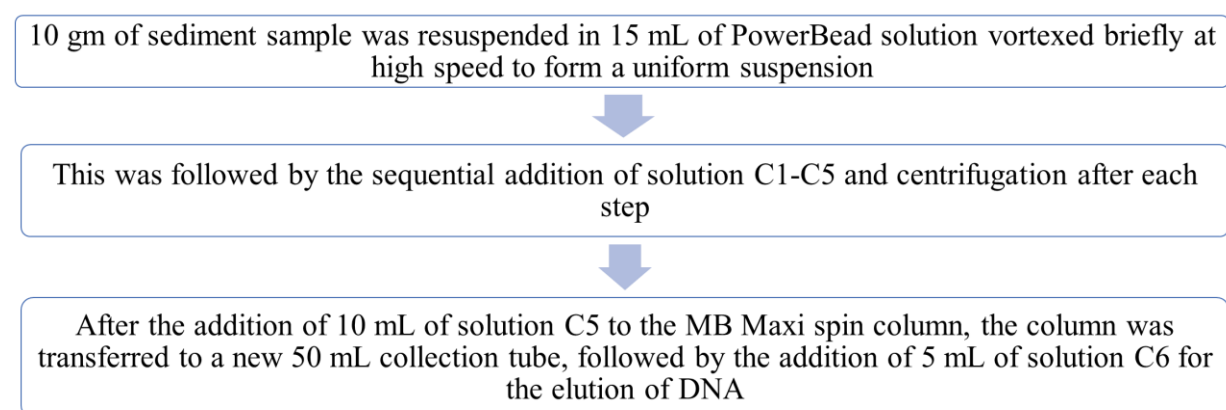


Fig. 13: A schematic flowchart illustrating the modified methodology for isolating total metagenomic DNA using DNeasy PowerMax Soil Kit (Qiagen).

2.1.14 DNeasy PowerMax Soil Kit (Qiagen) (Modified Protocol, Pmax_M)

In the modified protocol for the extraction of DNA from 10 grams of sediment, during the elution step, 1 mL of chilled C6 solution was placed onto the column and left at 4°C for 5-15 minutes, followed by elution of the DNA.

2.2 In-situ DNA amplification strategy

In-situ DNA amplification strategies were employed using Repli-G, MALBAC, and Long Amp methods to meet the required quantity of ~900 ng for nanopore sequencing.

2.21 Multiple Displacement Amplification Approach with Repli-G (Qiagen)

Approximately 10 ng of template DNA was combined with buffer D1 in a micro-centrifuge tube and incubated at room temperature for 3 minutes. Buffer N1 was then added to denature the DNA, followed by the addition of a master mix prepared on ice as per the manufacturer's protocol. The resulting solution was incubated at 30°C in a thermocycler for 16 hours, and the amplified product was heat-inactivated at 65°C for 3 minutes.

2.22 Quasi-linear based WGA Approach with MALBAC (Yikon Genomics)

Less than 1 µg of DNA was subjected to pre-amplification and exponential amplification steps. In the pre-amplification step, the pre-amp mix was added to the DNA sample and incubated in a thermocycler under specific conditions. This was followed by exponential amplification, where an amplification mix was added to the pre-amplification product and incubated on a thermocycler under specified conditions.

Pre-amplification: A total of 30 µL of the pre-amp mix was added to up to 5 µL of the DNA sample, centrifuged briefly, and incubated in a thermocycler.

Conditions of thermocycler

Cycles	Temperature (°C)	Time (s)
1	94	180
	20	40
	30	40
	40	30
	50	30
8	60	30
	70	240
	95	20

	58	10
1	4	∞
		82 min

Exponential amplification: An amplification mix of 30 μ L was added to 35 μ L of pre-amplification product and centrifuged briefly. The reaction mix was then incubated on a thermocycler following reaction conditions:

Conditions of thermocycler

Cycles	Temperature ($^{\circ}$ C)	Time (s)
1	94	30
	94	20
17	58	30
	72	180
1	4	∞
		74 min

2.23 Targeted amplification with 1-D Low input genomic DNA with PCR (SQK-LSK108), Oxford Nanopore Technologies

Amplification of whole metagenomic DNA using 1-D Low input genomic DNA with PCR (SQK-LSK108) involved transferring 100 ng of DNA into a microcentrifuge tube and subjecting it to an end repair step. The resulting DNA fragments were quantified using a Qubit fluorimeter and then subjected to adapter ligation using Ligation Sequencing Kit 1D (SQK-LSK108) (Oxford Nanopore Technologies, Oxford, UK). The adapter-ligated DNA was amplified using Long Amp Taq2X master mix (New England Biolabs), and the resulting product was purified using Ampure XP beads (Beckman Coulter).

2.3 Comparative assessment of the amplification methods and sequencing

The low DNA yield from sediments of upper stretch samples posed a significant sequencing challenge as compared with lower stretch samples, which yielded ample DNA. Therefore, DNA amplification was carried out for upper stretch samples to overcome this obstacle. However, to avoid potential bias resulting from the amplification process and also to ascertain the reliability of the amplification method for downstream taxonomic and

functional profiling, we evaluated one of the unamplified lower stretch samples from our previous study (Samson et al., 2023).

In this, the concentration of the lower stretch sample was diluted to 100 ng to mimic the upper stretch samples and then amplified with RepliG (RG) (Qiagen), MALBAC (MB) (Yikon Genomics), and PCR expansion (EXP-PCA001-Oxford Nanopore Technologies) + Long Amp (LA) (NEB), following manufacturer protocols. Further, the amplified DNA samples were indexed with a 1D Native barcoding genomic DNA kit (EXP-NBD103 and EXP-NBD 113). The library was prepared using a Ligation Sequencing Kit (SQK-LSK109) with certain modifications, as mentioned in our previous study (Samson et al., 2023). The final library was loaded onto the FLO-MIN-106D-R9 flow cell (ONT) placed in the MinION sequencing platform, and the run was continued up to 56-72 hours.

2.4 Data processing and quality check

The base-calling of the raw reads was carried out with Albacore (v2.3.4). Subsequently, the fastq files were subjected to qcat software v1.1.0 (ONT) for demultiplexing. The adapter sequences were trimmed with the Porechop tool (v 0.2.1). The datasets were normalized with the DESeq2 package. The data quality and stats were assessed using Nanostats in NanoPlot. The normality of the datasets was evaluated with the Shapiro-Wilk normality test (p-value ≤ 0.05), confirmed using a Q-Q plot. Further, the data was rarefied using the rarefy function in R. These rarefied datasets were used for downstream taxonomic and functional analysis.

3. Results and Discussion

3.1 Evaluation of the optimal DNA extraction method

The fourteen methods evaluated in this study shared similar principles involving sample preparation, lysis of the microbial cells, purification, precipitation, and elution of the nucleic acids. The initial DNA extraction results suggested that a small amount of sediment input did not yield adequate DNA quantities for metagenomics. This could be attributed to the various complex and intricate mineral matrices in the glacier-fed-rivers due to erosion and a limited occurrence of microbial biomass in the sample quantity (Lever et al., 2015). Consequently, the DNA extracts from all protocols (excluding Qiagen's, which are already optimized for large scale) resulted in an A_{260}/A_{280} ratio of ~1.2-1.6 (**Fig. 14A**).

Although a substantial DNA yield was obtained, the agarose gel electrophoresis of these DNA extracts showed a considerably sheared and smeared DNA, indicating its low quality,

which could not be used further for sequencing. However, among all the methods, only one involving total metagenomic DNA extraction from 10 gm of sediment sample using the modified protocol of DNeasy PowerMax Soil Kit (Qiagen) yielded DNA with an A260/A280 ratio of ~ 1.8 and yield of 30-60 ng in 50 μL (**Fig. 14B**). The agarose gel electrophoresis imaging showed no visible signs of shearing or smearing of the DNA, indicating the absence of contaminating substances that could hinder the library preparation step involved in the next-generation sequencing (NGS) (Healey et al., 2014) (**Fig. 14C**). While high-quality DNA was obtained from the DNeasy PowerMax Soil Kit (Qiagen), the concentration was not sufficient to fulfill the nanopore sequencing requirements of $\sim 1 \mu\text{g}$ of the initial input DNA. Therefore, the amplification of the DNA was the preferred approach.

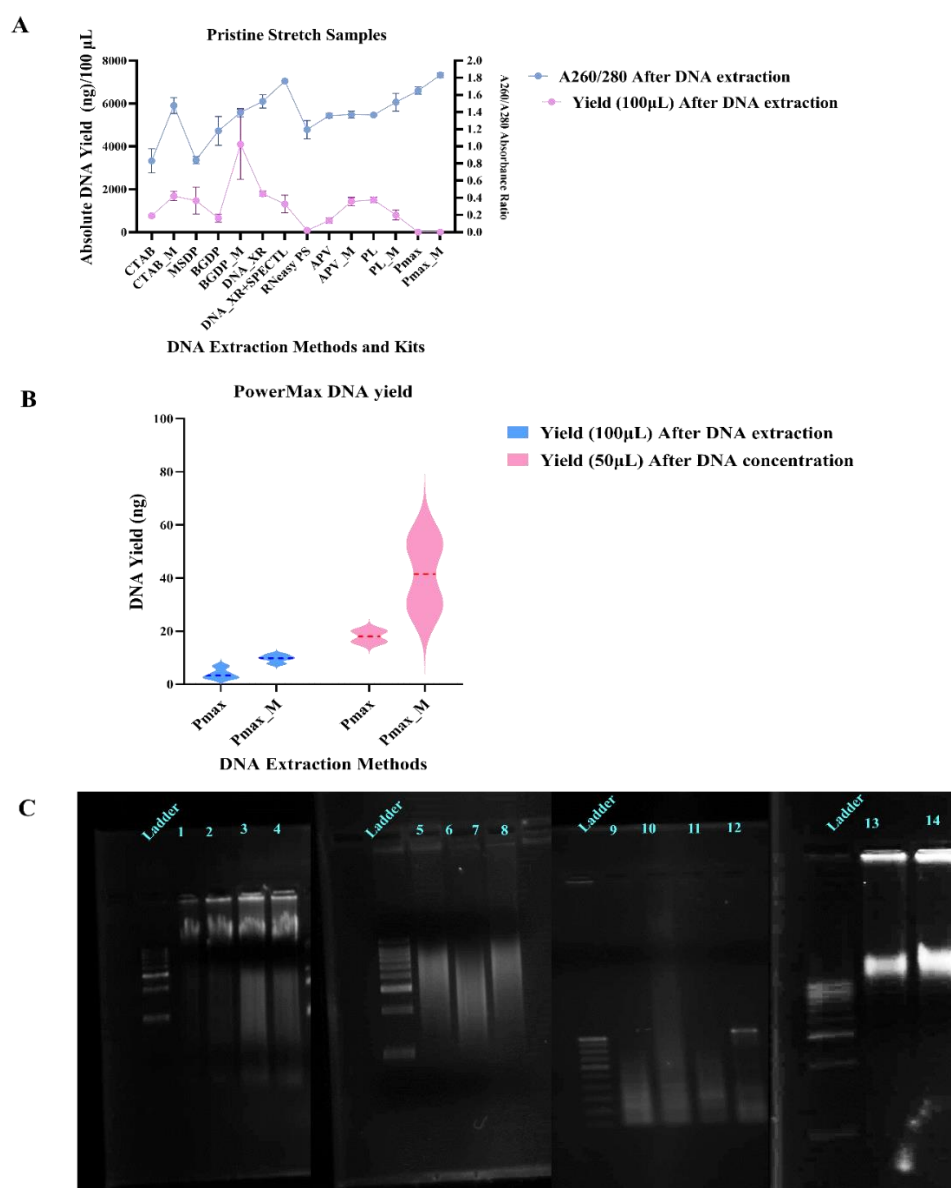


Fig 14: Evaluation of DNA concentrations obtained using different extraction protocols: A: The total yield of DNA (ng) in 100 μ L. B: Violin plots illustrating DNA yield (ng) after extraction using both the manufacturer's protocol and the modified protocol for the DNeasy PowerMax kit (Qiagen). C: Agarose gel electrophoresis image of DNA extracted using fourteen different classical and kit-based DNA extractions. Lanes 1-12 depict sheared and smeared DNA. Lanes 13 and 14 depict intact high-quality DNA.

3.2 Comparison of the amplification methods using a control sample

The MDA process of Repli-G involves isothermal amplification of the genomes. In this process, initially, random hexamers bind to denatured DNA, followed by Phi-29 polymerase-guided strand displacement synthesis (Pinard et al., 2006). While studies have shown the MDA method to have the least bias, its relative efficacy remains underexplored with complex metagenomic samples having low input of DNA (Ahsanuddin et al., 2017; Pinard et al., 2006). Recent studies have reported that MALBAC has less sequencing bias due to the introduction of a quasi-linear-amplification stage to the process (Chen et al., 2014; Liu et al., 2018). Amplification using MALBAC involves primers that are tailored to introduce particular sequences at the termini of the DNA amplicons, facilitating the formation of loops that do not undergo exponential copying. This looping strategy for amplicons helps alleviate the overrepresentation of more abundant templates, thereby diminishing amplification bias in contrast to MDA (Liu et al., 2018). However, certain studies have also reported considerably high positive rates and potential biases introduced by MALBAC with complex samples and those with high GC content (Hou et al., 2015; Voet et al., 2013).

Further, the 1D Low input genomic DNA with PCR (ONT) has primers that are complimentary to the adapters that are attached to the ends of the DNA during the end-repair step (Brown et al., 2017). Moreover, this protocol employs LongAmp® Taq DNA Polymerase (NEB), an optimized fusion of Taq and Deep Vent® DNA polymerases. This combination enables amplification of DNA fragments up to 30 kb, facilitating comprehensive amplification of the entire DNA sample ("LongAmp® Taq DNA Polymerase | NEB," n.d.). However, this particular amplification method has not been explored for amplification of metagenomic samples. Therefore, in order to understand the efficiency of these amplification methods and to apprehend the potential biases resulting from the amplification process, we initially evaluated one of the lower stretch samples from our previous study (Samson et al., 2023), having sufficient DNA concentration for direct sequencing on the MinION platform.

Subsequently, we adjusted its concentration to match that of the upper stretch samples by diluting it to 100 ng prior to its amplification using the aforementioned methods. The amplified samples were then sequenced on the MinION nanopore sequencing platform, followed by a comparative analysis with the native (unamplified) sample from our previous study.

Analysis of the bacterial diversity revealed no significant differences at the phylum level (**Fig. 15 A**). However, at the genus level, although the taxonomic profile of the top 10 bacterial genera appeared similar, significant differences (Two-Way ANOVA $p < 0.0001$) in the relative abundance were observed in the samples amplified by multiple displacement-based amplification methods of RepliG and MALBAC, respectively. The sample processed with a targeted amplification approach involving the use of PCR expansion (EXP-PCA001, ONT) coupled with Long Amp (LA) (NEB) showed a close taxonomic resemblance with the native sample (Two-Way ANOVA $p > 0.05$), as compared to RepliG and MALBAC. The genera that showed significant variation in terms of their abundance have been tabulated in **Table 2**.

Table 2: Comparative analysis of the relative abundance in percentage (%) of the top 10 bacterial genera that significantly differ across different amplification methods. G15S (unamplified sample), G15S_LA (Long Amp), G15S_RG (repliG), G15S_MB (MALBAC).

Bacterial Genera	G15S	G15S_LA	G15S_RG	G15S_MB
<i>Burkholderia</i>	14.39	14.20	7.83	6.02
<i>Geobacter</i>	13.11	12.04	24.84	23.94
<i>Methylibium</i>	8.36	9.53	1.57	12.02
<i>Anaeromyxobacter</i>	8.16	9.41	13.74	19.96
<i>Polaromonas</i>	7.56	7.09	3.69	2.30

Analysis of the archaeal diversity revealed a significant difference in the relative abundances at the phylum (Fig. 15C) as well as genus level (Fig. 15D) with the sample amplified by Repli-G and MALBAC method. The phyla and genera that showed significant variation in terms of their abundance have been tabulated in **Table 3**.

Table 3: Comparative analysis of the relative abundance in percentage (%) of the archaeal diversity at phylum and genus level that significantly differs with the amplification methods.

Archaeal Diversity	G15S	G15S_LA	G15S_RG	G15S_MB
Phyla				
<i>Thaumarchaeota</i>	62.42	65.68	80.98	37.98
<i>Euryarchaeota</i>	32.03	29.09	15.57	45.54
<i>Crenarchaeota</i>	5.11	4.65	3.28	15.68
Genera				
<i>Nitrosopumilus</i>	70.53	58.59	27.67	30.73
<i>Methanosarcina</i>	10.25	15.52	25.89	10.60
<i>Cenarchaeum</i>	7.46	10.79	21.85	9.30
<i>Thermococcus</i>	2.15	3.10	4.59	6.27
<i>Pyrococcus</i>	1.916	2.69	4.033	9.52

Moreover, analysis of viral diversity at the order and family level also revealed that the relative abundances of viral taxa from the LongAmp-based method aligned more closely with the unamplified sample (Fig. 15 E, F). However, the Repli-G and MALBAC methods displayed a significant variation, with a majority of reads from Repli-G being assigned to the unclassified viral fraction ($p < 0.01$). The viral order and families that showed significant variation in terms of their abundance have been tabulated in **Table 4**.

Table 4: Comparative analysis of the relative abundance in percentage (%) of the viral diversity at order and family level that significantly differs with the amplification methods.

Viral Diversity	G15S	G15S_LA	G15S_RG	G15S_MB
Order				
<i>Caudovirales</i>	59.11	54.01	16.66	66.29
<i>Herpesvirales</i>	34.25	32.84	6.4	3.3
<i>Unclassified</i>	5.52	10.94	75.64	28.09
Family				
<i>Mimiviridae</i>	27.10	32.65	6.66	25
<i>Siphoviridae</i>	23.49	21.42	8	7.5
<i>Podoviridae</i>	13.25	15.30	1.33	10
<i>Myoviridae</i>	12.65	12.24	61.33	32.5
<i>Mimiviridae</i>	27.10	32.65	6.66	25

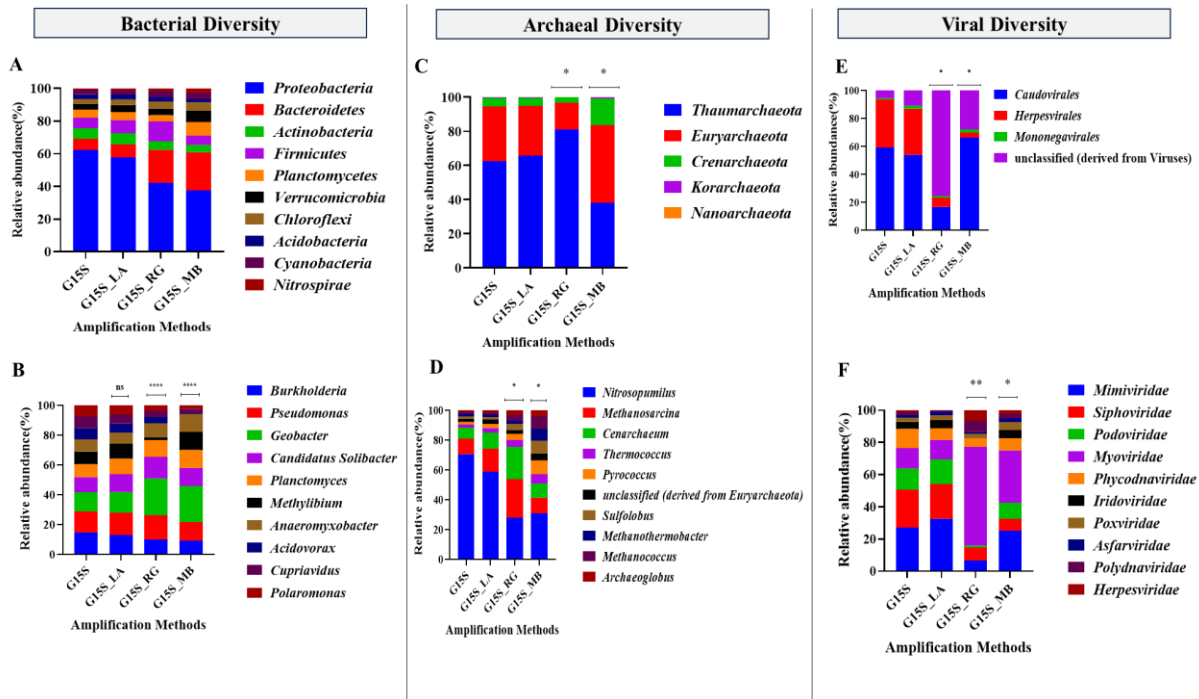


Fig. 15: Graphical illustrations showing the comparison of the taxonomic profiles obtained through amplified methods. A: Bacterial diversity of top 10 phyla; **B:** Bacterial diversity of top 10 genera; **C:** Archaeal diversity at phylum level; **D:** Archaeal diversity of top 10 genera; **E:** Viral diversity at order level; **F:** Viral diversity at of top 10 families.

Among the three amplification methods evaluated, it was noted that the sample amplified with PCA+Long Amp exhibited an overall taxonomic resemblance close to that of the native sample (Two-way ANOVA, $p > 0.05$). In contrast, RepliG and MALBAC displayed significant variations in taxonomic composition (Two-way ANOVA, $p < 0.05$). Therefore, PCA+Long Amp was considered the preferable method for further use in amplifying upper stretch samples.

4. Conclusion

Overall, this study is the first-ever report to present an efficient extraction and amplification method to sequence the microbiome in samples with less DNA concentration. This method holds promise for conducting shotgun-based whole metagenomic analyses of other glacier-fed streams and forelands. Nevertheless, further investigations are necessary to validate the efficacy of the Long Amp-based targeted amplification across a diverse range of samples with

low input DNA, thereby providing a comprehensive understanding of the promising capabilities of this method.

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3.2 The Microbial Diversity and Antimicrobial Potentials Along the Pristine Stretch

Abstract

Background

Glacier-fed rivers are referred to as ecological hotspots and harbor a diverse array of microbial communities, each portraying distinct trophic and biogeochemical functions that largely remain unexplored. The Ganges River in India is a glacier-fed river; however, it remains elusive for its microbial community composition over different seasons and the associated antimicrobial potentials.

Rationale

Only a limited number of studies have focused on the functional potentials of the microbiome and the special properties of non-putrefying (self-cleansing) rejuvenation of the Ganges River. However, these studies are restricted to the lower stretch of this riverine system. Therefore, there is a lack of a holistic understanding of the microbiome and its functional potential along the pristine stretch of the glacier-fed Ganges.

Results

The predominant bacterial phyla during the dry season were *Proteobacteria* and *Actinobacteria*, while *Bacteroidetes* and *Firmicutes* were abundant in the post-monsoon season ($p < 0.05$). Members of *Euryarchaeota* were the major taxa of the archaeal diversity contributing to the methane flux in this glacier-fed River. Analysis for secondary metabolites revealed the presence of biosynthetic pathways for streptomycin, phenylpropanoid, penicillin, and cephalosporins. Furthermore, a predominance of phages from *Podoviridae*, *Myoviridae*, and *Siphoviridae* was observed with viral proteins mainly involved in the structural, molecular, and biological functioning of the phage lifecycle and host-phage relationships.

Significance

This study highlights the microbial diversity of Glacier-fed Ganges along its pristine stretch as a repository with functional potentials for the synthesis of secondary metabolite compounds with antimicrobial potentials. The present study provides a whole metagenome-based initial scientific insights with a plausible explanation for the special properties of the Ganges River. The data obtained from this study lays a foundational basis for understanding the microbiome and its functional potentials, together with expanding our understanding of the non-putrefying and rejuvenating aspects of this unique ecosystem.

1. Introduction

The glacier biomes serve as a bridge between the cryosphere, biosphere, hydrosphere, and pedosphere and are susceptible to the effects of climate change (Jacobsen et al., 2012). The glacier-fed, mountain-river ecosystems comprise streams that are headwaters to several riverine systems across the globe. Glacier-fed rivers or streams (GFR/S) are a significant component of the Earth's fluvial landscape. Being perennial, they are capable of sustaining the downstream ecosystems, including humans and animals, through the supply of their freshwater all year long (Battin, Tom J; Ezzat, Leïla; Peter, Hannes; Bourquin et al., 2023). Oligotrophy, low water temperatures, and a unique microbiome characterize these GFRs. Therefore, they are referred to as ecological hotspots and harbor a diverse array of microbial communities, each portraying distinct trophic and biogeochemical functions that largely remain unexplored (Busi et al., 2022; Garcia-Lopez et al., 2021).

River Ganges (Ganga) is the national River of India and is among the crucial glacier-fed rivers of the Himalayan ranges. The Ganges originates as River Bhagirathi (the source stream of the Ganges) from the Gomukh, the snout of the Gangotri glacier. It traverses ~2500 km and drains into the Bay of Bengal in the Sunderban Delta. The melting of Himalayan permafrost gives rise to the sediment load, which gushes out alongside the waters, thereby contributing to the remarkable physical properties of the water at Gomukh (Khairnar, 2016). The pristine stretch of the Ganges is marked from its origin, Gomukh, to Rishikesh and is characterized by limited civilization scattered along its catchment area. The lower stretch of the Ganges is marked by its debouchment from Himalayan regions to Gangetic plains, with dense civilization along its catchment area (Bhutiani et al., 2014). This majestic riverine system is referred to as a lifeline for a vast multitude of populations, as it fulfills various sustenance needs of people and also serves as an embodiment of spirituality and culture (Bhutiani et al., 2016; Dutta et al., 2020).

The Ganges River has been revered for its non-putrefying (self-cleansing) and special healing properties for centuries (Khairnar, 2016). Ernest Hankin, in 1896, gave an experimental basis demonstrating the antibacterial property of the Ganges River's water against *V. cholerae*. In 1918, Félix d'Hérelle termed the factor conferring this exceptional property to the Ganges as 'bacteriophage' (Abedon et al., 2011). Previous microbiological-based studies on the Ganges River have described the isolation of bacteria resistant to antibiotics, heavy metals, and fecal coliforms (Ali et al., 2021; M Vortmann, 2015). Recent studies have used amplicon and shotgun metagenome-based high throughput sequencing platforms to delineate the resistome and

virulome at specific locations and the lower stretch spanning 1500 km (Behera et al., 2020b; Kumar et al., 2021; Reddy and Dubey, 2019; Samson et al., 2023a; Zhang et al., 2018). There are only limited studies that have focused on the functional potentials of the microbiome (Behera et al., 2020a; Rajput et al., 2022; Rout et al., 2024) and the special properties of non-putrefying (self-cleansing) and rejuvenation (Dutta et al., 2020; Dwivedi et al., 2020; Nautiyal, 2009; Samson et al., 2019) of the Ganges River. However, these studies are restricted to the lower stretch of this riverine system. Consequently, the microbial diversity and its contribution to the special properties along the pristine stretch remain elusive.

There is an interrelationship between microbes and their distinctive functions within the ecosystem. Several studies on glacier-fed rivers or streams have focused on bacterial and archaeal components through amplicon-based sequencing (Battin, Tom J; Ezzat, Leïla; Peter, Hannes; Bourquin et al., 2023; Besemer et al., 2013; Fell et al., 2017; Wilhelm et al., 2013). However, in the case of the Ganges River along its pristine stretch, there are no reports that provide insights into climate-driven alterations in the total microbial diversity due to the glacier retreat. Therefore, this study, for the first time, uses a new approach for extraction and amplification of the total metagenomic DNA from the sediments of the glacier-fed-Ganges River. The data obtained from this approach, combined with high throughput next-generation sequencing on the MinION platform (ONT), provides the first insights into the total microbiome of the glacier-fed-Ganges along its pristine stretch. Additionally, we have mapped the spatiotemporal heterogeneity of the microbiome and comprehended their ecological significance. Furthermore, based on the potentials of secondary metabolites, a rich bacteriophage diversity, and their functions, we have attempted to deliver shreds of scientific evidence that would expand our understanding of the microbial aspects conferring special properties of the glacier-fed River Ganges along its pristine stretch.

2. Methodology

2.1 Sampling and study site details

The entire course of the river Ganges in our study was broadly divided into two different stretches. The upper transect is from Gomukh to Rishikesh (~280 km), while Haridwar to Bhagalpur (~1500 km) marks the lower stretch (LS). The LS is characterized by the River Ganges debouching from the Himalayan region into the Gangetic plains and sufficing a diverse civilization alongside its basin. The origin of the Ganges River from Gomukh (snout

of the Gangotri glacier) to Rishikesh is characterized as the upper/ pristine stretch. The current investigation focuses on a ~250 km long transect of the River Ganges for its microbiome and its properties, conferring the Ganges River with its special properties. A total of 69 sediment samples (depth ~40-50 cm) were collected from six locations over four years (2018-2020) as detailed in **(Fig 1)**. The sampling strategy was similar to our previous studies, which involved the collection of sediment samples from the River in triplicates from the left, center, and right banks (Samson et al., 2019).

It should be noted that the climate changes affected the rainfall period in Uttarakhand, India, in the year 2019-2020 till January (TWC India Edit Team, 2020), as a result of which the post-monsoon sampling for 2019 was carried out in February 2020. *In-situ* measurements of the environmental parameters like pH, dissolved oxygen (DO), electrical conductivity (EC), and temperature were carried out using respective probes **(Table 1)**. The samples collected were preserved on dry ice and immediately transported to the laboratory for further investigation.

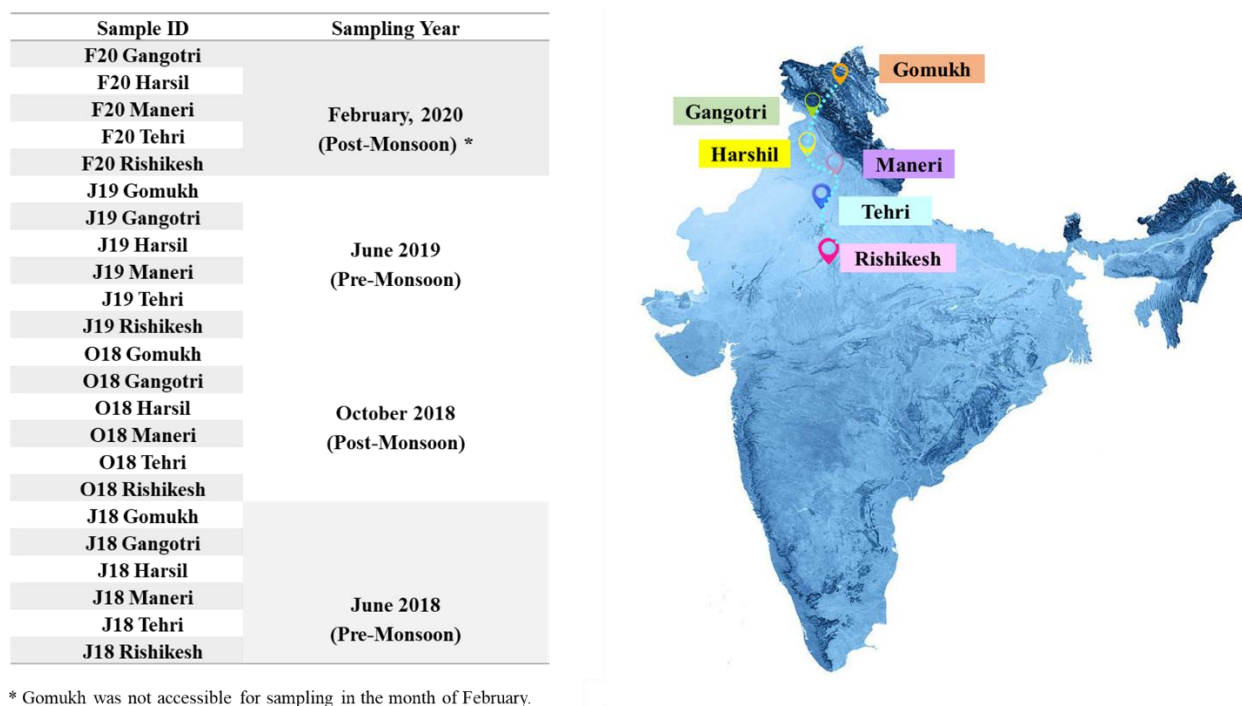


Fig. 1: Schematic illustration of the Ganges River detailing the sampling locations across four seasons, pre-monsoon (2018, 2019) and post-monsoon (2018, 2019).

Table 1: The environmental parameters such as temperature, pH, electrical conductivity (EC), and dissolved oxygen (DO).

Locations	Month and year of sampling	Season	pH	DO (mg/L)	Temperature (°C)
J18_Gomukh	Jun-18	Post-Monsoon	7.9	8.5	7
J18_Gangotri	Jun-18	Post-Monsoon	7.2	8.7	9
J18_Harsil	Jun-18	Post-Monsoon	7.6	10.5	11
J18_Maneri	Jun-18	Post-Monsoon	8.1	9.7	12
J18_Tehri	Jun-18	Post-Monsoon	8.4	9	12.5
J18_Rishikesh	Jun-18	Post-Monsoon	8	10.9	15
O18_Gomukh	Oct-18	Pre-Monsoon	7.07	8.6	5
O18_Gangotri	Oct-18	Pre-Monsoon	7.13	8.7	6
O18_Harsil	Oct-18	Pre-Monsoon	8	9	5
O18_Maneri	Oct-18	Pre-Monsoon	7.36	9.5	8
O18_Tehri	Oct-18	Pre-Monsoon	7.8	8.9	9.5
O18_Rishikesh	Oct-18	Pre-Monsoon	8.65	9.8	12
J19_Gomukh	Jun-19	Post-Monsoon	7.6	8	7.3
J19_Gangotri	Jun-19	Post-Monsoon	7.77	8.4	9
J19_Harsil	Jun-19	Post-Monsoon	8.2	8.8	10
J19_Maneri	Jun-19	Post-Monsoon	8.19	9.2	11
J19_Tehri	Jun-19	Post-Monsoon	8	9	12
J19_Rishikesh	Jun-19	Post-Monsoon	8.5	9.6	14
F20_Gangotri	Feb-20	Pre-Monsoon	7.57	8.1	2
F20_Harsil	Feb-20	Pre-Monsoon	8.4	8.3	4
F20_Maneri	Feb-20	Pre-Monsoon	8.1	9.5	5
F20_Tehri	Feb-20	Pre-Monsoon	8	9.2	7
F20_Rishikesh	Feb-20	Pre-Monsoon	8.5	10.5	9

2.2 Metagenomic DNA Extraction and shotgun sequencing

Prior to the DNA extraction, the sediment samples from the left, center, and right banks of the River were pooled together according to the locations. Based on the results obtained from the optimization studies of DNA extraction and amplification, we chose Long Amp enzyme coupled with 1-D Low input genomic DNA with PCR (SQK-LSK109 with EXP-PCA001), ONT as the method of choice for amplification of the DNA for the upper stretch samples. Briefly, in this process, 100 nanograms (ng) of DNA from each of the samples was end-

prepped using Ultra II End-Prep buffer and Ultra II End-Prep enzyme mix (New England Biolabs (NEB)). The resulting DNA was quantified using a Qubit fluorimeter (Thermo Fisher Scientific). The end-prepped DNA fragments were further taken for adapter ligation using a PCR Expansion kit (EXP-PCA001). The adapter-ligated DNA was then amplified using Long Amp Taq2X master-mix (NEB), and the Ampure XP beads (Beckman Coulter) were used for the purification of the resultant amplified product. Further, the amplified DNA samples were indexed with 1D Native barcoding genomic DNA kit (EXP-NBD103 and EXP-NBD 113). The library was prepared using a Ligation Sequencing Kit (SQK-LSK109) with certain modifications, as mentioned in our previous study (Samson et al., 2023a). The final library was loaded onto the FLO-MIN-106D-R9 flow cell (ONT) placed in the MinION sequencing platform, and the run was continued up to 56-72 hours.

2.3 Data processing and quality check

Processing of the raw reads and their quality assessment was carried out as per our previous study (Samson et al., 2023b). Briefly, base-calling of the raw reads was carried out with Albacore (v2.3.4). Subsequently, the fastq files were subjected to qcat software v1.1.0 (ONT) for demultiplexing. The adapter sequences were trimmed with the Porechop tool (v0.2.1). The datasets were normalized with the DESeq2 package. The data quality and stats were assessed using Nanostats in NanoPlot. The normality of the datasets was evaluated with the Shapiro-Wilk normality test ($p\text{-value} \leq 0.05$), confirmed using a Q-Q plot. Further, the data was rarefied using the rarefy function in R. These rarefied datasets were used for downstream taxonomic and functional analysis.

2.4 Microbial community structure and statistics

The metagenomic datasets were annotated for taxonomic and functional composition using default parameters in MG-RAST (E value: $1 \times e^{-5}$ and 60% of sequence identity). Additionally, the KRAKEN-2 tool was used for improved taxonomic resolution (Wood et al., 2019). GraphPad Prism v9.5 was used to prepare illustrations. Differences in the abundance of microbial diversity with respect to seasonal changes were evaluated using Welch's t-test with Bonferroni correction ($p\text{-value} \leq 0.05$) in STAMP (Statistical Analysis of Taxonomic and Functional Profiles) software v2.1.3 (Parks et al., 2014).

Taxonomic diversity over a spatio-temporal scale was ascertained using alpha and beta diversity measures. Prior to the analysis, the removal of singletons was ascertained from the

(Operational Taxonomic Unit) OTU file, and the OTU table was rarefied to ensure equal sampling depth. Alpha diversity metrics of Observed OTUs, Shannon diversity index, and Simpson's index were computed with the phyloseq package in R (McMurdie and Holmes, 2013). Beta diversity analysis using the Bray-Curtis dissimilarity matrix was carried out to understand compositional differences between the samples. The significance of the dissimilarity measure was determined through ANOSIM and PERMANOVA (Adonis test p-value <0.05). The similarities and differences between the samples with reference to the season (explanatory variable) and microbial diversity of the sampling locations (response variables) were visualized using the Principal Coordinate Analysis (PCoA) plot.

2.5 In-silico predictive functional annotation for bioactive potentials of untapped microbial diversity

The fastq files uploaded in MG-RAST were used for functional annotation. The metabolic potentials of microbes in each dataset were analyzed by assigning KEGG Orthology (KO) numbers obtained from known reference hits, and annotation of the metabolic pathways with respect to the biosynthesis of secondary metabolites was done using KEGG pathway analysis. Additionally, predictive *in-silico* functional analysis of the datasets to comprehend phage-related functions was achieved using Subsystems and KEGG databases in MG-RAST.

2.6 Data Availability

The metagenomic data of this study is publicly accessible through MG-RAST under the project titled 'Ganga_UpperStretchdata_NCL2022'. Additionally, this data has been submitted as bio project **PRJNA1101206** to the Sequence Read Archive (SRA) portal.

3. Results and Discussion

3.1 Microbial Diversity Measures

Shannon, Observed, and Simpson's diversity indices were used to compute the alpha diversity of the total microbiome across four seasons. A greater number of distinct OTUs were noted for the pre-monsoon season. However, this observation was not statistically significant. In the case of the Shannon diversity and Simpson's index, a relative increase in the microbial community was evidenced by a higher richness and evenness in the pre-monsoon samples.

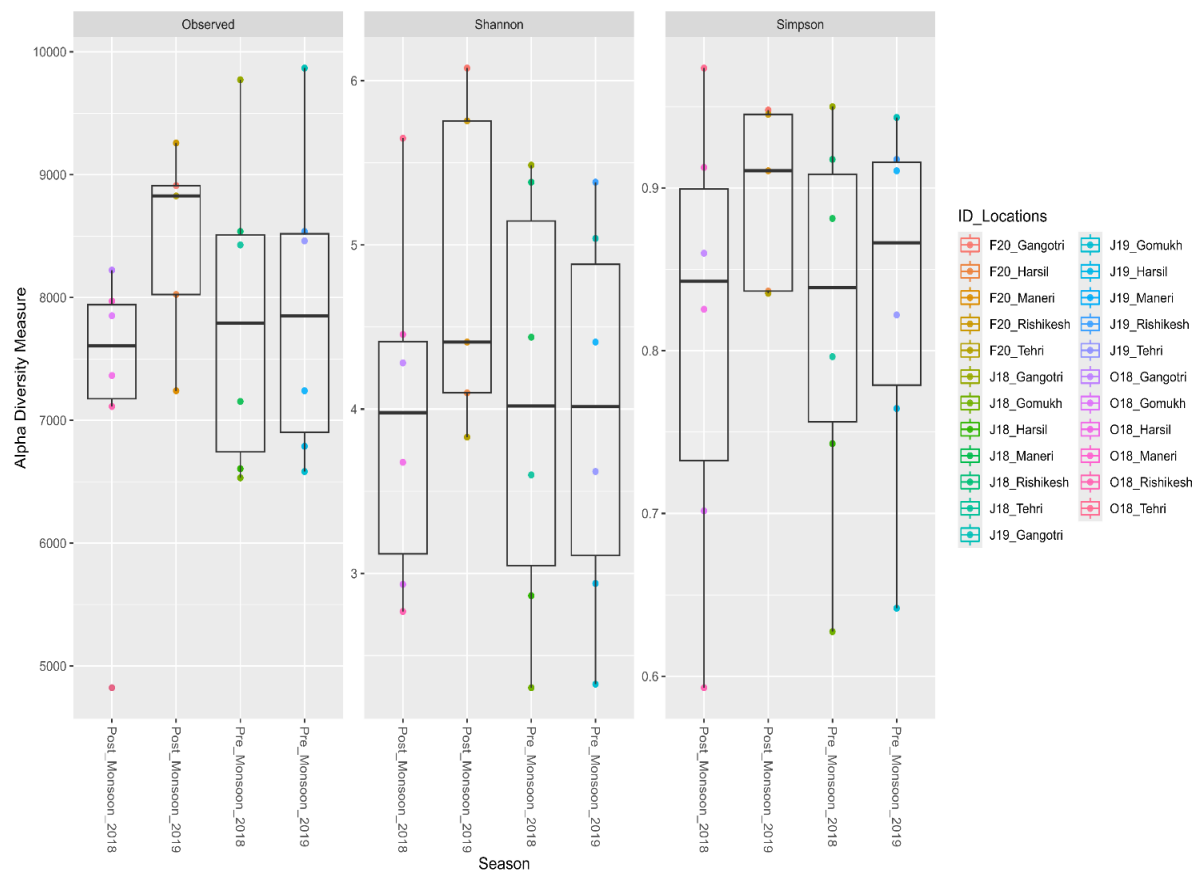


Fig. 2: Alpha diversity of the total microbial community for four years along two different seasons (pre-monsoon and post-monsoon).

The changes in the microbial community structure in response to the seasons (explanatory variable) were comprehended in the Beta diversity using the Bray-Curtis dissimilarity matrix. A higher dissimilarity between the pre-monsoon and the post-monsoon samples on axis 1, with 29% variation (Adonis test $p < 0.05$), indicated a significant influence of seasonal variation on the total microbial diversity.

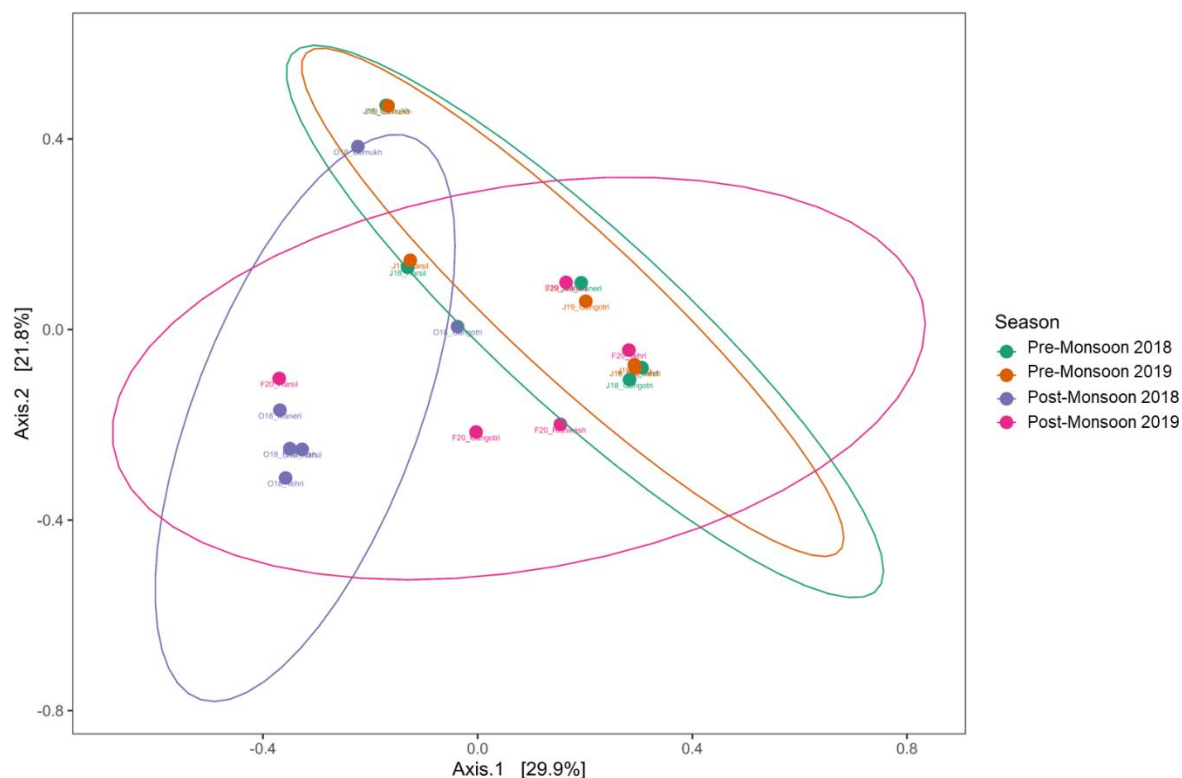


Fig. 3: Beta diversity of the total microbial community for four years along two different seasons (pre-monsoon and post-monsoon).

3.2 Bacterial community composition

Throughout four years of sampling encompassing pre and post-monsoon seasons, a total of 65 bacterial phyla were observed across all the locations. The top fifteen most abundant phyla that contribute to the total bacterial diversity have been illustrated in **Fig. 4A**. Glacier-fed riverine systems are dynamic due to the temporal fluctuations that dictate the microbial community structure (Wilhelm et al., 2013). A distinct taxonomic variation in the bacterial communities was observed in the wake of the seasonal shift. The *Proteobacteria* ($69 \pm 6.6\%$), *Actinobacteria* ($6 \pm 5.46\%$), and *Verrucomicrobia* ($4 \pm 1.23\%$) were the most prevalent phyla during the dry seasons (pre-monsoon) in both the years (2018-19) ($p < 0.05$). In the case of the wet seasons (post-monsoon), a higher relative abundance of *Bacterioidetes* ($42 \pm 9.16\%$) and *Firmicutes* ($32.8 \pm 11.26\%$) was observed ($p < 0.05$) (**Fig. 4 B, C**). These observations are consistent with other aquatic ecosystems like rivers and lakes, wherein *Proteobacteria* and *Actinobacteria* have been reported as the typical freshwater phyla that increase in the dry season (Guo et al., 2024; Krishna et al., 2020; Sun et al., 2017; Wilhelm et al., 2013). A considerable increase in the *Proteobacteria* phylum during the dry season is linked to its

copiotrophic character influenced by nutrient availability and organic carbon (Krishna et al., 2020). The dry season in the Uttarakhand state of India attracts an influx of tourists for trekking, rafting, and religious dips in this riverine system, resulting in increased anthropogenic inputs and contributing to the nutrient load.

Actinobacteria phylum is characterized by exceptional stress tolerance, which renders them the ability to survive in extreme habitats such as glaciers, permafrost, and hot springs. The mycelial form of *Actinobacteria* creates favorable conditions in the sediments by mobilization of Carbon, Nitrogen, and Phosphorous pools, thereby contributing to the C, N, and P cycle in the pre-monsoon season (Bhattacharya et al., 2022). Additionally, the prevalence of *Bacterioidetes* and *Firmicutes* in the wet seasons corroborates with the study of Št'ováček et al., wherein an increase in these phyla has been observed during heavy rain and lower temperatures, consequently resulting in a decrease of the relative abundance of *Actinobacteria* and *Verrucomicrobia* (Št'ováček et al., 2017). The spatial distribution of the top 15 abundant bacteria remains relatively consistent at the phylum level ($p>0.99$; ANOVA with BH correction).

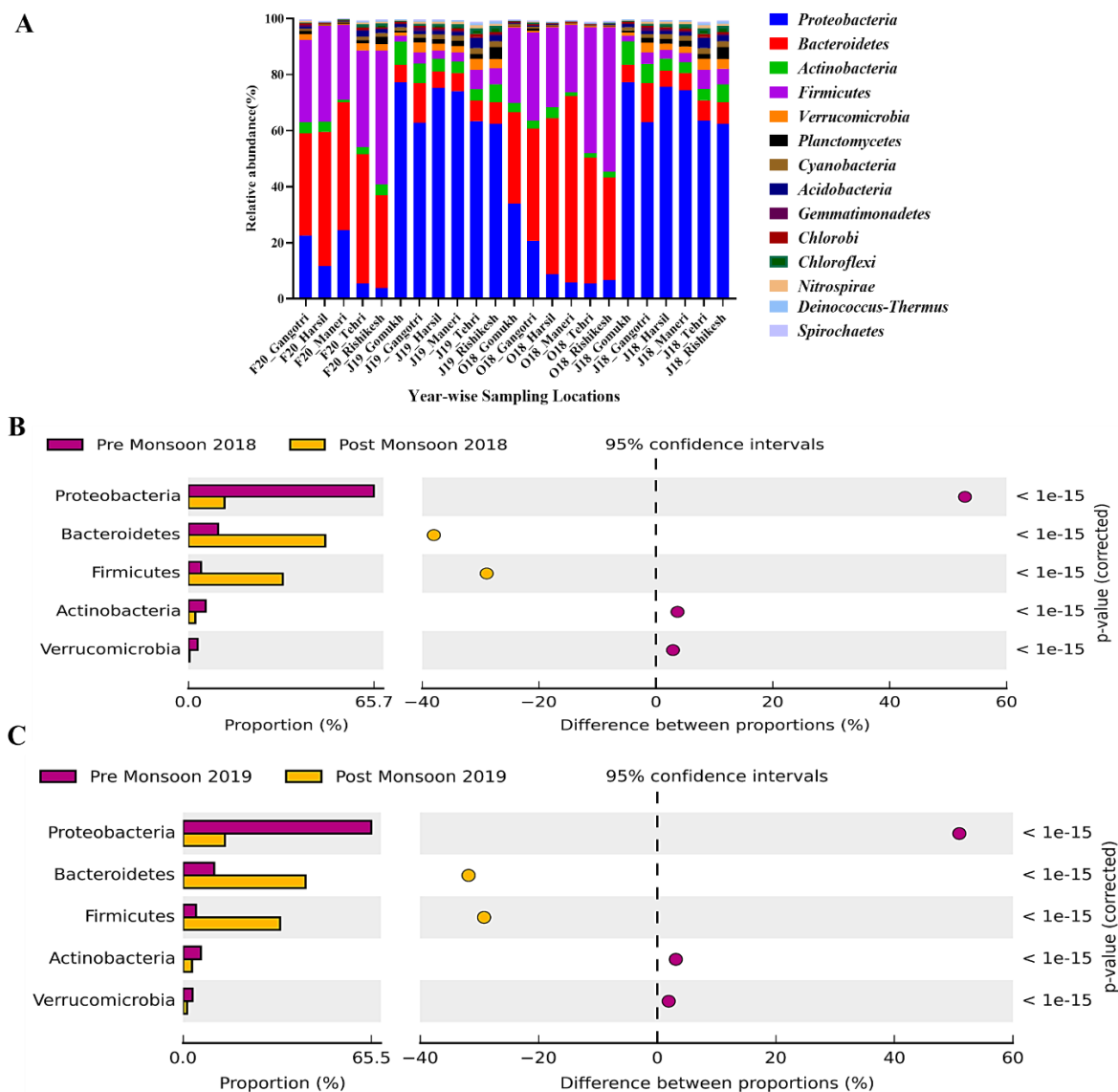


Fig. 4: Spatio-temporal variations in the bacterial phyla composition along the pristine stretch of the River Ganges. (A) Spatial diversity of top 15 phyla; **(B)** Temporal variations at phylum level in 2018 **(C)** Temporal variations at phylum level in 2019. **(D)** Spatial diversity of top 15 genera; **(E-F)** Temporal variations at genus level in 2018 **(E)** 2019 **(F)**.

At the genus level, a predominance of oligotrophic and lithotrophic bacteria such as *Thiobacillus* ($10.2 \pm 4.32\%$), *Sideroxydans* ($9.7 \pm 3.57\%$), *Burkholderia* ($8.5 \pm 4.32\%$), *Streptomyces* ($6.06 \pm 4.23\%$), and *Gallionella* ($4.6 \pm 2.19\%$), was observed at all the locations (**Fig 5A**). At the genus level, the bacterial diversity was not significantly affected by the spatial variation ($p > 0.99$; ANOVA with BH correction). On the contrary, the bacterial diversity at the genus level was considerably influenced by the seasonal (temporal) variation ($p > 0.05$; Welch's t-test and Bonferroni correction). *Pseudomonas*, *Burkholderia*, and *Sideroxydans* were more

prevalent in the pre-monsoon season. Likewise, an increase in the proportion of sequences for *Streptomyces*, *Thiobacillus*, *Acidovorax*, and *Polaromonas* was observed in the post-monsoon season (Fig 5 B, C).

Ecological niches such as glaciers and permafrost are characterized by reducing conditions that offer favorable conditions for the sustainability of aerobes and anaerobes with acetoclastic, hydrogenotrophic, methanogenic, sulfate-reducing, Fe(III) reducing, and denitrifying abilities (Margesin and Miteva, 2011). The origin of the Ganges River (Gangotri glacier in the Himalayan ranges) comprises stressful environments characterized by cold temperatures and low-nutrient oligotrophic conditions (Bhattacharya et al., 2022). *Thiobacillus* and *Sideroxydans* are known for the anaerobic oxidation of iron or thiosulphate coupled with nitrate reduction in cold temperatures. This microbial-mediated nitrate-dependent Fe(II) oxidation contributes to the biogeochemical cycling of iron (Lopez-Fernandez et al., 2023). The availability of iron is crucial for several vital enzymes and cytochromes responsible for the production of secondary metabolites such as antimicrobial compounds, siderophores, etc. (Salwan and Sharma, 2020).

Additionally, there are limited studies with freshwater ecosystems, wherein *Burkholderia* and *Streptomyces* have been identified as the most predominant genera (Hamm et al., 2017; Passari et al., 2018). *Streptomyces* have been regarded as a repository of an array of bioactive natural products attributed to their diverse and complex secondary metabolism (Ma et al., 2020; Passari et al., 2018). The presence of *Streptomyces* in such stressed environments is indicative of their competitive survival through the production of certain antimicrobial compounds (Bhat et al., 2024). Therefore, investigating different species of *Streptomyces* growing in such high-altitude, oligotrophic, and low-temperature environments will provide an opportunity for isolation and study of their pharmacologically active secondary metabolites. Recent studies in the quest to explore novel microbial metabolites beyond *Actinobacteria* have shown the presence of biosynthetic gene clusters in the genomes of *Burkholderia* bacteria (Depoorter et al., 2021). Different species of *Burkholderia* are known to have beneficial effects aiding in plant growth, secretion of allelochemicals, and production of antibiotics, as well as siderophores (Elshafie and Camele, 2021).

Furthermore, we also observed the presence of genera such as *Methylobacillus* and *Pseudomonas*. Members from the genus *Pseudomonas* have been reported for the production of a wide range of heterocyclic antimicrobial compounds (Smirnov et al., 1997). The genus *Methylobacillus* comprises methylotrophs, which are reported to be responsible for the

production of certain essential phytohormones that influence plant growth. However, members of this genus remain underexplored in terms of the bioactive potentials of their specialized metabolites (Puri, 2019). Therefore, this whole metagenome sequencing-based information is the first report comprehending the spatiotemporal alterations in the unexplored bacterial community composition along the pristine stretch of the Ganges River over four years. This data is crucial and would be helpful in the isolation of promising strains and the bioprospection of their biosynthesis potentials.

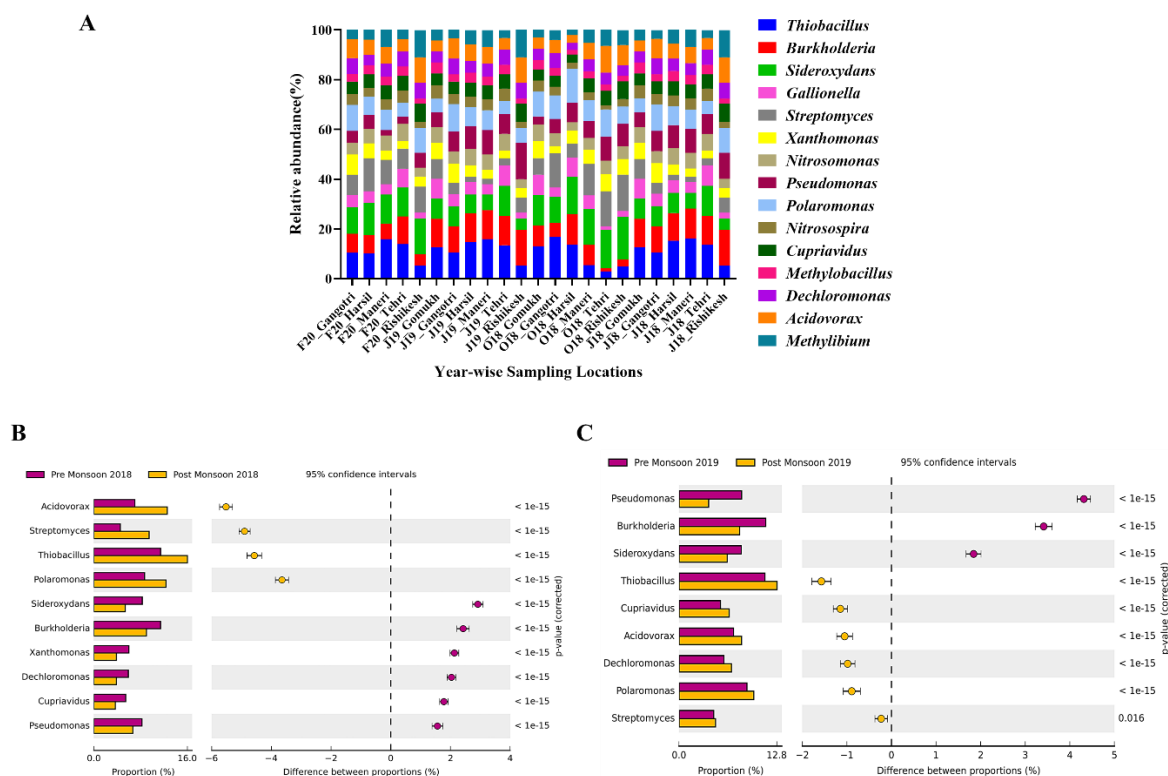


Fig. 5: Spatio-temporal variations in the bacterial genera composition along the pristine stretch of the River Ganges. (A) Spatial diversity of top 15 genera ; (B) Temporal variations at genus level in 2018 (C) Temporal variations at genus level in 2019.

3.3 In-silico functional analysis of the Metabolic Potentials of the Microbial Community

The metagenomic analysis of diverse microbial communities in freshwater environments provides a platform for uncovering novel metabolic genes. As glacier-fed rivers are unique ecological niches of microbial diversity, it becomes necessary to investigate the functional potentials of microbes (Besemer et al., 2013). Analysis revealed several metabolic functions like metabolism of amino acids, carbohydrates, energy, glycan synthesis, lipids, cofactors and

vitamins, biosynthesis of secondary metabolites, terpenoids and polyketides, nucleotide and xenobiotic biodegradation and metabolism (**Fig 6A**). However, to gain deeper insights and provide scientific reasoning toward the remarkable healing properties of the Ganges River, we focused our analysis primarily on the genes involved in the biosynthesis of secondary metabolite compounds. The analysis of secondary metabolites' biosynthesis aspect annotated pathways for the biosynthesis of compounds that exhibit antimicrobial activity. A significant fraction was assigned to Streptomycin biosynthesis ($70.25 \pm 8.11\%$), followed by phenylpropanoid biosynthesis (15.78 ± 11.93), and biosynthesis of penicillin and cephalosporins ($7.79 \pm 2.34\%$) (**Fig 6B**). The use of antibiotics like streptomycin, penicillin, and cephalosporins has been reported in the treatment of broad-spectrum bacterial infections in wounds, skin, and tissue. To date, there are limited studies that provide experimental validation to substantiate the healing touch of the River Ganges (Khairnar, 2016; Samson et al., 2023b; Tyagi and Dubey, 2020). However, to the best of our knowledge, there are no reports that provide insights into the microbiome and the associated biosynthetic gene clusters in the River Ganges. Therefore, the presence of microbes with the potential to produce such antimicrobial secondary metabolites could be attributed to one of the special properties of the River Ganges that could contribute to its healing touch.

Further, we explored the enzymes and proteins involved in the biosynthesis of secondary metabolites. Analysis revealed a predominance of dTDP -glucose-4,6 hydratase ($23.17 \pm 4.84\%$), glucose-1-phosphate thymidyltransferase (14.82 ± 2.47), and betaglucosidase ($12.21 \pm 8.2\%$) enzymes (**Fig 6C**). 4,6-Dehydratases catalyze the initial step in the deoxy sugar biosynthesis pathway. Deoxy (amino) sugars are crucial, especially in the biosynthesis of diverse macrolide antibiotics (Vara and Hutchinson, 1988). Another prevalent enzyme, glucose-1-phosphate thymidyltransferase, is involved in the biosynthesis of dTDP-L-rhamnose, a cell wall constituent of several bacteria, involved in adaptability to harsh environments and virulence. However, L-rhamnose is among the rare monosaccharides that are not commonly encountered in nature. Their limited occurrence makes them valuable, and therefore, the exopolysaccharides of bacteria containing such rare sugars signify an interesting source of isolation (Mistou et al., 2016). Recently, rhamnose-rich bacterial polysaccharides have been reported for their potential biological activities for applications in cosmetics, the food industry, and pharmaceuticals (Roca et al., 2015). Furthermore, another important enzyme observed in the BGC was betaglucosidase, which is fundamental for microbes to adapt to cold environments and plays a crucial role in the carbon flux (Tiwari et al., 2014). Additionally,

enzymes involved in the synthesis of penicillin and cephalosporins were also observed. Therefore, the presence of enzymes with bioactive potentials along the pristine stretch of the Ganges River opportunely their bioprospection.

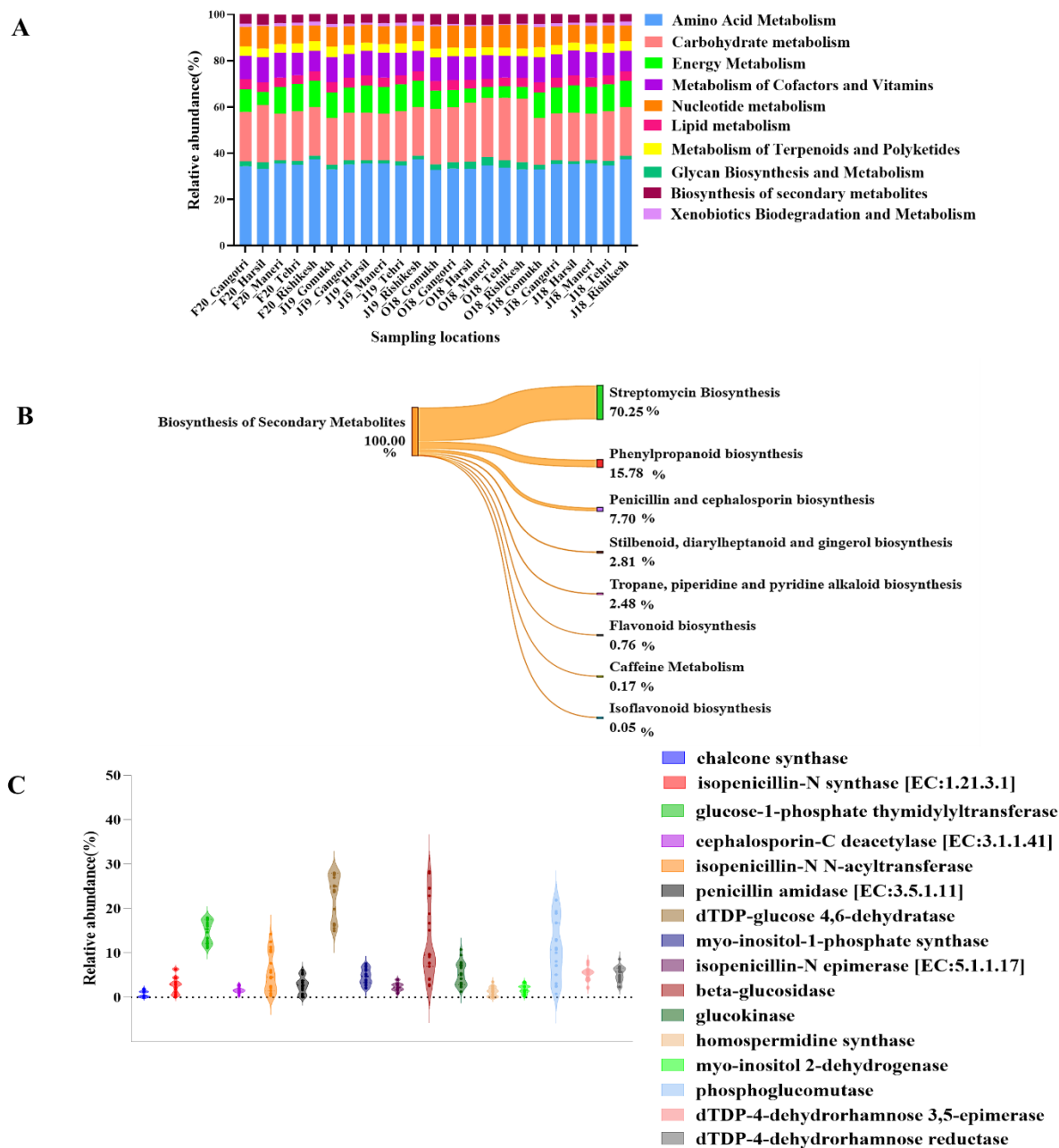


Fig. 6: KEGG-based classification of reads assigned to metabolism fraction. (A) The sub-metabolic profile with the relative abundance of reads allocated to different types of metabolism. **(B)** Sankey plot illustrating biosynthesis of secondary metabolites; **(C)** Enzymes involved in the biosynthetic gene cluster along the pristine stretch of the Ganges.

3.4 Archaeal community composition

The Ganges River has not been explored for its archaeal diversity along its pristine stretch. In view of this, we analyzed our metagenomic datasets to delve into the archaeal diversity. The phylum *Euryarchaeota* was observed to be more prevalent in all the samples with a mean relative percentage of ($82 \pm 11.43\%$), followed by *Crenarchaeota* ($5.35 \pm 2.5\%$) and *Thaumarchaeota* ($4.8 \pm 10.2\%$) (**Fig 7A**). Although the proportion of *Euryarchaeota* was higher in the post-monsoon season, it was the predominant phyla in both seasons ($p < 0.05$) (**Fig 7B, C**). *Euryarchaeota* comprises methanogenic archaea, anaerobic methanotrophs, and halophilic archaea. The soils of glacier forelands have been regarded as sinks for methane, wherein the glacier melting affects the methane flux (Hofmann et al., 2016). This indicates that glacial meltwater changes the methane sink to a methane source, thereby governing the shift in archaeal diversity from methanogens to methanotrophs (Xing et al., 2022). The *Thaumarchaeota* and *Crenarchaeota* group consists of ammonia-oxidizing archaea (AOA), which are essential for nitrification processes in aquatic biomes (Wang et al., 2020). *Crenarchaeota* phyla comprise hyperthermophilic, sulfur-metabolizing archaea (Qin et al., 2023). However, members of these phyla have also been reported to be prevalent in different cold habitats, which include high Arctic lakes, Alpine and Antarctic soils, Himalayan cold deserts, and Alpine permafrost (Margesin and Miteva, 2011).

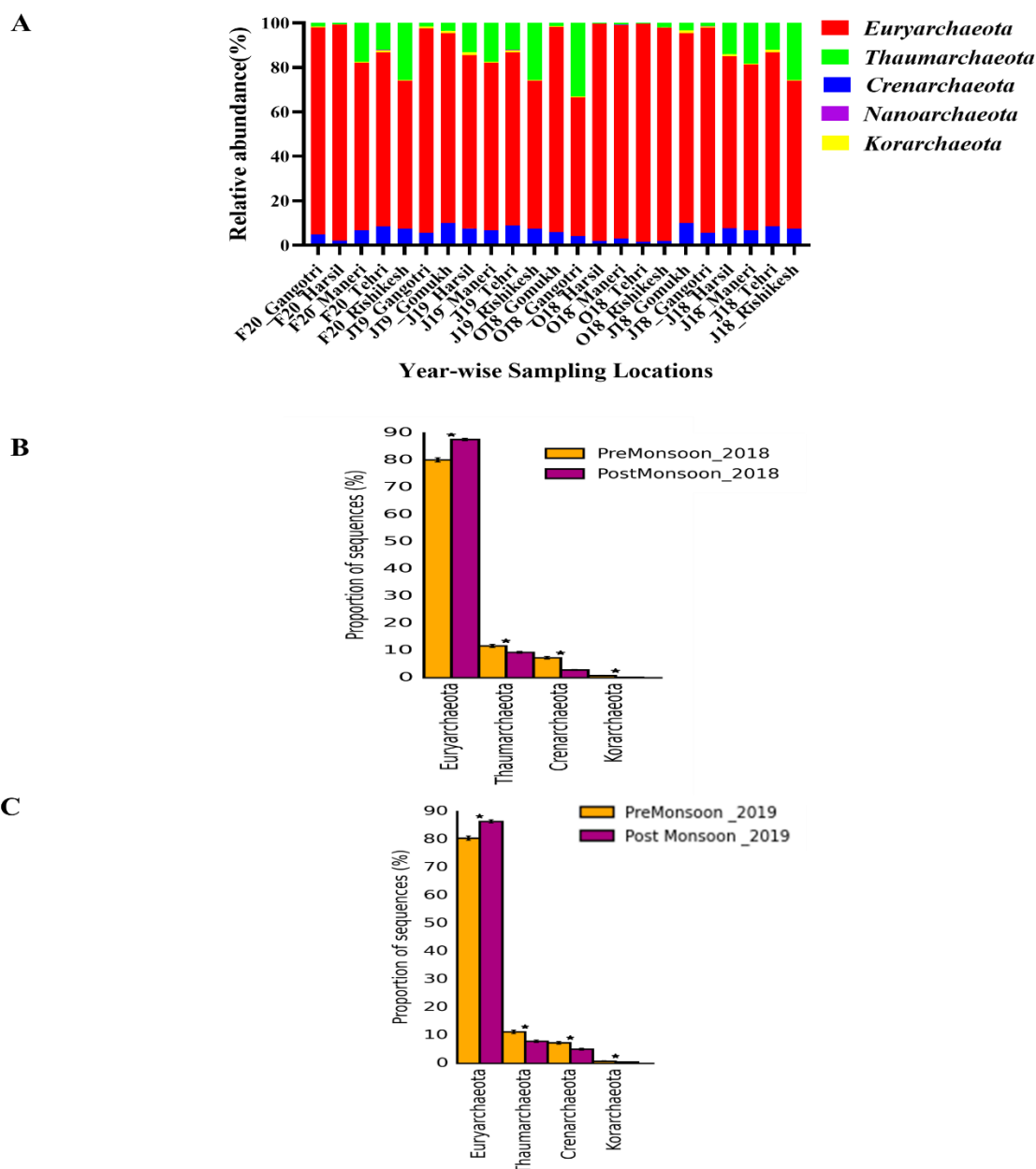


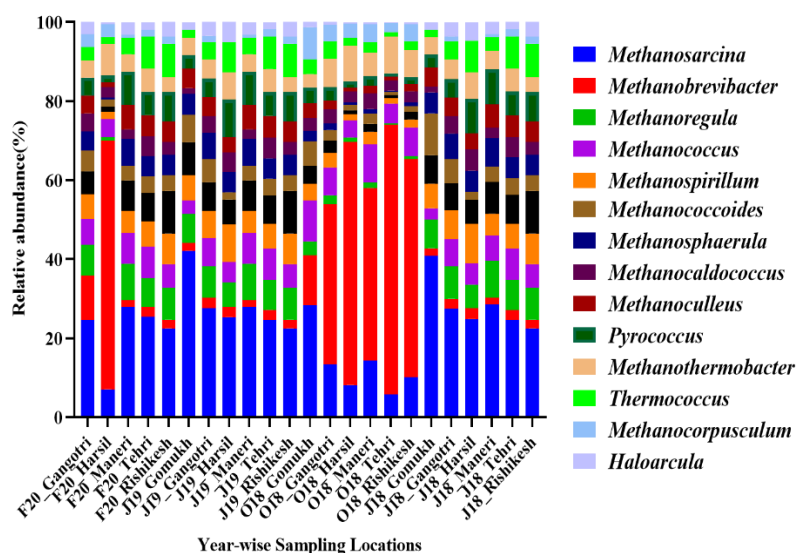
Fig. 7: Spatio-temporal variations in the archaeal phyla composition along the pristine stretch of the River Ganges. (A) Spatial diversity of archaeal phyla; (B) Temporal variations at phylum level in 2018 (C) Temporal variations at phylum level in 2019.

At the genus level, a substantial proportion of the members of *Euryarchaeota* like *Methanosarcina* ($20.46 \pm 9.45\%$), *Methanococcus* ($6.2 \pm 1.82\%$), and *Methanobrevibacter* ($5.8 \pm 24.09\%$) (**Fig 8A**) was observed. These methanogenic archaea are strictly anaerobic chemolithotrophs, and their substrate utilization is limited to acetate, formate, methylamines, and methanol (Qin et al., 2023). Additionally, species of these genera have been reported from

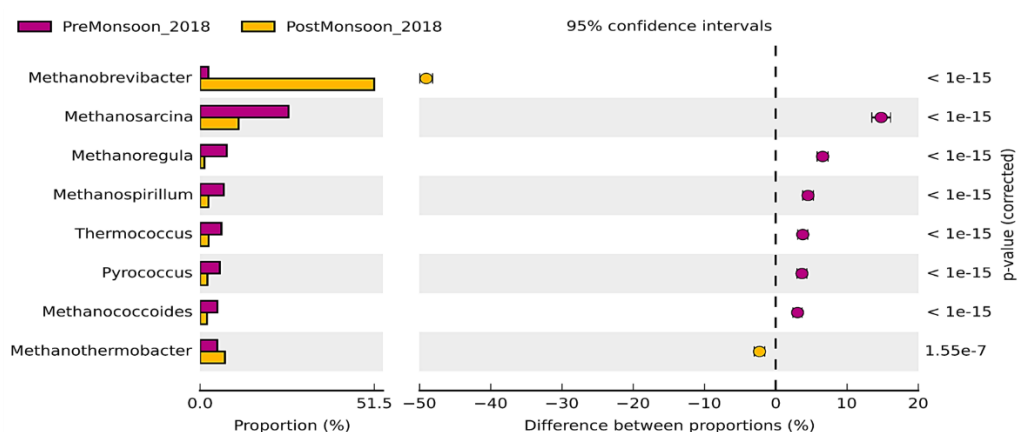
mesophilic, thermophilic, and hyperthermophilic environments. However, recent studies have shown a remarkable stress tolerance (pH, temperature, salinity, desiccation, oxygen exposure) in methanogenic archaea from cold habitats as opposed to their non-permafrost counterparts (Morozova and Wagner, 2007). Therefore, the information obtained through this study could contribute toward expanding the understanding of the ecology of methanogenic archaea along the Gangotri glacier and the consequent Ganges basin along its pristine stretch. This data could also be helpful for the isolation of potential methanogens as energy carriers for a possible replacement of fossil fuels.

Interestingly, although methanogenic archaea predominated in the pre-monsoon and post-monsoon seasons, the proportion of methylotrophic, sulfur-metabolizing, and thermophilic archaeal genera such as *Methanococcoides*, *Pyrococcus*, and *Thermococcus* increased in the dry season ($p < 0.05$), indicating the need for increased temperature for their prevalence (**Fig 8 B, C**). The presence of such thermophiles in such psychrotolerant environments enables opportunities for microbial ecologists to conduct a comprehensive assessment of the novel physiological, adaptive, and molecular mechanisms of these thermophilic archaea for their metabolic potentials to delineate thermal limits of the microbial life (Milojevic et al., 2022). To the best of our understanding, this is the first comprehensive report of archaeal community composition and their ecological significance along ~250 km transect of the pristine stretch of the River Ganges over four years.

A



B



C

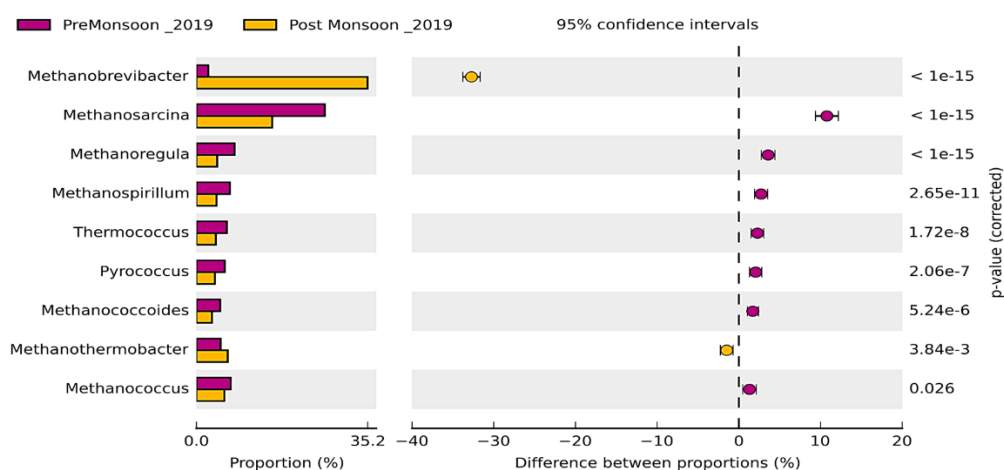


Fig. 8: Spatio-temporal variations in the archaeal genera composition along the pristine stretch of the River Ganges. (A) Spatial diversity of top 15 archaeal genera; (B) Temporal variations at genus level in 2018 (C) Temporal variations at genus level in 2019.

3.5 Viral and Bacteriophage Diversity and Predictive In-silico Functional Analysis

Natural environments have a heterogeneous composition of cellular microorganisms such as protozoa, algae, fungi, bacteria, and archaea, which fosters favorable conditions for the sustenance of extensive viral diversity. River Ganges is one such unique ecological niche to a wide variety of microbial life, and therefore, is a hub to a rich viral diversity that remains elusive. Since ancient times, this riverine system has been recognized for its non-putrefying properties, primarily ascribed to its bacteriophages. In this study, we have made an effort to investigate the dsDNA viral diversity in the River Ganges from Gomukh to Rishikesh over two seasons for four years. A notable proportion was assigned to the *Caudovirales* ($71.27 \pm 12.08\%$) order, followed by unclassified viruses ($16.90 \pm 10.06\%$). There are limited studies with riverine ecosystems wherein *Caudovirales* accounted for numerical superiority (Cai et al., 2016; Ge et al., 2013; Lu et al., 2022). A predominance of *Caudovirales*, which encompasses families of tailed bacteriophages, is indicative of a rich bacteriophage diversity in the Ganges River.

Conversely, in several other studies with freshwater systems, a significant fraction of viral reads consist of unknown or unclassified sequences. This could be attributed to the intrinsic diversity of the virosphere, nucleic acid extraction approach, amplification method, sequencing platform, and the limitations in the availability of the reference databases. This leads to a situation wherein a considerable fraction of the viral diversity in the environmental metagenomes remains unclassified (Palermo et al., 2021; Solonenko et al., 2013). In our data, yet another considerable fraction was assigned to unclassified viruses. This observation aligns with the detection rates of 6.75% and 28.67% of dsDNA viral abundance in various aquatic metagenomes (Cai et al., 2016). However, certain virome studies with lakes of the Arctic and Antarctic have shown ssDNA viruses and unclassified viruses to be predominant (De Cárcer et al., 2015). Therefore, to have a holistic understanding of the virosphere (DNA and RNA viruses) of the Ganges River along its pristine stretch, further studies are warranted. Nevertheless, the presence of numerous unclassified viruses in the Ganges River opens avenues for investigating its untapped viral diversity for various potential applications.

Further classifying the viral reads at the family level revealed a predominance of bacteriophage families like *Podoviridae* ($31.70 \pm 9.99\%$), *Myoviridae* ($30.42 \pm 13.61\%$), and *Siphoviridae* ($15.79 \pm 8.55\%$), followed by the family of giant viruses infecting protists- *Mimiviridae* ($3.89 \pm 4.35\%$). The viral community structure was dismally affected along the

spatial (location-wise) scale (ANOVA $p > 0.05$ with BH correction) (**Fig 9A**). However, temporal variations had a substantial influence on the viral diversity (Welch's t-test, $p < 0.05$) (**Fig 9B, C**). Bacteriophages are known to shape the microbial community structure in the environment through virus-host dynamics. The predominance of bacteriophage families indicated that bacteria were the most important host species. Analysis of the seasonal comparison of viral families indicated an increase in *Podoviridae* and *Siphoviridae* in the pre-monsoon (dry) season. This indicates that the phages belonging to these families primarily comprise members that infect the *Bacteroidetes* and *Firmicutes* groups, as their prevalence is observed to decrease in the dry season. In contrast, the proportion of *Myoviridae* significantly increased during the wet season ($p < 0.05$), suggesting the presence of phages that essentially govern the abundance of the members from the *Proteobacteria* group. This observation could be one of the plausible reasons leading to the decreased prevalence of *Proteobacteria* in the post-monsoon season (**Fig 3B, C**). Therefore, it could be stated that the phage activity along the pristine stretch of the Ganges River is sequentially triggered by environmental changes, which consequently affects the pool of phages that actively replicate and lyse their cognate hosts across seasons and contribute toward shaping the host community dynamics, ecology and biogeochemistry of this riverine system.

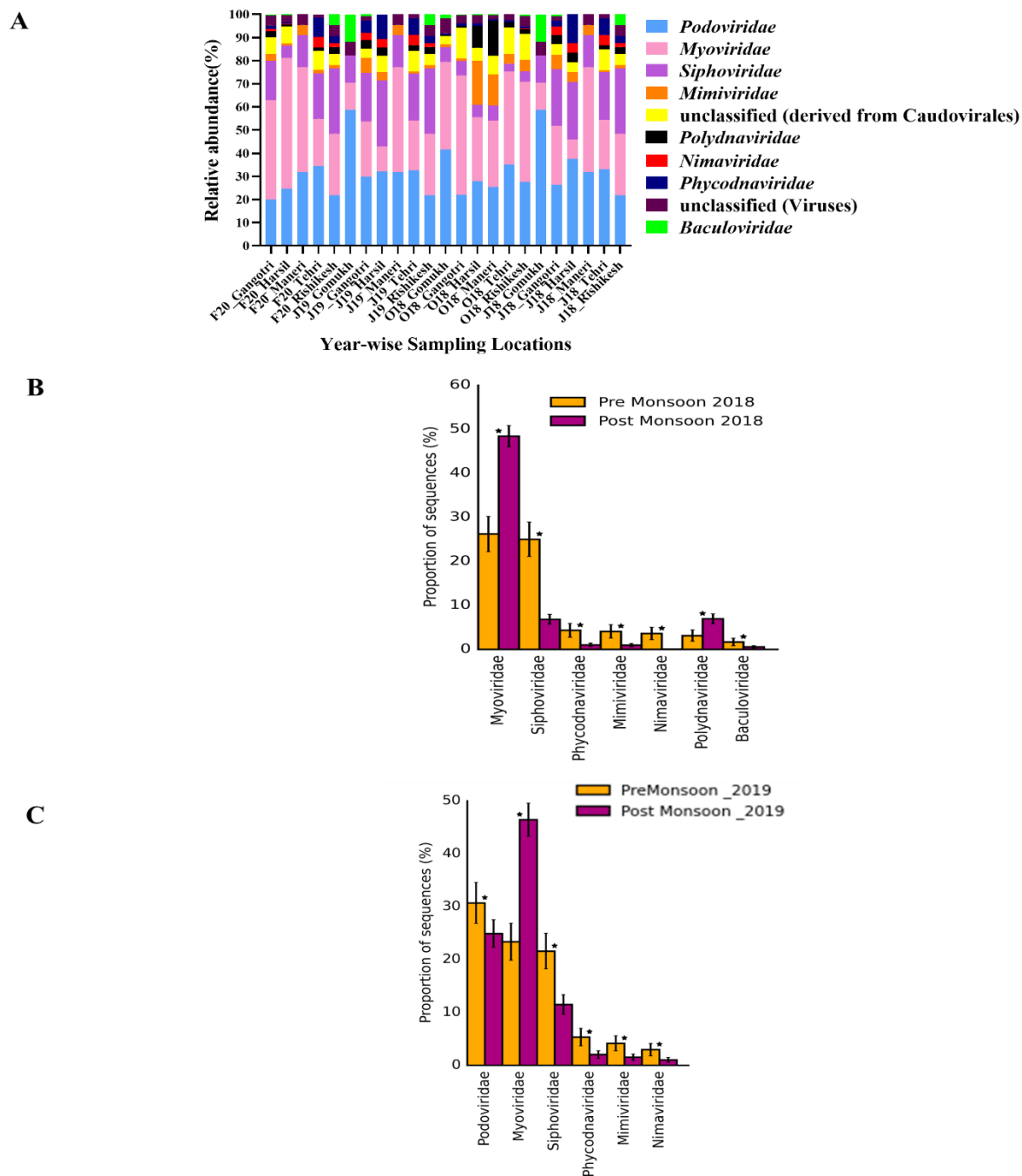


Fig. 9: Spatio-temporal variations in the viral family composition along the pristine stretch of the River Ganges. (A) Spatial diversity of top 10 viral families; (B) Temporal variations at family level in 2018 (C) Temporal variations at family level in 2019.

As the Ganges River is well-known for its non-putrefying properties, we analyzed our metagenomic datasets for the presence of bacteriophages from all twenty-three locations over four years. Analysis revealed the presence of bacteriophages against several putrefying bacteria and potentially pathogenic bacteria, affecting human, animal, and environmental health, as illustrated in **(Fig 10 A, B)**. It is noteworthy that the pristine stretch has bacteriophage diversity with untapped potential against the ESKAPEE group of pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* sp., and *Escherichia coli*). The presence of a plethora of bacteriophage repertoire for putrefying and potentially pathogenic bacteria along the pristine stretch of the Ganges River could be correlated with its special properties (non-putrefaction and healing touch). Drawing from this data, the Ganges River emerges as a promising ecological niche to be explored for the isolation of potentially novel bacteriophages along its pristine transect, which could further be used for diverse applications spanning one health.

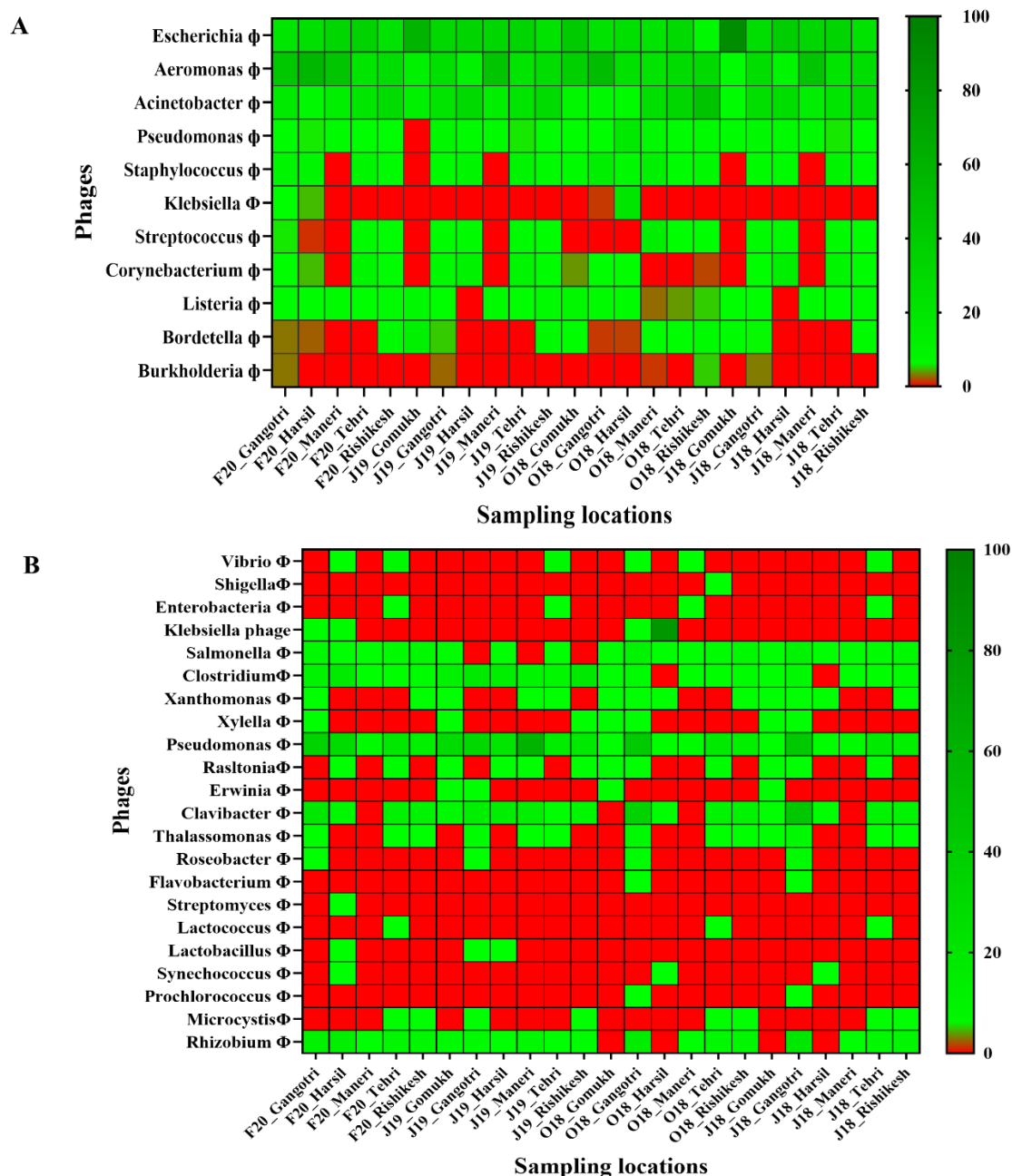
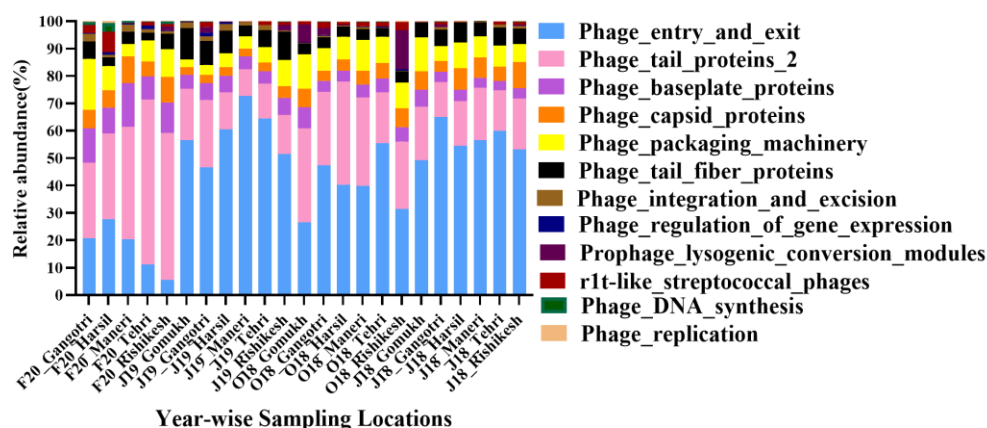


Fig. 10: Bacteriophage diversity along the pristine stretch of the River Ganges. (A) Bacteriophage diversity against potentially pathogenic bacteria at the human-animal-environmental interface **(B)** Bacteriophage diversity against putative putrefying and pathogenic bacteria.

Additionally, to comprehend phage-related and host-phage-linked functions, predictive *in-silico* functional annotation of the datasets was conducted using Subsystems and KEGG databases in MG-RAST. Analysis revealed the presence of phage-associated functions pertaining to their lifecycle, biological process, molecular function, and structural assembly (**Fig. 11A**). The prevalence of hits against phage-related proteins indicates a robust presence of a host-phage relationship. In our previous study with the anthropogenically impacted (lower) stretch of the Ganges river, which spans ~1500 km, a predominance of proteins involved in the lysogenic lifecycle of phages was observed (Samson et al., 2023b). However, the functional profile in the pristine stretch reflects phage structural and entry and exit-related proteins, indicative of the presence of lytic phages or temperate phages that have initiated the lytic cycle. This could be correlated with the predominance of narrow and broad-spectrum lytic viruses belonging to the *Podoviridae* and *Myoviridae* families at the pristine stretch.

In our previous study, wherein the anthropogenically impacted stretch was comprehended for its total microbiome, the viral diversity varied significantly. The predominant viral families therein were giant viruses that infect protists, i.e., *Mimiviridae* ($31.68 \pm 11.16\%$), and phages from *Siphoviridae* (primarily temperate phages) ($19.09 \pm 5.06\%$), *Myoviridae* (broad-spectrum lytic phages) ($11.23 \pm 4.44\%$), and *Phycodnaviridae* (algae infecting phages) ($7.38 \pm 2.83\%$) families (Samson et al., 2023b). The presence of a higher diversity of lytic phages in the pristine stretch, compared to the lower stretch, presents an opportunity for their exploration in various applications within the One Health domains. Therefore, this riverine system offers a unique niche with a substantial eco-diversity of its autochthonous bacteriophages to gain insights into their fundamental and applicative aspects. Interestingly, our investigation also revealed hits for sequences associated with phage-associated lytic activities (including endolysin, endopeptidases, lysozyme, and amidase) (**Fig. 11B**). These enzymes hold potential for their use in therapy, diagnostics, and various biotechnological applications. Given that this study serves as an initial exploration of phage diversity and its associated functions, further research is warranted to thoroughly investigate and characterize the biological and genetic potentials of phages for diverse applications.

A



B

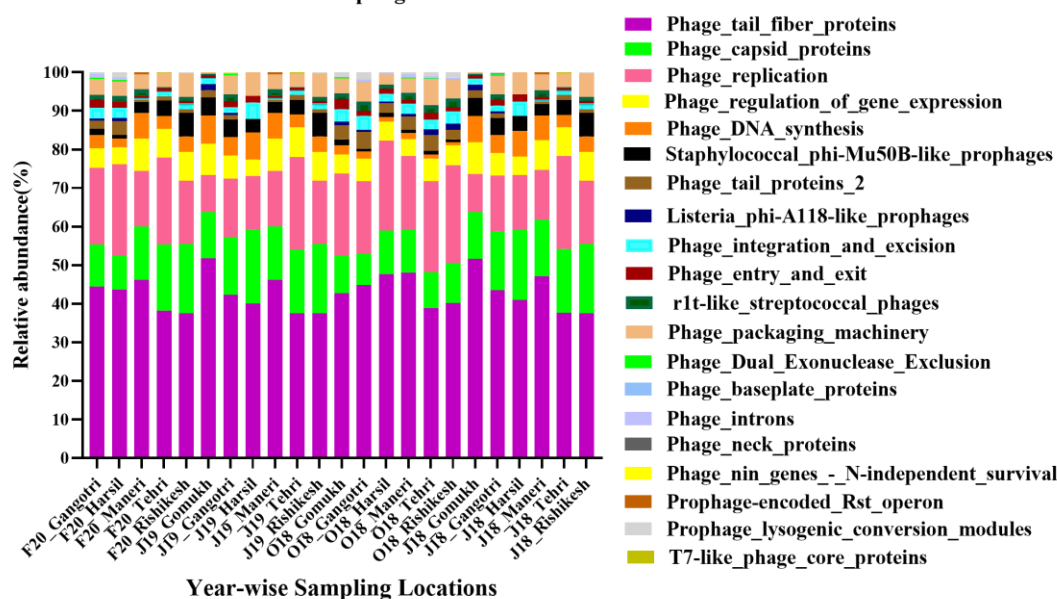


Fig. 11: (A) *In-silico* prediction of functional annotations for hits targeting host-phage-related proteins. (B) Prediction of phage-associated lytic cassette functions along the pristine stretch of River Ganges using MG-RAST.

4. Conclusion

Overall, the present study is the first-ever report that underscores the compositional heterogeneity of microbial diversity along a spatiotemporal gradient of the pristine stretch of the Ganges River. The strength of our research lies in the approach used to dissect the microbiome and its functional potential along this stretch. The results highlighted the microbial diversity of this Glacier-fed River as a repository with functional potentials for the synthesis of secondary metabolite compounds with antimicrobial potentials. Additionally, a rich bacteriophage diversity with lytic potentials toward putative putrefying and potentially pathogenic bacteria opportune their use under one-health canopy. Therefore, this study provides whole metagenome-based initial scientific insights with a plausible explanation for the special properties of the Ganges River. However, further studies that demonstrate the bioactive potentials of these microbes, including phages, are warranted to have a holistic understanding of the promising capacities of untapped microbial diversity and their contribution toward the special properties of the Ganges.

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Chapter 4

*Harnessing the potentials of lytic phages
from the pristine stretch of the Ganges
River for environmental applications*

4.1 Characterization of a Novel Tequatrovirus Phage from the Pristine Stretch of The Ganges River in Reducing Bacterial Load from Sewage

Abstract

Background

The key bacterial pathogens in sewage that cause environmental and health problems are *E. coli*, *Salmonella*, *Shigella*, and *Pseudomonas*. The current WW treatment includes a combination of physical, chemical, and biological methods for eliminating microbial contamination. However, the rise of antimicrobial resistance (AMR) in bacteria has considerably challenged the treatment of microbial pollution in WW. Therefore, emerging alternatives are necessary to tackle the existing antibiotic resistance crisis and safeguard human and animal health by shielding environmental health through the ‘one health’ approach.

Rationale

The metagenomic analysis has revealed the presence of promising lytic phages within the pristine stretch, targeting putative putrefying and potentially pathogenic bacteria. Nevertheless, the extent of lytic phage diversity along this stretch remains unexplored in terms of its potential environmental applications.

Results

This study is the first report of a novel broad-spectrum phage (ϕ ERS-1) isolated from a pristine stretch of the Ganges River in the biocontrol of *E. coli*, resistant to 3rd-and 4th generation cephalosporins and aztreonam. The ϕ ERS-1 could reduce the abundance of *E. coli* cells by $8.22 \log_{10} \text{ CFU/mL} \leq 24 \text{ hrs}$. Additionally, ϕ ERS-1 disrupted the biofilm of *E. coli* with a reduction of $3.88 \log_{10} \text{ CFU/mL}$. Further, ϕ ERS-1 could inhibit biofilm by multiple strains of *E. coli* and multiple genera (*E. coli*, *S. boydii*, and *P. aeruginosa*). The phage ϕ ERS-1 reduced bacterial counts in raw sewage by $2 \log_{10} \text{ CFU/mL}$ and $4 \log_{10} \text{ CFU/mL}$ reduction in coliform-enriched sewage in ≤ 24 hours.

Significance

Our approach of using a polyvalent lytic phage as a green alternative for bacterial biocontrol of sewage water highlights the importance of the potential applications of such broad-spectrum bacteriophages to improve the quality of the effluent and disposal of sludge in the environment. This approach can significantly impact the delivery of sustainable development goals 6: clean water and sanitation, 3: good health and well-being, and 11 sustainable cities and communities.

1. Introduction

The treatment and recycling of wastewater (WW) for non-potable purposes has been recognized progressively as a sustainable alternative as it is a cost-effective option to tackle the existing water scarcity crisis. Reusing domestic WW allows sustainable use of water resources for agriculture or environmental benefits, contributing to the circular economy (Hernández-Crespo et al., 2022). Considering the load of inorganic and organic discharges in the sewage, the conventional indicators include estimating the carbon pollutants and nitrogen and phosphorus content. However, in recent times, biological indicators have been at the center of attention for sewage treatment and reuse (Al-Gheethi et al., 2018). The raw (untreated) WW comprises various pathogenic bacteria, which include the members of *Enterobacter* sp., *Staphylococcus aureus*, *Klebsiella* sp., *Acinetobacter* sp., *Escherichia coli*, *Enterococcus* sp., *Proteus* sp., *Salmonella* sp., *Shigella* sp., *Pseudomonas aeruginosa*, and *Citrobacter* sp. Such microbial contamination in water has a detrimental effect on the public health (Soliman et al., 2023).

The key bacterial pathogens in sewage that cause environmental and health problems are *E. coli*, *Salmonella*, *Shigella*, and *Pseudomonas*. The contamination of these microbes in the environment occurs through intestinal and extra-intestinal routes (Jang et al., 2017). Therefore, biological indicators should be more focused on reflecting the connotation of sewage recycling better and providing an answer to the current water-environmental sanitation practices. The current WW treatment includes a combination of physical, chemical, and biological methods for eliminating microbial contamination. (Qian et al., 2022). However, the rise of antibiotic resistance in bacteria has considerably challenged the treatment of microbial pollution in WW. Therefore, to tackle the existing antibiotic resistance crisis, there is a need to safeguard human and animal health by shielding environmental health through the ‘one health’ approach (Garvey, 2020).

E. coli members are an essential indicator in evaluating the extent of environmental pollution. Additionally, *E. coli* has been identified as a key enteric pathogen responsible for diarrhoeal disease due to its transmission through contaminated food, water, soil, surfaces, and hands (Navab-Daneshmand et al., 2018). The fate of *E. coli* strains as commensals or pathogens (expressing virulence factors) depends upon a complex balance between the host's status and expression of the virulence determinants. Certain signature characteristics of pathogenic strains of *E. coli* include adhesion, biofilm formation, toxin production, and evasion of host defense mechanisms. The environmental transmission of *E. coli* and its pathotypes include animal

wastes, manure, WW, and sewage sludge exiled from WW treatment plants (Osińska et al., 2023). Besides, the presence of *E. coli* in treated WW remains a considerable public health concern due to the high expense of removal via traditional techniques employed in sewage treatment plants. Therefore, to address the issue of AMR and control microbial contamination, bacteriophages as green biocontrol agents have re-emerged (Soliman et al., 2023).

Recent studies have highlighted the extended ability of phages to control bacterial populations in various fields of agriculture, aquaculture, biomedicine, the food industry, and wastewater treatment. However, there are limited studies detailing the use of a cocktail of phages in their natural or engineered form to treat the WW for the reduction of bacterial load and the elimination of waterborne pathogenic bacteria (Beheshti Maal et al., 2015; Bhargava et al., 2023; Grami et al., 2022; Jassim et al., 2016; Periasamy and Sundaram, 2013; Withey et al., 2005). Most of the phages are host-specific and facilitate the killing of their host through lysis, followed by the release of the phage progeny. Moreover, phages, being abundant in nature, make them ideal candidates for their use in a variety of applications spanning various domains under one health canopy (Samson et al., 2024). However, a major limitation of the use of phages in environmental settings is their narrow host range. Isolation of phages that could lyse multiple hosts effectively is challenging. However, this constraint is overcome by combining various monovalent phages as a cocktail or engineering naturally occurring phages to extend their host spectra. Combining different phages may often lead to antagonistic outcomes and cause a bacteriostatic effect instead of bactericidal ones (Zhou et al., 2022). Therefore, isolation and development of phages with a broad-spectrum host range is essential.

The Ganges River is India's national river and is well known for its unique properties of 'self-cleansing (non-putrefying) and special healing' since the ancient past (Khairnar, 2016). The waters of the River Ganges have a rich history of demonstrating antibacterial properties, first studied by Ernst Hankin against *Vibrio cholerae* in 1896 (Hankin, 1896). However, a detailed insight into these antibacterial aspects was provided by Félix d'Hérelle, who discovered and termed the antagonist microbe of *V. cholerae* as a 'bacteriophage' (D'Herelle, 1917).

It is well-known that despite tremendous anthropogenic activities and their related pollution load, the Ganges can rejuvenate itself rapidly (Paul, 2017; Reddy and Dubey, 2019; Samson et al., 2019; Zhang et al., 2018). This fact has also been witnessed recently during COVID-19 lockdown times with remarkably clean water even at the most polluted sites (Dutta et al., 2020). However, there are limited studies with this riverine system wherein novel virulent phages were isolated from its waters against MDR strains of *K. pneumoniae* (Sundaramoorthy et al., 2021)

and *P. aeruginosa* (Rathor et al., 2022). Our recent study on the sediments of the river Ganges along its 1500 km has identified the repertoire of bacteriophages and their associated host-phage functions against putative human, plant, and putrefying pathogens (Samson et al., 2023). Therefore, this unique aquatic ecosystem provides an opportunity to bioprospect its untapped and unique phage diversity for its potential applications. Given this, the present study was initiated to explore the untapped phage diversity from the pristine stretch of the river Ganges to isolate novel phages and explore their potential for use as biocontrol agents against *E. coli*, facilitating improved environmental health.

2. Materials and Methods

2.1 Bacterial strain and antimicrobial susceptibility profile

Escherichia coli (ATCC 8739) was the bacterial host used in this study. The genomic identity of the isolate was confirmed using the MinION Mk1C Nanopore sequencer (Oxford Nanopore Technologies). The resistance profile of the isolate was ascertained with the VITEK 2 system (bioMérieux) using two different sets of antimicrobial susceptibility testing (AST) cards, AST-N235 and AST-N281, having a defined set of parameters as recommended by the Clinical and Laboratory Standards Institute (CLSI).

2.2 Isolation, enrichment, and propagation of *Escherichia coli* phage ERS-1

Water-saturated sediment samples of the Ganges River from the Harshil (31°02'18.0"N 78°44'22.7" E) location were used as the source of phage isolation against the primary host *E. coli* (ATCC 8739). A different approach was used for the isolation of bacteriophages. The sediment samples (n=3, 50 gm) were vortexed at maximum speed for 30 minutes at room temperature. The sediment was allowed to settle briefly while the sediment-laden water (~40 mL) was equilibrated with 10 mL of SM buffer for 1 hour in an incubator at 37°C with a speed of 180 rpm. The resulting mixture was removed and centrifuged (Eppendorf centrifuge, 5804 R) at 6000×g for 10 mins. The supernatant (~40 mL) was then passed through a combination of 0.45µm 0.22µm of Polyether sulfone (PES) membrane-based syringe filters (Hi-Media), respectively, and 10 mL of filtrate from each set of the syringe filters was used for enrichment.

A total of 5mL of double-strength broth of soybean-casein digest broth (SCDB) (MH011-500G, Hi-Media) and 5 mL of exponential phase culture of *E. coli* (host) was added to 20 mL of the filtrate obtained from the previous step. The enrichment flasks were incubated at 37°C for 24 h with shaking (180rpm). The presence of lytic phages in the samples was

confirmed by observing clear zones (plaques) against the bacterial lawn through the qualitative spot assay. The quantitative enumeration of phages in the enriched lysate was done using the soft agar overlay method. A single, well-isolated plaque was picked and suspended in SM (Sodium-Magnesium) buffer (NaCl 5.8g/L, MgSO₄·7H₂O 2g/L, Tris-HCl (pH7.4) 50mL, autoclaved distilled water 950mL). The phage titer of the released phages from the single plaque was determined in triplicates over four times by making serial dilutions and infecting the log-phase culture of *E. coli*, followed by double-layer agar plating and incubation at 37 °C for 16-18 h. Purification and concentration of ϕ ERS-1 was carried out with Amicon® Ultra-15 centrifugal filter with Ultracel100 kDa membrane (Merck Millipore) (Sun et al., 2014). The multiplicity of infection (MOI) (Kasman et al., 2002) and Poisson distribution for predicting the probable number of infected cells in a population (P_0) (Abedon, 2023) were calculated using the following formula:

MOI = Number of plaque forming units (PFU)/mL ÷ Number of Colony forming units (CFU)/mL

$$(P_0) = 1 - e^{-MOI}$$

A high titer phage stock of the purified phage lysate containing 10¹⁷ PFU/mL was made using replicates of a total of 30 confluent lysis plates using replicates of a total of 30 confluent lysis plates flooded with 7mL of SM buffer and incubated at 15°C with shaking (100 rpm) for 4 hours followed by ultracentrifugation at 14000×g for 30 mins at 4°C and subsequent filtration of the lysate with 0.2 μ m PES syringe filters (Bonilla and Barr, 2018). Enumeration of the high titer phage stock was done using the double-layer agar overlay method. The purified high-titer phage stock was stored at 4°C.

2.3 Characterization of *Escherichia coli* phage (ϕ) ERS-1

2.3.1 Morphological features

The morphology of ϕ ERS-1 was visualized using Jeol JEM-F2100 high resolution-transmission electron microscope (HR-TEM) at 200 kV and imaged with a Xarosa emsis camera coupled to the microscope. To obtain a detailed (3D) view of the phage morphology, HR-TEM was used in STEM (Scanning Transmission Electron Microscopy) mode, enabling scanning images at nanometer (nm) resolution. Briefly, 1 mL of the high titer stock of ϕ ERS-1 was centrifuged at 12,000 ×g for 30 mins, and the pellet was resuspended with 10 μ L of SM buffer. A total of 4 μ L of the sample was placed on a lacey formvar/carbon, 200 mesh, copper grid followed by staining with VitroEase™ Methylamine Tungstate Negative Stain (Thermo

Fisher Scientific), following the manufacturer's protocol. Morphological classification of phage was done following the guidelines recommended by the International Committee on Taxonomy of Viruses (ICTV) (Turner et al., 2023).

2.3.2 Phage adsorption rate and one-step-growth curve

To understand how fast phage virions can adsorb a target bacterial host cell, an adsorption experiment was performed as described by (Heineman and Bull, 2007) with certain variations. The host cell culture at the exponential phase was mixed with the fresh phage lysate at an MOI of 0.1 and incubated at 37°C, 100 for 30 mins. After 5 minutes, an aliquot was drawn and centrifuged to pellet the adsorbed fraction of the phage each time. At the same time, the supernatant was filtered using a 0.2 µm PES syringe filter to obtain the fraction of unadsorbed phages. The fractions were plated using the agar overlay method to get total phage (N_{total}) and free phage (N_{free}) densities, respectively. The assay was carried out in three replicates, and the adsorption curve as a function of the percentage of adsorbed and unadsorbed phages versus time was plotted using GraphPad Prism v9.5.1. The adsorption rate (α) for each time point was calculated as:

$$(\alpha) = \ln \left(\frac{N_{total}}{N_{free}} \right) / \text{adsorption time (t)}$$

One one-step growth curve assay for ϕ ERS-1 was carried out as described by (Jagdale et al., 2019) with few modifications. Briefly, fresh phage lysate of ERS-1 at a MOI of 0.1 was added to the *E. coli* cells (10^8 cells/mL) and allowed to adsorb for 20 mins at 37°C, 100 rpm. The adsorbed phages were centrifuged at $10,000 \times g$ for 5 min, and subsequently, the bacteria-phage complex (pellet) was resuspended in 2 mL of SCDB and incubated at 37°C, 100 rpm for 100 mins. A 100 µL aliquot was collected every 10 minutes, followed by plaque assay to determine the phage count (PFU/mL) at each time interval. The assay was carried out in triplicates.

2.3.3 Evaluation of pH and temperature stability

The stability of ϕ ERS-1 under different acidic and basic conditions was assessed as described by (Oliveira et al., 2020). Briefly, the pH of the SM buffer was adjusted to 3, 5, 7, 9, and 11, followed by adding 100µL of the phage suspension (10^{17} PFU/mL) to 900 µL of SM buffer with respective pH. The samples were incubated for four hours at 37°C, 100 rpm in a dry bath, followed by plaque assay. For determining the thermal stability, suspensions of 100µL of the phage (10^{17} PFU/mL) in 900 µL of SM buffer were made and incubated at a diverse set

of temperatures (4, 15, 25, 37, 45, 55, and 65°C) for four hours at 100 rpm followed by measuring the phage titer with plaque assay. The assays for pH and thermal stability of ϕ ERS-1 were carried out at two independent times in triplicates. GraphPad Prism v9.5.1 was used to compute the statistical significance of the results using repeated measures (RM) one-way analysis of variance (ANOVA). Additionally, multiple comparisons with a false discovery rate (FDR correction) and a p-value of 0.05 were used to compare the mean between the two groups.

2.3.4 Evaluation of the Lytic Spectrum

The host range of ϕ ERS-1 was evaluated using in-house bacterial host cultures of *Escherichia coli* O157:H7 (ATCC 43888), *Shigella boydii* (ATCC 9207, 8700), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* (ATCC 12011, 13314), *Staphylococcus aureus* (ATCC 6538), *Listeria monocytogenes* (ATCC 19112), *Enterococcus faecalis* (ATCC 19443) *Yersinia enterocolitica* (ATCC 27729). Details of the susceptibility profile of each host strain with phage ERS-1 have been tabulated (**Table 1**).

2.3.5 DNA extraction, Genome Sequencing, and Analysis of ϕ ERS-1

A high-titer phage stock was used to isolate DNA from phage ERS-1. The DNA was extracted using PureLink Viral RNA/DNA Mini Kit (Thermo Fisher Scientific), following the manufacturer's protocol. A 16S rRNA gene amplification was done to ascertain that the extracted DNA was devoid of host DNA. The PCR products were examined on 0.8% agarose gel electrophoresis along with controls and standard molecular weight markers. The gel was visualized using the BioEra Gel Documentation System.

The phage DNA concentration was quantified on a Qubit 4 Fluorometer (Invitrogen) using a dsDNA HS (High Sensitivity) assay kit fluorometer (Invitrogen). Library preparation of the samples for whole genome sequencing was carried out with Ligation Sequencing Kit (SQK-LSK114) and Native Barcoding Kit (SQK-NBD 114.24) as per the manufacturer's instructions. The library was loaded onto flow cell R10.4.1(Oxford Nanopore Technologies), and the sequencing run was carried out for ~56 hours using the MinION Mk1C sequencing platform.

The reads were processed from the sequencing data for quality control and adapter trimming using FastQC (Galaxy Version 0.25.1+Galaxy0) and Porechop (Galaxy Version 0.2.4+galaxy0). The genome assembly was performed using Flye, a *de novo* genome assembler v 2.9.1. Validation of sequence homology for the generated assembly with known phage sequences was done with NCBI nucleotide BLAST(BLASTN). Further, the phage genome annotation of the putative proteins was predicted with Rapid Annotation using the Subsystem

Technology (RAST) annotation server (<https://rast.nmpdr.org>). The GenBank file generated from RAST was used in Proksee for in-depth analysis and visualization of the phage genome.

Additionally, an open reading frame (ORF) finder from NCBI (<https://www.ncbi.nlm.nih.gov/orffinder/>) was used to identify the protein-coding sequences in the phage genome. Further, VipTree was used to understand genome-wide similarities between ϕ ERS-1 and other reference viral genomes (Nishimura et al., 2017). Whole genome Average nucleotide identity (ANI) to ascertain genetic relatedness between ERS-1 and other closely related phages was computed with OAT v0.93.1 (Orthologous Average Identity Tool) software (Lee et al., 2016). PhaBOX, a comprehensive web tool for phage identification, taxonomic classification, and prediction of phage lifestyle and its host, was used for an integrated phage analysis. Furthermore, intergenomic comparisons were made to ascertain relatedness between the phages using the Virus Intergenomic Distance Calculator (VIRIDIC) (Moraru et al., 2020).

2.4 Antibiofilm Potentials of ϕ ERS-1

2.4.1 Quantification and Visualization of Biofilm Inhibition Spectrum of ϕ ERS-1

A mixed culture biofilm inhibition assay was performed to understand ϕ ERS-1's effect in inhibiting broad-spectrum biofilm. In this experiment, the biofilm from mixed cultures was formed in a 35 mm treated tissue culture dish (Hi-Media) that supports cell adherence as described previously with certain modifications (Duarte et al., 2021). The assay was divided into four sets, each having five replicates of control and treated groups as follows:

Set I (Single species biofilm of Gram-negative bacterium- *E. coli*)

Set II (Multi-strains biofilm of Gram-negative bacteria- *E. coli*) (ATCC 8739, 25922, 43888)

Set III (Multi-genera biofilm of Gram-negative bacteria- *E. coli*, *S. boydii*, *P. aeruginosa*)

Set IV (Single species biofilm of Gram-positive bacterium- *S. aureus*)

The overnight grown cultures of the above bacteria were diluted in SCDB (1×10^8 CFU/mL) and inoculated in a tissue culture dish. For each set of the treated group, 0.1 MOI of ϕ ERS-1 was immediately added, while the control group was devoid of the phage treatment. Three replicates of each set were incubated for 24 h at 37°C. Subsequently, the biofilms were washed with 1X-phosphate-buffered saline (PBS), pH 7.4 (Hi Media) to remove the planktonic cells, followed by quantifying three of the replicates crystal violet (CV) assay as described by (Matysik and Kline, 2019). Briefly, the biofilms were stained with 0.1% CV for 15 mins and air-dried. The dye was solubilized with acetic acid 33% (v/v), and 200 μ L aliquots were transferred from each replicate into a 96-well microtiter plate (Tarsons) followed by measuring

the absorbance at 595 nm with BioTek Synergy H1 microplate reader (Agilent Technologies). The microscopic examination of the biofilm was done under 100× using an OLYMPUS optical microscope U-CMAD3 T7 coupled with Lumenera Infinity 1 camera. GraphPad Prism v9.5.1 was used to compute the statistical significance of the results using RM-one-way ANOVA with multiple comparisons (FDR correction) and a p-value of 0.05.

2.4.2 Time-dependent evaluation of cell viability of *E. coli* biofilm

In-vitro efficacy of ϕ ERS-1 was assessed against *E. coli* ATCC 8739 in biofilm control through inhibition and disruption assays using Film Tracer™ LIVE/DEAD® Biofilm Viability kit (Invitrogen). The experiment was carried out as described by (Mulani et al., 2022) and divided into two sets as follows:

Set I: Biofilm Inhibition (ϕ ERS-1 treatment at 0 hr. followed by imaging the replicates at 6, 12, and 24 hrs.).

Set II: Biofilm Disruption (ϕ ERS-1 treatment after 24 hrs. on pre-formed biofilm and imaging the replicates at 6, 12, and 24 hrs.).

For biofilm inhibition, the overnight grown host culture (*E. coli*) cells were diluted in SCDB (1×10^8) and inoculated in a tissue culture dish, followed by the addition of ϕ ERS-1 at 0.1 MOI. CFU was determined after each time point of 6, 12, and 24 hours.

For biofilm disruption, the overnight grown host culture (*E. coli*) cells were diluted in SCDB (1×10^8), inoculated in a tissue culture dish, and allowed to form biofilm for 24 hours. The planktonic cells were washed, and ϕ ERS-1 was added to the pre-formed biofilm at 0.1 MOI. Untreated cells of *E. coli* served as culture control. CFU was determined after each time point of 6, 12, and 24 hours. Statistical analysis of the results was performed in GraphPad Prism v9.5.1 using RM-one-way ANOVA with multiple comparisons and a p-value of 0.05.

For each time point, three replicates were used. The cells were washed with 1X-PBS (Hi Media) and stained with a mixture of SYTO® 9 and propidium iodide (PI) stains from Film Tracer™ LIVE/DEAD® Biofilm Viability kit following manufacturers protocol. The stained dishes were visualized with an inverted confocal laser scanning microscope (CLSM) (Leica Stellaris 5, DMI8) using a 20× objective. The fluorescence from live and dead bacteria was visualized using excitation wavelengths of 488 nm (SYTO® 9) and 588 nm (PI), respectively. Additionally, to understand the effect of phage treatment on the biofilm morphology, the control and 24 h sample of disruption were imaged using Field emission scanning electron microscopy (FESEM, Nova Nano SEM 450).

2.5 *Bacteriophage-based biocontrol for reduction of bacterial counts from raw sewage*

2.5.1. Sample collection and details of the sampling site

Phytorid sewage treatment plant (STP) (18° 32.8246' N, 73° 48.8338' E) situated at CSIR-National Chemical Laboratory, India, with a capacity of 0.15 million liters per day (MLD) was chosen for sampling of untreated wastewater. A total of 1 L (n=3) of untreated wastewater samples were collected in a sterile bottle by grab-sampling method. The samples were brought immediately to the lab and processed for experimental setup.

2.5.2. Evaluation of bacterial biocontrol by ϕ ERS-1 in untreated raw sewage

The raw sewage (untreated wastewater) samples from Phytorid STP were divided into test and control groups. For the test group (100 mL aliquot of the raw sewage was challenged with 1 mL of high titer phage stock of ϕ ERS-1 (10^{17} PFU/mL), while the control group was devoid of any treatment. The experiment was carried out in triplicates, and the flasks from each group were incubated at 37°C for 24 hours, followed by recording the results as CFU/mL, while the reduction in the bacterial counts was calculated in the form of log reduction and percent reduction as described previously (Bashir et al., 2022).

2.5.3. Time kill assay for evaluation of bacterial biocontrol by ϕ ERS-1 at lab-scale

A time-kill assay was performed to harness the broad-spectrum potentials of ϕ ERS-1 in improving the reusability of wastewater and associated environmental health. The experimental setup comprised of two sets as follows:

Set I: Unenriched group (Test: 100 mL raw sewage +1 mL ϕ ERS-1), (Control: 100 mL raw sewage).

Set II: Coliform enriched group (Test: 100 mL raw sewage +10 mL Mc Conkey broth + 1 mL ϕ ERS-1), (Control: 100 mL raw sewage + 10 mL Mc Conkey broth).

The experiment was performed in three replicates, and aliquots from each set were drawn at 0, 6, 12, and 24 hours, followed by serial dilutions of the aliquots and plating onto selective media of Brilliant Green Bile Lactose Broth agar (BGLB-A) (Hi-Media), and Eosin-methylene blue agar (EMB-A). For each time point, the bacterial count was determined as CFU/mL, and the efficacy of ϕ ERS-1 in reducing the bacterial counts was calculated through log reduction and (%) reduction as described by (Bashir et al., 2022). Additionally, at each time point, live-dead cell staining of the aliquots was carried out as described above and visualized under 20 $\times$ objective using CLSM. Statistical analysis of the results was performed in GraphPad Prism v9.5.1 using two-way ANOVA with multiple comparisons (FDR correction) and a p-value of 0.05 for statistical significance.

3. Results and Discussion

3.1 Isolation, identification, and growth parameters of ϕ ERS-1

Amongst the 15 isolated phages, *Escherichia* phage (ϕ) ERS-1 showed a broad-spectrum lytic activity against *E. coli*, *P. aeruginosa*, *S. boydii*, *Y. enterocolitica* and partial lysis with cloudy plaques for *S. aureus*, and therefore, was selected for detailed characterization in this study. The host range of ϕ ERS-1 has been tabulated (**Table 1**).

Table 1: Lytic spectra of ϕ ERS-1

Name of the bacterial strain	ATCC accession number	Source of isolation (by ATCC)	Lysis by ϕ ERS-1
<i>Escherichia coli</i>	8739	feces	Clear lysis zone
<i>Escherichia coli</i>	25922	Clinical isolate	Clear lysis zone
<i>Escherichia coli</i> O157; H7	43888	feces	Clear lysis zone
<i>Pseudomonas aeruginosa</i>	9027	Outer ear infection	Clear lysis zone
<i>Yersinia enterocolitica</i>	27729	Blood	Clear lysis zone
<i>Shigella flexneri</i>	12022	NA	Clear lysis zone
<i>Shigella boydii</i>	8700	NA	Clear lysis zone
<i>Shigella boydii</i>	9207	NA	Clear lysis zone
<i>Staphylococcus aureus</i>	6538	Lesion	Turbid plaques
<i>Enterococcus faecalis</i>	19443	NA	Turbid plaques
<i>Acinetobacter baumannii</i>	19606	Urine	No lysis
<i>Klebsiella pneumoniae</i>	700603	Urine	No lysis
<i>Salmonella enterica</i>	12011	NA	No lysis
<i>Listeria monocytogenes</i>	19112	Spinal Fluid	No lysis

The antimicrobial resistant profile of *E. coli* (ATCC 8739) assessed through VITEK-2 suggested its resistance toward third and fourth-generation cephalosporins (3GC, 4GC), and beta-lactam class of antibiotics, namely ceftazidime, cefepime, and aztreonam. The World Health Organization (WHO) declared 3GCs and 4GCs as antimicrobials of critical importance for human and animal health in 2019. However, due to an increase in the prevalence of plasmid-encoded β -lactamases in *E. coli*, members of this group have become resistant to these crucial antimicrobials (Kang et al., 2022). The genome annotation of *E. coli* (ATCC 8739) showed the

presence of β -lactamase enzymes (EC 3.5.6.2) responsible for multidrug resistance toward extended-spectrum beta-lactam antibiotics such as penicillins, cephalosporins, and aztreonam. Additionally, type 1 fimbriae are among the essential virulence factors in pathogenic *E. coli* strains and are known to promote their survival by antibiotic evasion. Besides, this strain was also found to possess resistance mechanisms (enzymes and efflux pumps) for multiple antibiotics (**Table 2**). Therefore, the isolation of phage for improved management of *E. coli* and control of resistance transfer for 3, 4 GCs, and multidrugs highlights the importance of this study.

Table 2: Genome annotation of *E. coli* ATCC 8739 detailing various resistance mechanisms and virulence factors

Sr. No	Resistance Functions
1	Bacteriophage N4 adsorption protein A
2	Bacteriophage N4 adsorption protein A
3	Baseplate assembly protein J
4	Baseplate assembly protein V
5	Biofilm PGA outer membrane secretin PgaA
6	Biofilm PGA outer membrane secretin PgaA
7	Biofilm PGA synthesis auxiliary protein PgaD
8	Biofilm PGA synthesis deacetylase PgaB (EC 3.-)
9	Biofilm PGA synthesis deacetylase PgaB (EC 3.-)
10	Biofilm PGA synthesis N-glycosyltransferase PgaC (EC 2.4.)
11	Biofilm PGA synthesis N-glycosyltransferase PgaC (EC 2.4.)
12	biofilm regulator BssR
13	Capsular polysaccharide synthesis enzyme CpsA, sugar transferase
14	Capsular polysaccharide synthesis enzyme CpsB
15	Capsular polysaccharide synthesis enzyme CpsB
16	Capsular polysaccharide synthesis enzyme CpsC, polysaccharide export
17	Capsular polysaccharide synthesis enzyme CpsD, exopolysaccharide synthesis
18	Capsular polysaccharide synthesis enzyme CpsD, exopolysaccharide synthesis
19	CFA/I fimbrial auxiliary subunit
20	CFA/I fimbrial chaperone

21	CFA/I fimbrial chaperone
22	CFA/I fimbrial major subunit
23	CFA/I fimbrial minor adhesin
24	CFA/I fimbrial minor adhesin
25	CFA/I fimbrial subunit C usher protein
26	CFA/I fimbrial subunit C usher protein
27	Cobalt/zinc/cadmium efflux RND transporter, membrane fusion protein, CzcB family
28	Cobalt/zinc/cadmium efflux RND transporter, membrane fusion protein, CzcB family
29	Cobalt/zinc/cadmium efflux RND transporter, membrane fusion protein, CzcB family
30	Cobalt/zinc/cadmium efflux RND transporter, membrane fusion protein, CzcB family
31	Cobalt-zinc-cadmium resistance protein
32	Cobalt-zinc-cadmium resistance protein CzcA; Cation efflux system protein CusA
33	Cobalt-zinc-cadmium resistance protein CzcA; Cation efflux system protein CusA
34	Cobalt-zinc-cadmium resistance protein CzcA; Cation efflux system protein CusA
35	Cobalt-zinc-cadmium resistance protein CzcA; Cation efflux system protein CusA
36	Fimbriae usher protein StcC
37	Fimbriae usher protein StcC
38	Fimbriae usher protein StfC
39	Fimbriae usher protein StfC
40	Fimbriae usher protein StfC
41	Fimbriae-like adhesin SfmH
42	Fimbriae-like adhesin SfmH
43	Fimbriae-like adhesin SfmH
44	Fimbriae-like adhesin SfmH
45	Fimbriae-like periplasmic protein SfmF

46	Fimbrial periplasmic chaperone SfmC
47	Fimbrial protein YadL
48	Fimbrial protein Yad-like
49	Fimbrial protein YadM
50	Fimbrial protein YadN
51	Macrolide export ATP-binding/permease protein MacB (EC 3.6.3.-)
52	Macrolide export ATP-binding/permease protein MacB (EC 3.6.3.-)
53	Macrolide-specific efflux protein MacA
54	Macrolide-specific efflux protein MacA
55	Multidrug resistance protein A
56	Multidrug resistance protein A
57	Multidrug resistance protein A
58	Multidrug resistance protein A (ErmA)
59	Multidrug resistance protein B
60	Multidrug resistance protein D
61	Multidrug resistance-like ATP-binding protein mdlB
62	Multidrug translocase MdfA
63	Multidrug transporter MdtA
64	Multidrug transporter MdtB
65	Multidrug transporter MdtB
66	Multidrug transporter MdtB
67	Multidrug transporter MdtB
68	Multidrug transporter MdtC
69	Multidrug transporter MdtC
70	Multidrug transporter MdtD
71	Multidrug transporter MdtD
72	Multidrug transporter MdtD
73	Multidrug-efflux transporter, major facilitator superfamily (MFS) (TC 2.A.1)
74	Multidrug-efflux transporter, major facilitator superfamily (MFS) (TC 2.A.1)
75	Multidrug-efflux transporter, major facilitator superfamily (MFS) (TC 2.A.1)

76	Multimodular transpeptidase-transglycosylase (EC 2.4.1.129) (EC 3.4.-.-)
77	Multimodular transpeptidase-transglycosylase (EC 2.4.1.129) (EC 3.4.-.-)
78	Multimodular transpeptidase-transglycosylase (EC 2.4.1.129) (EC 3.4.-.-)
79	Multimodular transpeptidase-transglycosylase (EC 2.4.1.129) (EC 3.4.-.-)
80	Multiple antibiotic resistance protein MarA
81	Multiple antibiotic resistance protein MarA
82	Multiple antibiotic resistance protein MarB
83	Multiple antibiotic resistance protein marC
84	Multiple antibiotic resistance protein MarC
85	Multiple antibiotic resistance protein MarR
86	N-3-oxohexanoyl-L-homoserine lactone quorum-sensing transcriptional activator @ N-3-oxooctanoyl-L-homoserine lactone quorum-sensing transcriptional activator
87	Penicillin-binding protein 2 (PBP-2)
88	Penicillin-binding protein 2 (PBP-2)
89	Penicillin-binding protein AmpH
90	Penicillin-binding protein AmpH
91	Penicillin-insensitive transglycosylase (EC 2.4.2.-) & transpeptidase PBP-1C
92	Polymyxin resistance protein ArnC, glycosyl transferase (EC 2.4.-.-)
93	Polymyxin resistance protein ArnT, undecaprenyl phosphate-alpha-L-Ara4N transferase; Melittin resistance protein PqaB
94	Polymyxin resistance protein ArnT, undecaprenyl phosphate-alpha-L-Ara4N transferase; Melittin resistance protein PqaB
95	Polymyxin resistance protein ArnT, undecaprenyl phosphate-alpha-L-Ara4N transferase; Melittin resistance protein PqaB
96	Polymyxin resistance protein PmrD
97	Polymyxin resistance protein PmrG; Ais protein
98	Polymyxin resistance protein PmrJ, predicted deacetylase
99	Polymyxin resistance protein PmrL, sucrose-6 phosphate hydrolase
100	Polymyxin resistance protein PmrM
101	Polymyxin resistance protein PmrM
102	Two-component response regulator CreB

103	Two-component response regulator CreC
104	Two-component response regulator CreC
105	Two-component response-regulatory protein YehT
106	Two-component sensor protein RcsC (EC 2.7.3.-)
107	Two-component sensor protein RcsC (EC 2.7.3.-)
108	Two-component sensor protein RcsD (EC 2.7.3.-)
109	Two-component system response regulator OmpR
110	Two-component system response regulator OmpR
111	Two-component system response regulator QseB
112	type 1 fimbriae adaptor subunit FimF
113	type 1 fimbriae adaptor subunit FimF
114	type 1 fimbriae adaptor subunit FimF
115	type 1 fimbriae adaptor subunit FimG
116	type 1 fimbriae adaptor subunit FimG
117	type 1 fimbriae anchoring protein FimD
118	type 1 fimbriae anchoring protein FimD
119	type 1 fimbriae anchoring protein FimD
120	type 1 fimbriae anchoring protein FimD
121	type 1 fimbriae major subunit FimA
122	Type I restriction-modification system, DNA-methyltransferase subunit M (EC 2.1.1.72)
123	Type I restriction-modification system, specificity subunit S (EC 3.1.21.3)
124	Type I restriction-modification system, specificity subunit S (EC 3.1.21.3)
125	Type I secretion outer membrane protein, TolC precursor
126	Type I secretion outer membrane protein, TolC precursor
127	Type I secretion system ATPase, LssB family LapB
128	Type I secretion system ATPase, LssB family LapB
129	Type I secretion system ATPase, LssB family LapB
130	Type I secretion system ATPase, LssB family LapB
131	Type I secretion system ATPase, LssB family LapB
132	Type II restriction enzyme, methylase subunits
133	Type II restriction enzyme, methylase subunits

134	Type II restriction enzyme, methylase subunits
135	Type III secretion bridge between inner and outermembrane lipoprotein (YscJ,HrcJ,EscJ, PscJ)
136	Type III secretion chaperone protein for YopD (SycD)
137	Type III secretion cytoplasmic protein (YscF)
138	Type III secretion inner membrane protein (YscQ,homologous to flagellar export components)
139	Type III secretion inner membrane protein (YscT,HrcT,SpaR,EscT,EpaR1,homologous to flagellar export components)
140	Type III secretion inner membrane protein (YscU,SpaS,EscU,HrcU,SsaU, homologous to flagellar export components)
141	Type III secretion inner membrane protein (YscU,SpaS,EscU,HrcU,SsaU, homologous to flagellar export components)
142	Type III secretion protein EprH
143	Type IV fimbrial assembly protein PilC
144	Type IV fimbrial assembly, ATPase PilB
145	Type IV pilin PilA
146	Type IV pilus biogenesis protein PilM
147	Type IV pilus biogenesis protein PilN
148	Type IV pilus biogenesis protein PilN
149	Type IV pilus biogenesis protein PilO
150	Type IV pilus biogenesis protein PilP
151	Type IV pilus biogenesis protein PilP
152	Type IV pilus biogenesis protein PilQ
153	Virulence factor MviM
154	Virulence factor VirK

The plaque morphology of ϕ ERS-1 with this host comprised clear plaques encircled in a translucent halo (**Fig 1A**). This type of plaque morphology could be attributed to the occurrence of polysaccharide depolymerases in either the structural proteins or tail fibers of ϕ ERS-1. These enzymes are non-lytic and are known to cleave the extracellular polysaccharides of

bacteria, reducing their virulence (Rice et al., 2021). This preliminary observation suggested that ϕ ERS-1 could have potential antibiofilm activity.

A detailed morphological characterization of the purified high titer stock of ϕ ERS-1 was carried out with HR-TEM and STEM, which revealed typical "T4- like" features of myovirus. The morphological architecture of ϕ ERS-1 comprised of a prolate head (length, 114 ± 5 nm; width, 66 ± 3 nm) showing an icosahedral symmetry, a collar with whiskers, a contractile tail (length, 90 ± 10 nm; width, 15 ± 2 nm), a small baseplate with short spikes, and six long terminal (tail) fibers (**Fig 1B**). Based on the above morphological features, ϕ ERS-1 was classified into the class *Caudoviricetes* and family *Straboviridae*, as suggested by the recent taxonomic update of ICTV (Turner et al., 2023).

The titer of ϕ ERS-1 was determined as 4.8×10^{17} PFU/mL. An MOI of five or greater indicates the host cell being infected by more than one phage particle, thereby portraying the virulent ability of phage to set up a productive infection within the susceptible host (Abedon, 2023). The MOI for ϕ ERS-1 was calculated as 8.8, which could achieve a bacterial clearance of 99.98%, with a probability of infected cells being $P_0=0.998$. This indicates the virulent nature of ϕ ERS-1 and its capability of mounting a productive infection in *E. coli* (ATCC 8739) cells.

The binding of the phage to specific receptors on the bacterial cell surface initiates bacteriophage infection of the susceptible host. This event is termed phage adsorption, an essential parameter in deciding the application of a bacteriophage (Ge et al., 2020). The percentage of adsorbed fraction of ϕ ERS-1 particles was 50% up to 5 mins, which increased to >90% by 20 mins. Additionally, the percentage of free phages in the unadsorbed fraction decreased rapidly at 5 mins (~70%), and only ~10 % of free phages were observed from 20-30 mins (**Fig 1C**).

The fundamental nature of replication of ϕ ERS-1 in *E. coli* (ATCC 8739) was determined with a one-step growth experiment. This enables us to understand the duration of the different phases in the lifecycle and the burst size (yield of the viral cycle) (Adams and Wassermann, 1956). Phage ERS-1 showed a latent period of 20 mins and a burst size of 45 (± 5) phage particles per infected cell of *E. coli* (host) (**Fig 1D**).

Various external factors are known to affect phage persistence. However, according to Ackermann, tailed phages tend to be most stable under adverse external factors. The temperature is a crucial factor in determining the survival and infection cycle of the phage. This factor is also essential for phages' short-term and long-term storage to retain their activity (Jończyk et al., 2011). The temperature stability of ϕ ERS-1 was determined from 4°C to 65°C.

Interestingly, it was observed that cold conditions of 4 and 15 °C were found to be optimal temperatures as the phage titer increased from 10^{17} PFU/mL to 10^{20} PFU/mL. This could be because the phage was isolated from the Ganges River's upper stretch (Harshil location), which has a relatively cold temperature. An increase in the temperature from 37°C to 45°C resulted in a significant loss in the phage titer (ANOVA, $p < 0.001$). Additionally, a significant decline was observed in the phage titer at 55°C and 65°C (ANOVA, $p < 0.001$) (**Fig 1E**).

Another essential factor that limits the stability and activity of phages is the pH of the environment (Jończyk et al., 2011). The pH stability of ϕ ERS-1 was determined from pH 3 to 11. From the phage titration, pH 7 was the optimum pH for sustaining phage stability. On either side of the pH scale, it was observed that the phage titer reduced from 10^{17} PFU/mL to 10^{10} PFU/mL (pH 3) and 10^{12} PFU/mL (pH 5, pH 9), respectively (**Fig 1F**). However, a phage titer of 10^9 PFU/mL is considered a high titer (Bonilla and Barr, 2018). This suggests that ϕ ERS-1 continues to have an infectious nature for *E. coli* even at a diverse pH range. Additionally, in the environment, especially in wastewater, most microbes thrive at pH 6-9; therefore, most of the biological treatment of wastewater occurs at this pH range (Bouchaala et al., 2021). The ability of ϕ ERS-1 to mount infection to its target host at various ranges of temperature and pH substantiates its candidacy for use in wastewater treatment.

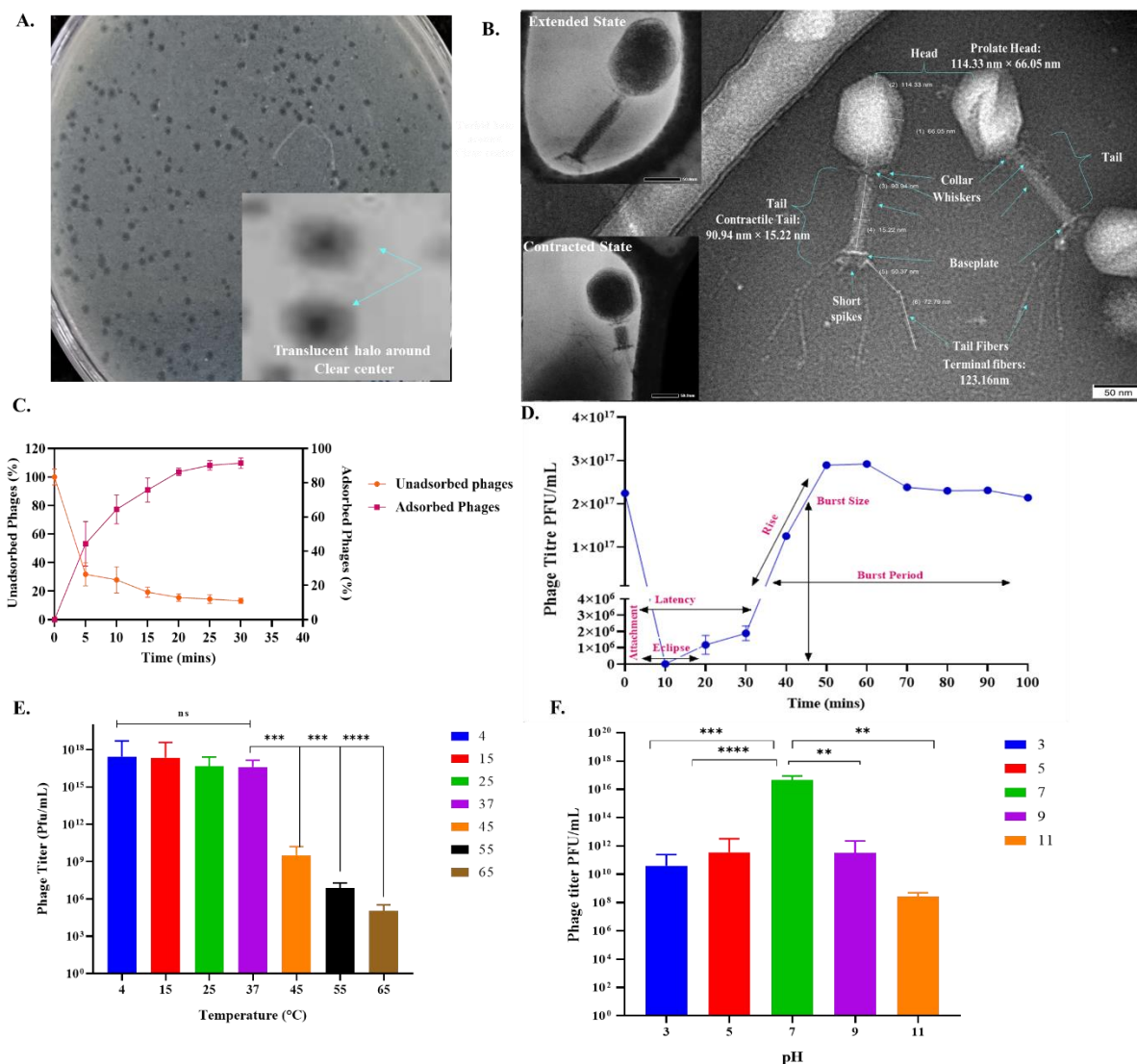


Fig. 1: Morphology and growth parameters of ϕ ERS-1. (A) Plaque morphology. The blue arrows point to the zoomed view of plaques, showing a clear center surrounded by a translucent halo. (B) HR-TEM micrographs of ϕ ERS-1 at 80000 $\times$, and scalebar of 50 nm. The inset is STEM images of ϕ ERS-1 in extended and contracted states. (C) Graphical representation of the percentage of adsorbed and unadsorbed phage particles of ϕ ERS-1 as a function of time. (D) One-step growth curve of ϕ ERS-1 with the phases of its lifecycle marked (pink). (E) Illustration of the effect of temperature on the stability of ϕ ERS-1. (F) Effect of various pH ranges on the stability of ϕ ERS-1. Data in the graph panels from C-F represent means of the experimental replicates, standard deviations (SD), and statistical significance of the data (*, **, ***, ****) using RM- one-way ANOVA. The acronym 'ns' implies no significant difference between the test groups.

3.2 Genome analysis, annotation, and proteome of ϕ ERS-1

The genome length of ϕ ERS-1 consists of 175,242 bp and 60.9% of G+C content. Analysis of ORFs revealed the presence of 796 ORFs, mainly comprising hypothetical proteins followed by phage proteins involved in defining phage structure, DNA replication, repair, packing, and lysis of the host. The details and position of various proteins in the genome of ϕ ERS-1 have been illustrated in **Fig 2A**. Phage lysins are considered highly efficient and evolved molecules that can digest the peptidoglycan in the cell wall of bacteria, thereby facilitating the release of viral progeny (Fischetti, 2005). From genome annotation using RAST and Prokka v1314.6 tool, it was evident that lysin was the key enzyme responsible for the host lysis by ϕ ERS-1. Further, from the orthoANI comparison of the genome of ϕ ERS-1 and closely related *Tequatrovirus* members, it was observed that ϕ ERS-1 has an ANI of 94.2% with *Escherichia* phage LH2, indicating close relatedness of its genome (**Fig 2B**). Additionally, the intergenomic similarities analysis with the VIRDIC tool revealed only 19% similarity of this phage with its closest relatives, indicating distant relatedness of the genome of phage ERS-1 (**Fig 2C**).

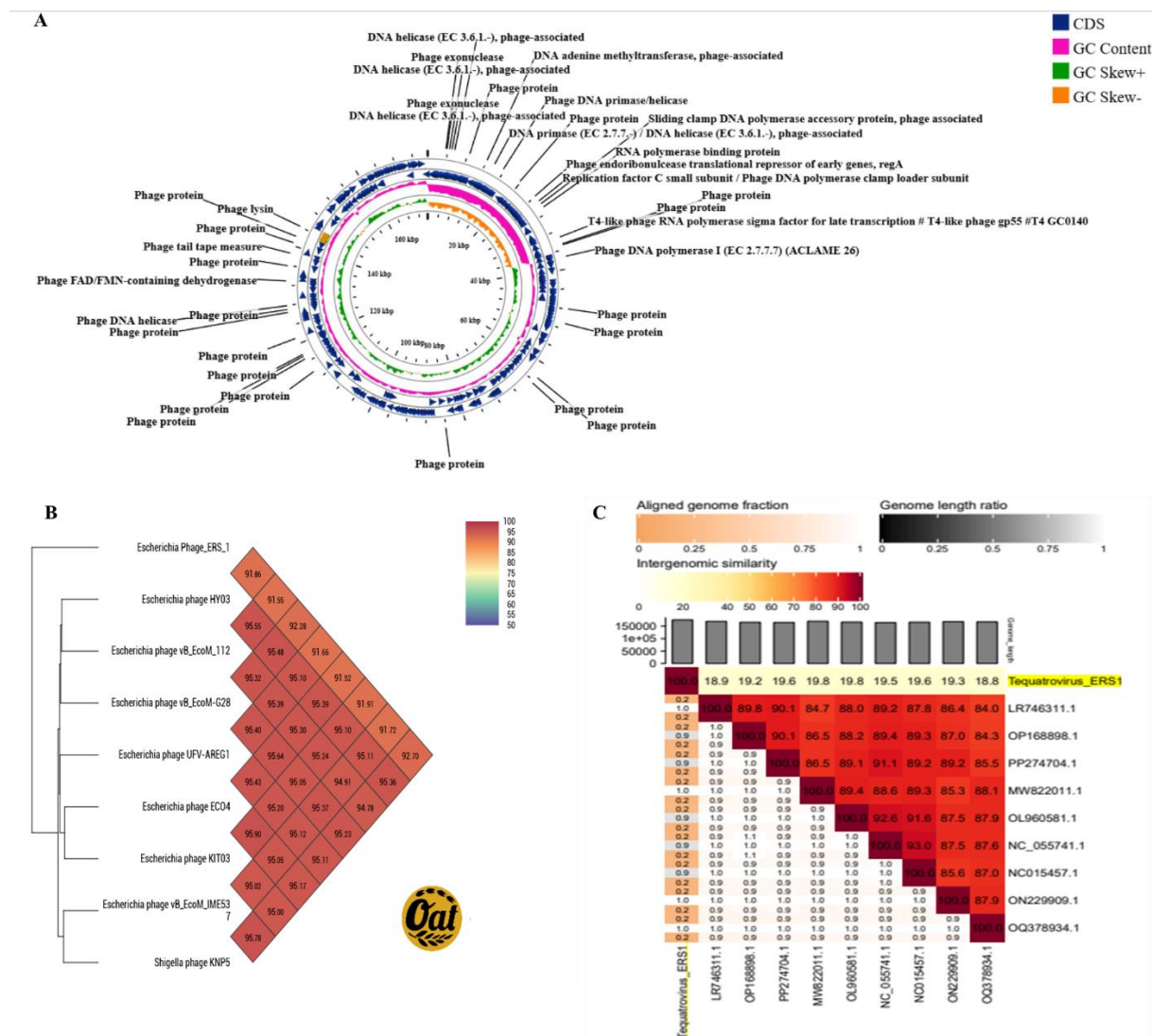


Fig. 2: Comparative genomic and phylogenetic analysis of ϕ ERS-1. (A) Circular genome map of ϕ ERS-1 created with Proksee viewer. The outer blue arrows are the RAST annotated protein-coding genes on the positive and negative strands of the DNA. The inner orange skew displays the total GC content, followed by GC skew information in the positive (green) and negative (purple) strands. (B) Heatmap of OrthoANI between ϕ ERS-1 and closely related *Tequatroviruses*, computed with OAT software. The color scale (top right) contains values defining the similarity percentage between the genomes. The numerical values of intergenomic genetic distance (from OAT software) are marked on the branches. (C) The heat map shows alignment indicators (left half) and intergenomic similarity values (right half) generated through VIRDIC.

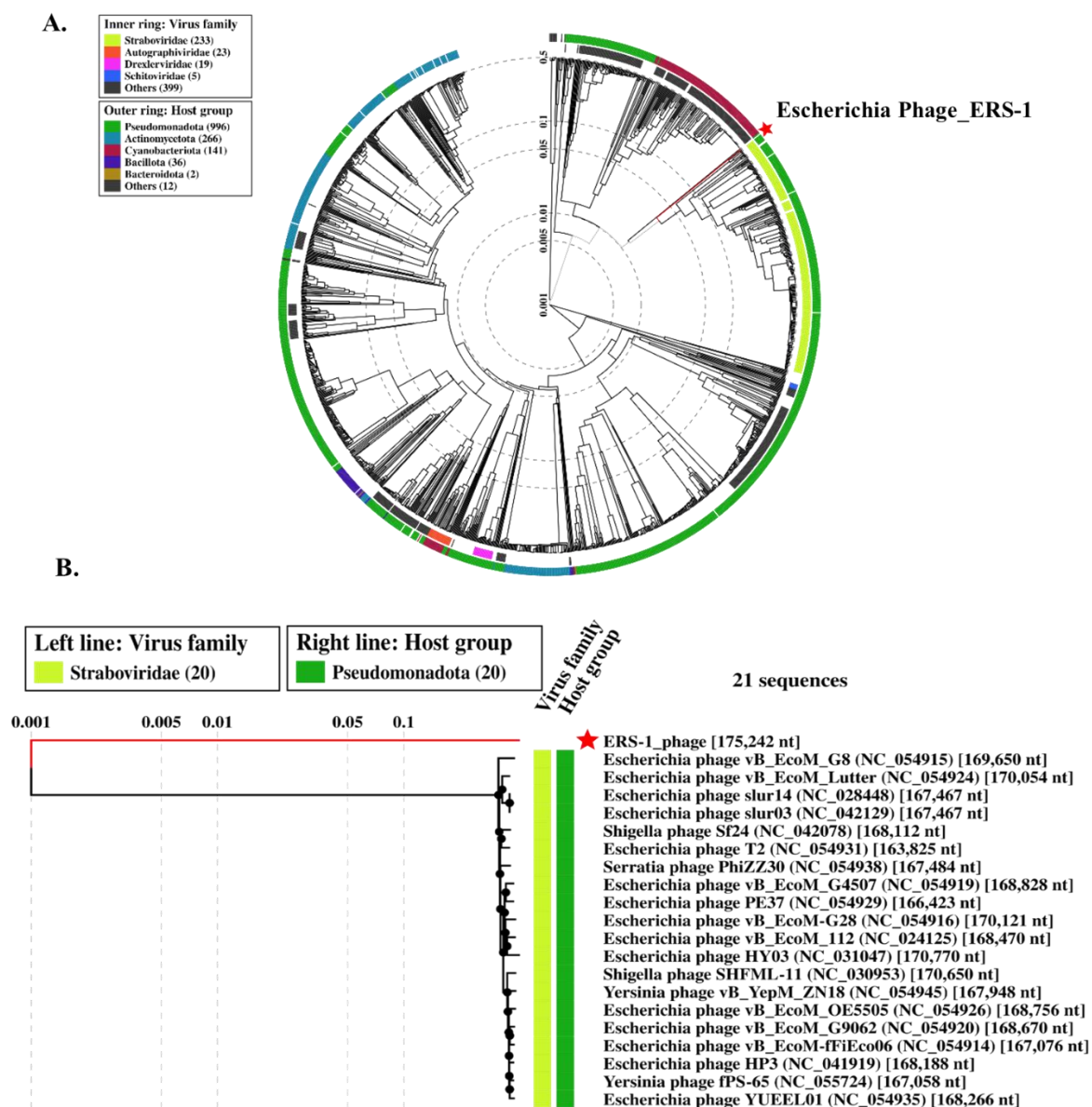


Fig. 3: Comparative amino-acid-based phylogenetic analysis of ϕ ERS-1. (A) A circular phylogenetic tree generated by VipTree for all 3036 reference genomes of phages related to ϕ ERS-1. The genome distance matrix of the phage under consideration and its relatives are calculated by BIONJ (an algorithm based on the distance for phylogeny reconstruction (Gascuel, 1997)). The midpoint of the tree marks the root. The outer-colored rings (host taxa) and the inner-colored rings (viral families). (B) Based on the SG scores, a rectangular phylogenetic tree of ϕ ERS-1 with its 21 closely related and 3 distantly related phage genomes. The red stars represent Escherichia phage ϕ ERS-1.

However, VIRIDIC is not able to capture similar relationships between the related viruses, which have regions of similarity of less than 65%, a limitation inherent to BLASTN. Therefore, protein-based analysis is recommended to clarify the phylogenetic relationships. Analysis of amino-acid sequences in the whole genome of ϕ ERS-1 with all the available reference phages and closely related phages was performed using VipTree. A total of 3036 reference sequences of the phage genomes were used to construct the phylogenetic tree (**Fig 3A**). The preliminary observation of the circular phylogenetic tree of ϕ ERS-1 was consistent with morphological features of 'T4 like myovirus'. Further, depending on the S_G scores of tblastx, a subset of 21 closely related phages and three distantly related phages were chosen to construct a rectangular phylogenetic tree (**Fig 3B**). The genome of ϕ ERS-1 clustered with some *Escherichia* phages, followed by *Shigella* phages, all belonging to *Tequatrovirus*, a genus of the *Straboviridae* family, according to ICTV. As per ICTV, the isolated phage(s) are classified as a member of the same genus if their identities of nucleotide sequences are >70% and a distinct species if ANI is $\leq 95\%$ (Bin Jang et al., 2019). The above nucleotide and amino-acid-based analysis of ϕ ERS-1 suggested that ϕ ERS-1 could be classified as a new member within the *Straboviridae* family and *Tequatrovirus* genus. The genome of this phage has been submitted to NCBI and has been assigned accession number PP337211.

The members of *Tequatrovirus* are known to infect *Escherichia*, *Enterobacteria*, *Shigella*, *Salmonella*, *Yersinia*, *Aeromonas*, *Burkholderia*, *Stenotrophomonas*, *Prochlorococcus*, *Synechococcus*, *Citrobacter* and *Staphylococcus* (<https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=10663>). However, it is noteworthy that there are no reports of *Tequatrovirus* infecting *P. aeruginosa* in the NCBI database. Hence, to our knowledge, this could be the first report detailing a complete genome of a *Tequatrovirus* member capable of infecting *P. aeruginosa* ATCC 9027.

3.3 Anti-Biofilm Potential of ϕ ERS-1.

3.3.1 Single, Multi-strain, Mixed-Genera Biofilm Inhibition Spectra

Several reports have suggested the occurrence of single species biofilm and mixed or multi-species/ mixed biofilms in nature. The most frequently encountered bacteria in the formation of biofilm in the environment, medicine, food industry, agriculture, and animal husbandry include members of *Escherichia*, *Shigella*, *Salmonella*, *Pseudomonas*, *Acinetobacter*, *Klebsiella*, *Enterococcus*, *Streptococcus*, and *Staphylococcus* (Burmølle et al., 2014; Denissen et al., 2022; Mouiche et al., 2019; Sharma et al., 2023; Touthik et al., 2022).

Given this, we evaluated the potentials of ϕ ERS-1 for inhibition of biofilm by single species, multiple strains, and multiple genera, using the information of its host range obtained through spot assay. Results of the CV assay indicated that ϕ ERS-1 could inhibit and reduce 96.17% of the biofilm mass produced by *E. coli* (ATCC 8739) (**Fig 4A, E**).

Interestingly, it was observed that the addition of ϕ ERS-1 to multiple strains of *E. coli* (ATCC 8739, 25922, 43888) (**Fig 4B, E**) and multiple genera (*P. aeruginosa* (ATCC 9027), *S. boydii* (ATCC 9207), and *E. coli* (ATCC 8739) (**Fig 4 C, E**) inhibited the formation of biofilm by 56.2% and 54% respectively. This is an important observation, as bacterial biofilms are more resistant to external factors and treatment by antimicrobial and chemical agents than their planktonic counterparts. The ability of ϕ ERS-1 to render the cells in their planktonic form could be attributed to the presence of polysaccharide depolymerases in the either free or bound state, which is defined by the semi-transparent halo around phage plaques as evident in (**Fig 1A**). The other plausible mechanism behind the broad-spectrum antibacterial and antibiofilm nature of ϕ ERS-1 could be the production of certain quorum-quenching enzymes or lipases. Quorum-quenching enzymes in phages are responsible for inhibiting quorum sensing by mixed species/genera biofilms composed of *P. aeruginosa* and *E. coli* (Liu et al., 2022; Pei and Lamas-Samanamud, 2014). On the other hand, lipases disperse biofilms by hydrolyzing lipids in the bacterial cell membrane (Azeredo et al., 2021). However, there is limited information about these enzymes. Therefore, to have a holistic understanding of the mechanism of bacterial lysis and broad host specificity, an in-depth study of phage-derived proteins of ϕ ERS-1 is necessary.

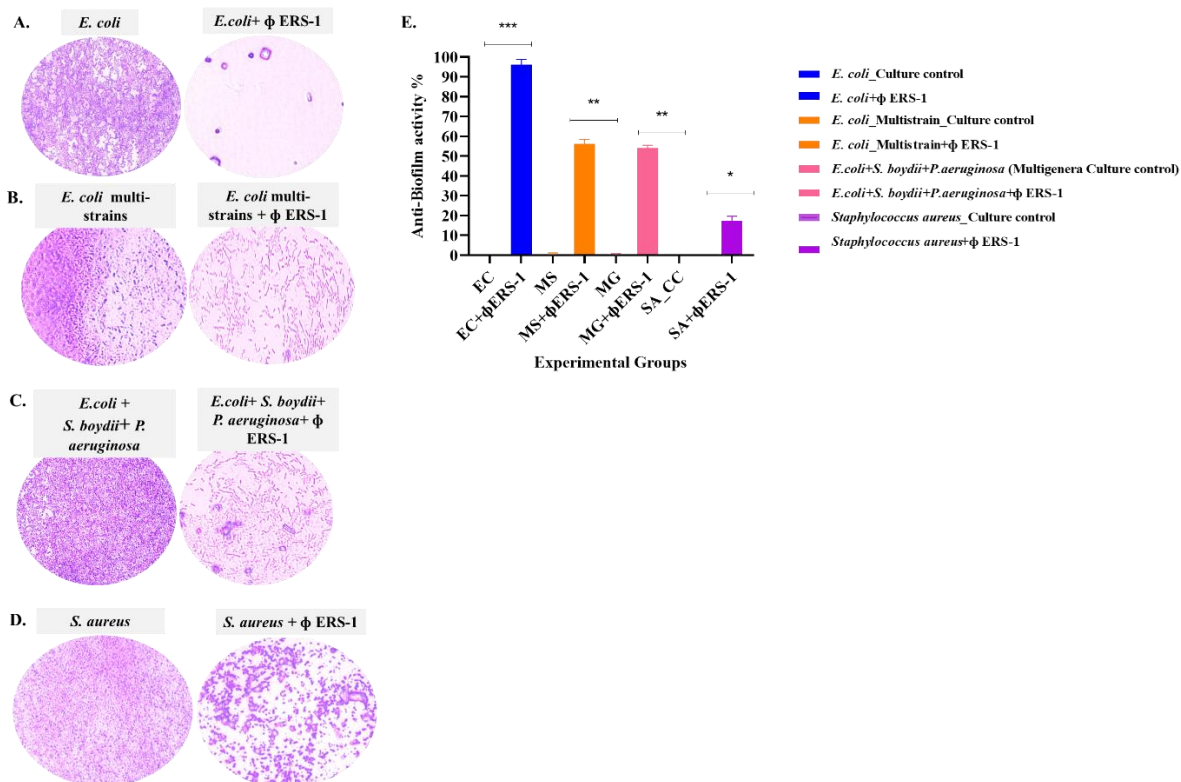


Fig. 4: Effects of ϕ ERS-1 in biofilm inhibition by single species, multi-strains, and multiple genera of bacteria with CV assay. Figure 4A-D are CV-stained microscopic images of biofilm, observed under an oil immersion lens (100 $\times$) after the control and treated groups were incubated for 24 hours. **(A)** Single species biofilm of *E. coli* culture control (left), *E. coli* treated with ϕ ERS-1 (right). **(B)** Multiple strains of *E. coli* mixed to form biofilm-culture control (left), multi-strain group treated with ϕ ERS-1(right). **(C)** Biofilm formation by multiple genera – culture control (left), multiple genera group treated with ϕ ERS-1. **(D)** Biofilm formation by *S. aureus*- culture control (left), and ϕ ERS-1 treated (right). **(E)** Quantification of biofilm formation with CV assay, followed by absorbance at 590 nm.

3.3.2 Time-dependent evaluation of *E. coli* biofilm inhibition and disruption

E. coli is a prominent fecal indicator in the waterways. The biofilm formation ability of *E. coli* on the surface of water treatment pipes and filters poses a significant challenge for treating and preventing this bacteria during disinfection and recycling practices of water and wastewater (Qian et al., 2022; Steven et al., 2022). From the genome annotation study, the biofilm composition of the *E. coli* host in this study was linear homopolymer poly-beta-1,6-N-acetyl-D-glucosamine (beta-1,6-GlcNAc; PGA). The *pgaABCD* operon's gene products are necessary for forming and maintaining biofilm structural stability in several enteric pathogenic *E. coli* (Itoh et al., 2008). Therefore, in this study, we evaluated the potentials of ϕ ERS-1 in

the prevention (inhibition) and dispersal (disruption) of biofilm by *E. coli* ATCC 8739 in a time-dependent manner.

The CLSM analysis of ϕ ERS-1 treated *E. coli* showed red fluorescence at 6, 12, and 24 hours, indicating the dead biomass of *E. coli* cells (**Fig 5 B-D**), as compared to the control (devoid of ϕ ERS-1 treatment) (**Fig 5A**). The presence of live biomass was ascertained through CFU counts of viable cells. Results indicated a significant inhibitory effect on biofilm formation at 6 hrs., which continued up to 24 hours (ANOVA, $p < 0.0001$) (**Fig 5D**). Previous studies have demonstrated the antibiofilm effect of *Tequatrovirus* against *E. coli* and *Salmonella*, with bacterial reduction from 2-5 logs within 6-24 hours (Liao et al., 2022; Zhou et al., 2022). However, in our study, for the first time, we report the potential of ϕ ERS-1 in reducing the abundance of *E. coli* cells from $6.14 \log_{10}$ CFU/mL at 6 hours to $8.22 \log_{10}$ CFU/mL at 24 hours, with a $\sim 100\%$ reduction in the total viable counts. This data suggests that ϕ ERS-1 poses an inhibitory effect that could be used for biological control of surface colonization by *E. coli* cells either through inhibition of their initial attachment or impediment of bacterial establishment on various surfaces.

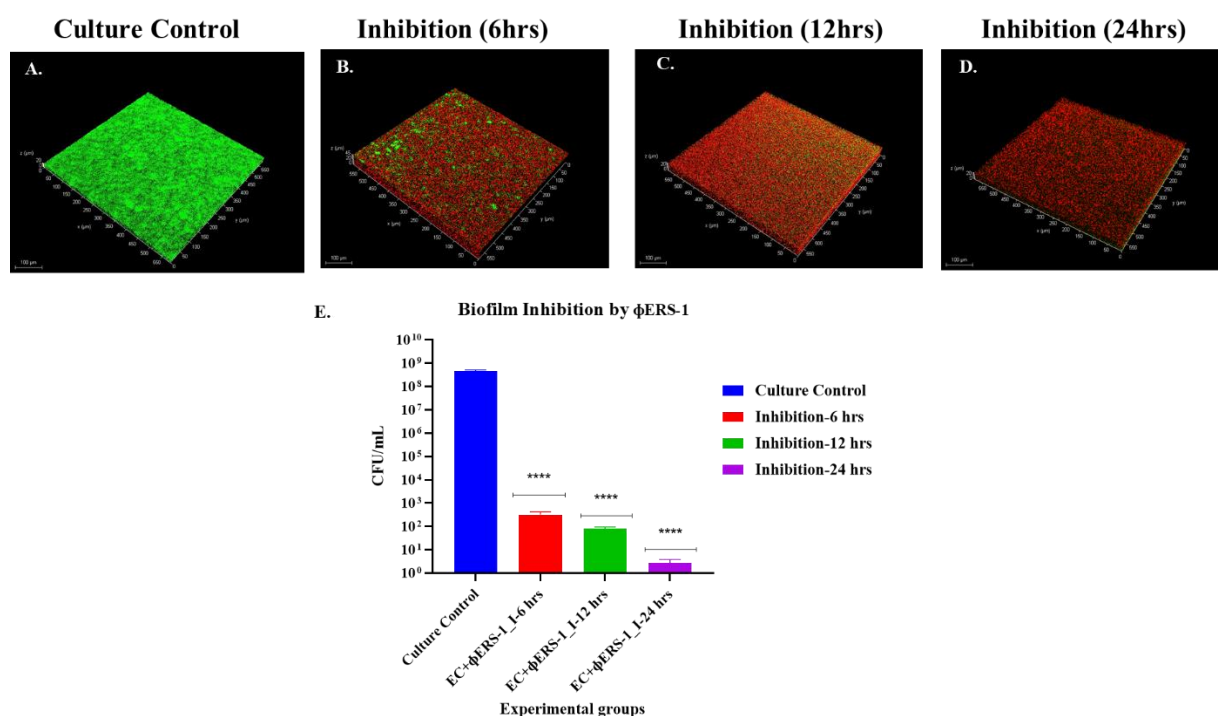


Fig. 5: Inhibitory effect of ϕ ERS-1 on biofilm formation by *E. coli* (ATCC 8739). Samples were observed under a Leica Stellaris 5, DMI8 microscope with a 20 $\times$ objective. The red fluorescence indicates dead *E. coli* cells, while the green fluorescence indicates viable cells at different time intervals. **(A)** CLSM micrograph of *E. coli* cells with no treatment, capable of

forming biofilm after 24 hours. **(B)** CLSM micrograph of ϕ ERS-1 treated group imaged after 6 hours. **(C)** CLSM micrograph of ϕ ERS-1 treated group imaged after 12 hours. **(D)** CLSM micrograph of ϕ ERS-1 treated group imaged after 24 hours. **(E)** Quantification of the viable cells as CFU/mL of the control (non-treated) and ϕ ERS-1 treated sets.

In natural environments, most biofilms are in their mature state, and therefore, to detach the biofilm, disruption of EPS polymers followed by planktonic cell dispersal is essential (Shrestha et al., 2022). Capsular polysaccharides as major virulence factors have been reported for *A. baumannii* (Wang et al., 2020), *K. pneumoniae* (García et al., 2019), and *E. coli* (Guo et al., 2017) in biofilm formation. Thus, the antibiofilm effect of ϕ ERS-1 in disintegrating matured or pre-formed biofilms was evaluated with CLSM and counts of viable cells using CFU/mL.

It was observed that the number of dead/ non-viable cells increased in the phage-treated group for 24 hours **(Fig 6A-D)**. This indicates a gradual disruption of *E. coli* biofilm by ϕ ERS-1. The total viable counts at each time point revealed that ϕ ERS-1 was capable of disrupting the pre-formed biofilm layer of *E. coli* with a reduction of 2.4 log₁₀ CFU/mL at 6 hours, followed by 2.70 log₁₀ CFU/mL at 12 hours, and 3.88 log₁₀ CFU/mL at 24 hours. These values indicate that ϕ ERS-1 can disrupt biofilm with a percent reduction of viable cells up to 99% with 6 hours of treatment. After 24 hours, the percent reduction of *E. coli* cells observed in the biofilm was 99.98% (ANOVA, p=0.0022). **(Fig 6 K)**.

Additionally, the FESEM imaging of the control and ϕ ERS-1 treated biofilm after 24 hours confirmed disruption of the pre-formed biofilms. The cells of the control set showed a well-defined, rod-shaped morphology embedded in a matrix of EPS **(Fig 6 E-G)**, while the ϕ ERS-1 (24 hrs.) treated set showed a distorted morphology within the biofilm **(Fig 6 H-J)**. The planktonic bacteria within biofilms are highly resistant to the action of antibiotics, disinfectants, and disruption by physical or chemical ways (Kovacs et al., 2023). However, the experimental evidence in this study suggests the efficacy of ϕ ERS-1 in biofilm disruption and its bacteriolytic effects against the planktonic cells, demonstrating its potential applications.

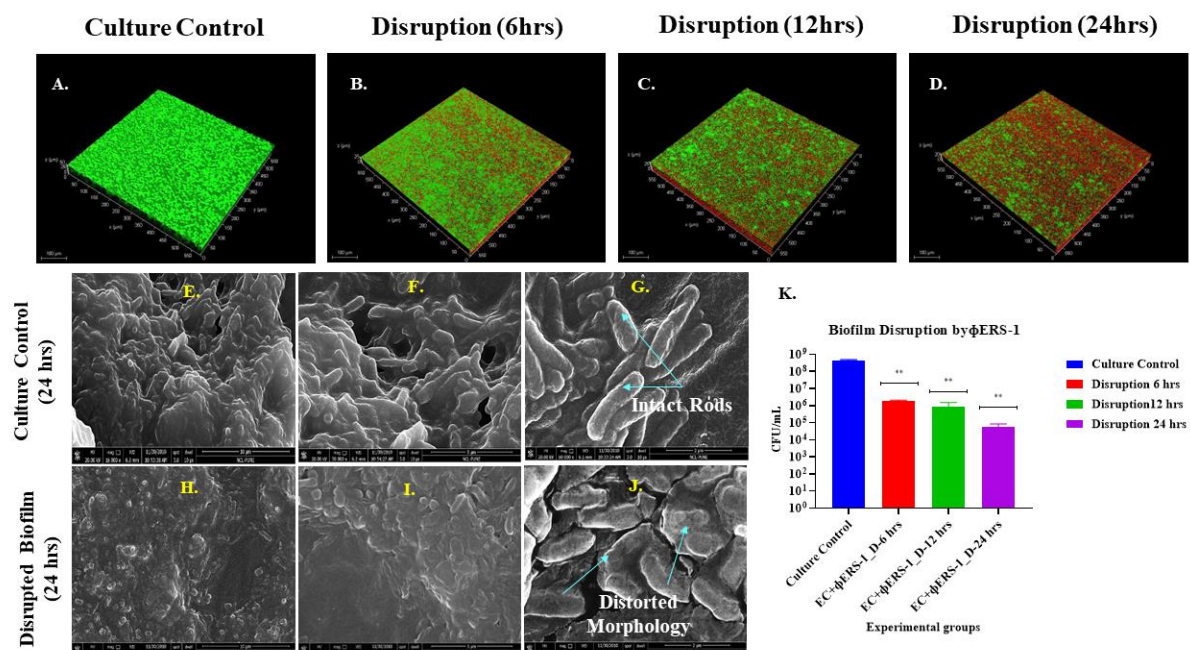


Fig. 6: Effect of ϕ ERS-1 on pre-formed/ matured biofilm of *E. coli* (ATCC 8739). The control and treatment sets visualized through CLSM with 20 $\times$ objective at specified time intervals are represented in the panels as (A) CLSM micrograph of culture control with pre-formed biofilm of *E. coli* cells devoid of any treatment. (B-D) CLSM micrograph of ϕ ERS-1 treated pre-formed biofilm imaged after 6 hours, 12 hours (C), and 24 hours (D). (E-G) FE-SEM images of control biofilm at different magnifications 16000 $\times$ (E), 30000 $\times$ (F), and 60000 $\times$ (G), showing intact rods immersed in the EPS matrix of the biofilm. (H-J) FE-SEM images of ϕ ERS-1 treated biofilm at different magnifications 16000 $\times$ (H), 30000 $\times$ (I), and 60000 $\times$ (J), showing disrupted EPS matrix and distorted morphology of compromised cells in the biofilm. (K) Quantification of the viable cells in disrupted biofilm as CFU/mL of the control (non-treated) and ϕ ERS-1 treated sets.

3.4 Bacteriophage-based reduction of bacterial counts from wastewater

The disinfection of the WW to make it safe is crucial to protecting the health of the environment, humans, and animals (Regulation EU 2020/741). Failure to do so could be responsible for different diseases because of various pathogens. According to this regulation, the reclaimed water is monitored based on various pathogen indicators. For bacteria: *E. coli*; for pathogenic viruses: coliphages; and protozoa: the spores of *Clostridium perfringens* or sulfate-reducing bacteria (Hernández-Crespo et al., 2022; Kadlec and Wallace, 2008). Therefore, considering the broad-spectrum host range, bacteriolytic, and antibiofilm potentials

of ϕ ERS-1, we focused its application on reducing the bacterial load from untreated wastewater/ raw sewage.

The initial screening results showed a significant reduction of the bacterial load of $2.27 \log_{10}$ CFU/mL in the ϕ ERS-1 treated group of the raw sewage after 24 hours ($p < 0.001$) (**Fig 7 A, B**). Further, a time-kill assay was performed to evaluate the magnitude of reduction in the viable coliform load in control and ϕ ERS-1-treated groups. The rationale behind plating the raw sewage and coliform enriched (in McConkey broth) on BGLB agar and EMB agar was differentiation and specific enumeration of the coliform bacterial load. Results after 24 -48 hours of incubation revealed a significantly lower number of viable bacteria in the ϕ ERS-1 treated group in both raw sewage (ANOVA, $p < 0.01$) and coliform enriched group (ANOVA, $p < 0.001$). Interestingly, in the set of ϕ ERS-1 treated raw sewage, after 24 hours, a 2- $2.4 \log_{10}$ reduction was observed compared to the control group. Notably, in the coliform enriched set of raw sewage, $4.2 \log_{10}$ CFU/mL reduction was observed in the ϕ ERS-1 treated group (**Fig 7 C-E**). Additionally, the CLSM imaging of aliquots also revealed an increase in the dead cells (red) over time in the ϕ ERS-1 treated group of unenriched and coliform-enriched sewage (**Fig 7 F**) compared to the control group. This indicates that ϕ ERS-1 could be used as a green approach to reduce the coliform counts.

There are few reports describing the use of phages in wastewater treatment to reduce the bacterial load and the use of phage cocktails to eliminate waterborne pathogenic bacteria (Grami et al., 2022; Jassim et al., 2016; Periasamy and Sundaram, 2013). However, to the best of our knowledge, this is the first study to highlight the use of single polyvalent, non-engineered (naturally occurring) phage ϕ ERS-1 to reduce the coliform load in wastewater. Also, this is the very first kind of study detailing an in-depth characterization, antibacterial, and antibiofilm potentials, together with lab-scale evaluation of reduction in coliform counts in raw sewage by a novel phage ϕ ERS-1 isolated from the untapped location of the Ganges River. However, a detailed understanding of the optimized phage dose and its effect on physicochemical and microbiological parameters before and after ϕ ERS-1 treatment would be necessary for its on-site application. Additionally, to overcome resistance by bacteria in the long run, combining various phages with ϕ ERS-1 or using phage-derived enzymes from ϕ ERS-1 would benefit its application under one health canopy, promoting human, animal, and environmental health.

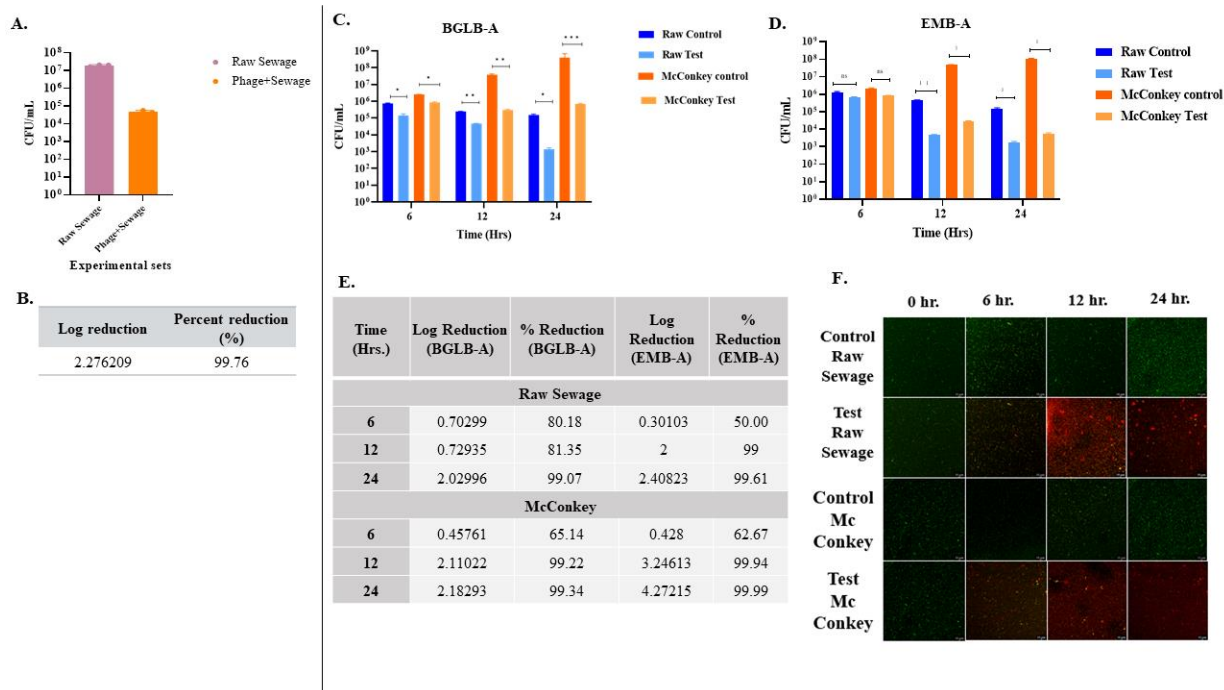


Fig. 7: Evaluation of ϕ ERS-1 in reduction of coliform counts. (A) Graphical representation of quantification of the viable cells in raw sewage as CFU/mL of the control (non-treated) and ϕ ERS-1 treated sets. (B) Log reduction and percentage reduction values indicate ϕ ERS-1 efficacy in reducing bacterial load in raw sewage after 24 hours. (C, D) Graphical representation of quantification of the viable cells in raw and coliform enriched sewage as CFU/mL of the control (non-treated) and ϕ ERS-1 treated sets on BGLB-agar (C), and EMB agar (D), as a function of time. (E) Log reduction and percentage reduction values indicate the time-dependent efficacy of ϕ ERS-1 in reducing bacterial load in raw and coliform-enriched (McConkey broth) sewage. (F) CLSM micrographs showing live (green) and dead (red) bacterial cells in the raw (unenriched) sewage and coliform-enriched (McConkey) sewage at different time intervals.

4. Conclusion

This study demonstrates the isolation and characterization of a novel phage ϕ ERS-1 from an untapped location of the Ganges River. The strength of our research lies in the novelty of phage and its bacteriolytic and antibiofilm potentials in reducing the bacterial load of environmentally relevant waterborne pathogens in their planktonic cells and biofilm form. The concept of controlling bacteria using phages and their enzymes without the aid of chemical or UV disinfection is a challenging one. However, the data also shows the efficacy of the newly isolated, high titer ϕ ERS-1 in controlling the bacterial (coliform) load in the sewage water and

possibly improving the water quality. Our approach of using polyvalent phage as a green alternative for bacterial biocontrol of sewage water highlights the importance of the potential applications of such broad-spectrum bacteriophages to improve the quality of the effluent and disposal of sludge in the environment. This approach can significantly impact the delivery of sustainable development goals 6: clean water and sanitation, 3: good health and well-being, and 11 sustainable cities and communities. Moreover, using lytic phages to improve wastewater quality would substantially reduce the load on other treatment methods, thereby contributing to low energy consumption levels, reduced use of harmful chemicals, and improved access to water reuse with reduced biological contamination, promoting a circular economy.

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4.2 Characterization of A Novel Pbunavirus Phage from the Pristine Stretch of The Ganges River and its Evaluation in Abatement of Multidrug-Resistant P. aeruginosa from Raw Sewage

Abstract

Background

The environment, especially the wastewater, can act as a reservoir of pre-existing resistant pathogens. Apart from MDR *E. coli*, high levels of *P. aeruginosa* have been reported in sewage water and WWTP sludge, consistent with levels found in hospital wastewater. Therefore, the treatment of raw sewage wastewater for abatement of MDR *P. aeruginosa* is essential for safe effluent disposal.

Rationale

With increased AMR and biofilm formation abilities, *P. aeruginosa* bugs are gaining resistance to the action of disinfectants, rendering them ineffective. The metagenomic analysis has revealed the presence of promising lytic phages within the pristine stretch, targeting putative putrefying and potentially pathogenic bacteria. However, the potential of these phages remains unexplored in mitigating MDR strains of *P. aeruginosa* from sewage water.

Results

The present study is the first report of a novel phage (ϕ PRS-1) isolated from a pristine stretch of the Ganges River as a potent disinfectant to tackle the MDR strain of *P. aeruginosa* PAO1 (ATCC 15692). The ϕ PRS-1 is a new species within the *Pbunavirus* genus. The ϕ PRS-1 at its 0.0000001 MOI could reduce the abundance of planktonic cells of PAO1 by 8.14 log₁₀ CFU/mL within 24 hours. The antibiofilm biofilm data of ϕ PRS-1 against PAO1 showed a log₁₀ reduction of 9.83 (inhibition) and 7.01 (disruption) after 24 hours. ϕ PRS-1 treatment of raw sewage combined sludge showed a 4.2 log₁₀ CFU/mL reduction in the *P. aeruginosa* counts, indicating its efficacy. Additionally, the *in-vitro* cytotoxic studies indicate no apparent cytotoxic effects exerted by this phage.

Significance

The use of a single, absolute lytic phage in the reduction of PAO1 from contaminated surfaces serves as a green approach to specifically target the MDR *P. aeruginosa* and also tackle the dissemination of the pathogen-associated AMR. This would aid in reducing the load of other chemical disinfectants, thereby contributing to the environmental sustainability and delivery of various SDGs like 3, 6, 11,14, and 15.

1. Introduction

Antimicrobial resistance (AMR) has been identified among the top ten global public health concerns by the World Health Organisation (WHO). AMR has also set a significant barrier to attaining universal healthcare, consequently hampering the delivery of various sustainable development goals (SDGs) like SDGs 2, 3, 6, 8, 11, 12, 14, and 15 (Ferdinand et al., 2023). Wastewater treatment plants (WWTPs) consistently receive a flow of human-associated antibiotic-resistant bacteria (ARB), antibiotic-resistance genes (ARG), antibiotics, disinfectants, and metals. These substances coexist within the treatment plants alongside the normal microbiota, creating ideal conditions for the emergence, accumulation, and spread of antibiotic resistance (Marutescu et al., 2023). The most concerning resistant pathogens recognized by the acronym ESKAPE encompasses *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (which may include *Escherichia coli*) (De Oliveira et al., 2020; Rice, 2008).

Several scientific studies have documented the existence of clinically significant ARBs and ARGs in the natural environment, such as freshwater and wastewater (Denissen et al., 2022; Nnadozie and Odume, 2019; Pazda et al., 2019). In fact, it's estimated that approximately 46.4% of bacteria isolated from hospitals, sewage plants, and pharmaceutical factories exhibit resistance to multiple antibiotics (Baquero et al., 2008). In developing nations, where water and sewage treatment infrastructure may be inadequate, there is a greater likelihood that water serves as a significant pathway for the transmission of resistant bacteria and ARGs originating from humans and animals (Aubertheau et al., 2017). Consequently, the environment, especially the wastewater, can act as a reservoir of pre-existing resistant pathogens or acquire ARGs from other bacteria associated with humans, animals, or environmental resistome (Kang et al., 2024; Kumar and Pal, 2018; Rodriguez-Mozaz et al., 2015). Apart from MDR *E. coli*, high levels of *P. aeruginosa* have been reported in sewage water and WWTP sludge, consistent with levels found in hospital wastewater (Slekovec et al., 2012). The presence of various virulence factors such as exopolysaccharide (EPS), flagella, and pili confer this bacterium with a remarkable fitness and ability to adapt and thrive in various environments. Additionally, the intrinsic and acquired antibiotic resistance, together with the biofilm-forming potentials of *P. aeruginosa*, have led to rising incidences of MDR strains with practically ineffective antibiotics availability to put an end to this bacterium (Chegini et al., 2021; Silva et al., 2023).

Studies have demonstrated that MDR *P. aeruginosa* strains discharged from hospital WWTPs and sewage waters into rivers and, therefore, pose a considerable public health risk to residents

living in contact with the contaminated water (Denissen et al., 2022; Magalhães et al., 2016; Marutescu et al., 2023). Moreover, high-risk *P. aeruginosa* clones have been identified throughout wastewater networks and recovered from treated water and downstream rivers (Marutescu et al., 2023; Slekovec et al., 2012). Some studies have reported the presence of MDR and extended drug-resistant (XDR) *P. aeruginosa* isolates in clinical and urban systems, indicating their potential to colonize sewer and drinking water plumbing systems (Müller et al., 2018). These resistant isolates have a considerable count in treated wastewater effluents and have been reported for their continuous release into natural water basins, primarily through sewage, and potentially spread to the soil via natural fertilizers (Hocquet et al., 2016; Kerr and Snelling, 2009; Marutescu et al., 2023; Slekovec et al., 2012). Therefore, the treatment of raw sewage wastewater for abatement of MDR *P. aeruginosa* is essential for safe effluent disposal. To address the issue of AMR and control microbial contamination, bacteriophages have resurfaced as eco-friendly biocontrol agents (Soliman et al., 2023).

The Ganges River in India has been renowned since ancient times for its unique attributes of self-cleansing (non-putrefying) and extraordinary healing. These unique antibacterial properties were first demonstrated by Ernst Hankin in 1896 against *Vibrio cholerae* in 1896. However, a comprehensive understanding of detailed insight into these antibacterial attributes was offered by Félix d'Hérelle, who coined the term bacteriophage for the antagonist microbe of *V. cholerae* (Samson et al., 2024). However, there are limited studies with this riverine system wherein novel virulent phages were isolated from its waters against MDR strains of *K. pneumoniae* (Sundaramoorthy et al., 2021) and *P. aeruginosa* (Rathor et al., 2022). Our recent study on the sediments of the river Ganges along its 1500 km has identified the repertoire of bacteriophages and their associated host-phage functions against putative human, plant, and putrefying pathogens (Samson et al., 2023). Therefore, this unique aquatic ecosystem provides an opportunity to bioprospect its untapped and unique phage diversity for its potential applications. Given this, the present study was initiated to explore the untapped phage diversity from the pristine stretch of the River Ganges to isolate novel phages, characterize them, and explore their potential for tackling the MDR *P. aeruginosa* from raw sewage wastewater in combination with the sludge to facilitate their improved management at the environmental, animal and human interface.

2. Materials and Methods

2.1 Bacterial strain and antimicrobial susceptibility profiling

Pseudomonas aeruginosa PAO1 (ATCC 15692) (PAO1) was the bacterial host used in this study. The genomic identity of the isolate was ascertained with the MinION Mk1C Nanopore sequencer (Oxford Nanopore Technologies). The antimicrobial resistance profile of the isolate was determined using the VITEK 2 system (bioMérieux). Two distinct sets of antimicrobial susceptibility testing (AST) cards were used, namely AST-N235 and AST-N281. The AST profiles were generated following the prescribed parameters recommended by the Clinical and Laboratory Standards Institute (CLSI).

2.2 Isolation, enrichment, and propagation of phage PRS-1 targeting PAO1

Phage isolation against the primary host PAO1 (ATCC 15692) was carried out using water-saturated sediment samples obtained from an untapped location of the Ganges River at Maneri (30°45'14" N 78°33'34" E). The rationale behind using samples from pristine stretches of the Ganges was to explore and harness the potential of phages from the unexplored glacier-fed riverine stretch. A distinct method was employed to isolate bacteriophages against PAO1. The sediment samples (n=3, 50 gm each) were vortexed at maximum speed for 30 minutes at room temperature. After a brief period of settling, the sediment-laden water (~50 mL) was equilibrated with 50 mL of SM buffer for 4-5 hours in an incubator set at 37°C at 120 rpm. Subsequently, the resultant mixture was centrifuged (Eppendorf centrifuge, 5804 R) at 6000×g for 10 minutes, and the supernatant (~50 mL) was then filtered sequentially through 0.45µm and 0.22µm Polyether sulfone (PES) syringe filters (Hi-Media). Ten milliliters of filtrate from each set were selectively used for the enrichment of potent phages against PAO1.

A total of 5 mL of PAO1 culture at the brink of log and stationary phase was grown in soybean-casein digest broth (SCDB) (MH011-500G, Hi-Media). Subsequently, 10 mL of the filtrate acquired from the initial enrichment step was inoculated with this culture. The enrichment flasks were placed in an incubator set at 37°C, 180 rpm for 24 hours. The presence of lytic phages in the samples was confirmed by the qualitative spot assay, wherein clear zones (plaques) against the bacterial lawn were observed.

The quantitative enumeration of phages within the enriched lysate was executed using the soft agar overlay method. A singular, well-isolated plaque was selected and reconstituted in autoclaved SM (Sodium-Magnesium) buffer, comprising NaCl (5.8g/L), MgSO₄.7H₂O (2g/L), Tris-HCl (pH 7.4) (50mL), and distilled water (950mL). The phage titer of the released phages

from the individual plaque was ascertained. Each set of plaque was serially diluted and infected with 100 μ L of the log-stationary phase culture of PAO1 (6.5×10^8 CFU/mL). The host-phage mixture was plated using the double-layer agar plating method and incubated at 37 °C for 16-18 hours. Each set was titrated in triplicate across three independent iterations to ascertain the phage titer accurately. Furthermore, the purification and concentration of ϕ PRS-1 were accomplished using the Amicon® Ultra-15 centrifugal filter equipped with an Ultracel 100 kDa membrane. The multiplicity of infection (MOI) and Poisson distribution for predicting the probable number of infected cells in a population (P_0) were calculated using the following formula (Abedon, 2023)

$$\text{MOI} = \text{Number of plaque forming units (PFU)/mL} \div \text{Number of Colony forming units (CFU)/mL}$$

$$(P_0) = 1 - e^{-\text{MOI}}$$

A high titer phage stock of the purified phage lysate containing 10^{10} PFU/mL was made using replicates of a total of 20 confluent lysis plates flooded with 7mL of SM buffer and incubated at 15°C with shaking (100 rpm) for 3-4 hours followed by ultracentrifugation at $14000 \times g$ for 30 mins at 4°C and subsequent filtration of the lysate with 0.2 μ m PES syringe filters. Enumeration of the high titer phage stock was done using the double-layer agar overlay method. The purified high-titer phage stock was stored at 4°C.

2.3 Characterization of PAO1 phage (ϕ) PRS-1

2.3.1 Morphological features

The morphology of ϕ PRS-1 was visualized using Jeol JEM-F2100 high resolution-transmission electron microscope (HR-TEM) at 200 kV and imaged with a Xarosa emsis camera coupled to the microscope. To obtain a detailed (3D) view of the phage morphology, HR-TEM was used in STEM (Scanning Transmission Electron Microscopy) mode, enabling scanning images at nanometer (nm) resolution. Briefly, 1 mL of the high titer stock of ϕ PRS-1 was centrifuged at $12,000 \times g$ for 30 mins, and the pellet was resuspended with 10 μ L of SM buffer. A total of 4 μ L of the sample was placed on a lacey formvar/carbon, 200 mesh, copper grid followed by staining with VitroEase™ Methylamine Tungstate Negative Stain (Thermo Fisher Scientific), following the manufacturer's protocol. Morphological classification of phage was done following the guidelines recommended by the International Committee on Taxonomy of Viruses (ICTV) (Turner et al., 2023).

2.3.2 Phage adsorption rate and one-step-growth curve

An adsorption experiment was performed with certain modifications to understand how fast phage virions can adsorb a target bacterial host cell. The host cell culture at the exponential phase was mixed with the fresh phage lysate at an MOI of 0.1 and incubated at 37°C, 100 for 30 mins. After 5 minutes, a 1 mL sample was drawn and centrifuged to pellet the adsorbed fraction of the phage each time. At the same time, the supernatant was filtered using a 0.2 µm PES syringe filter to obtain the fraction of unadsorbed phages. The fractions were plated using the agar overlay method to get total phage (N_{total}) and free phage (N_{free}) densities, respectively. The assay was carried out in three replicates, and the adsorption curve as a function of the percentage of adsorbed and unadsorbed phages versus time was plotted using GraphPad Prism v9.5.1. The adsorption rate (α) for each time point was calculated as:

$$(\alpha) = \ln \left(\frac{N_{\text{total}}}{N_{\text{free}}} \right) / \text{adsorption time (t)}$$

The determination of the one-step growth curve assay for ϕ PRS-1 was carried out as described by Jagdale et al., 2019 with certain modifications (Jagdale et al., 2019). Briefly, the fresh phage lysate of PRS-1 at an MOI of 0.1 was added to the PAO1 cells (10^8 cells/mL) and allowed to adsorb for 15 mins at 37°C, 100 rpm. The adsorbed phages were centrifuged at $10,000 \times g$ for 5 min, and subsequently, the bacteria-phage complex (pellet) was resuspended in 2 mL of SCDB and incubated at 37°C, 100 rpm for 100 mins. A 100 µL sample was collected every 10 minutes, followed by plaque assay to determine the PFU/mL at each time point. The assay was carried out in triplicates.

2.3.3 Evaluation of pH and temperature stability

The stability of ϕ PRS-1 under different acidic and basic conditions was evaluated as described by (Oliveira et al., 2020) (Oliveira et al., 2020). Briefly, 900 µL SM buffer with varied pH ranges adjusted to 3, 5, 7, 9, and 11 were inoculated with 100µL of the phage suspension (10^{10} PFU/mL), respectively, followed by 4 hours incubation at 37°C, 100 rpm in a dry bath, and the subsequent plaque assay. Similarly, for thermal stability evaluation, suspensions of 100 µL of the phage (10^{10} PFU/mL) were introduced in 900 µL of SM buffer and incubated at a diverse set of temperatures (4, 15, 25, 37, 45, 55, and 65°C) for 4 hours at 100 rpm followed by measuring the phage titer with plaque assay. The assays were carried out at three independent times in triplicates. The statistical significance of the assays was computed in GraphPad Prism v9.5.1 using repeated measures (RM) one-way analysis of variance (ANOVA). Additionally, multiple

comparisons with a false discovery rate (FDR correction) and a p-value of 0.05 were used to compare the mean between the two groups.

2.3.4 Evaluation of the Lytic Spectrum of ϕ PRS-1

The host range of ϕ PRS-1 was evaluated using in-house bacterial host cultures of *Pseudomonas aeruginosa* (ATCC 15692), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* O157:H7 (ATCC 43888), *Shigella boydii* (ATCC 9207, 8700), *Salmonella enterica* (ATCC 12011, 13314), *Staphylococcus aureus* (ATCC 6538), *Listeria monocytogenes* (ATCC 19112), *Enterococcus faecalis* (ATCC 19443) *Yersinia enterocolitica* (ATCC 27729). Details of the susceptibility profile of each host strain with phage PRS-1 have been tabulated in **Table 1**.

Besides this, the lytic spectra of ϕ PRS-1 were evaluated on *P. aeruginosa* isolates obtained from clinical and environmental (sewage treatment plants) sources. Prior to the host range determination, these isolates were initially screened for their antimicrobial susceptibility using various classes of antibiotics. For each of the *P. aeruginosa* isolates, the multiple antibiotic resistance index (MARI) was determined using the protocol followed by Cristina et al., 2021 (Cristina et al., 2021). Based on the MAR index, if the isolate displayed resistance against antibiotics from over three distinct classes, it was categorized as MDR (Magiorakos et al., 2012; Sandhu, 2016). Subsequently, the MDR isolates of *P. aeruginosa* were evaluated for the host range of ϕ PRS-1 through initial spot assay. After the qualitative spot assay, the virulence of phage was determined on MDR *P. aeruginosa* isolates through the efficiency of plating (EOP). In this, the phage titer at terminal dilution on the test strain was divided by the titer of the same phage on its host (isolation) strain. Further, based on the EOP value derived from the phage titer of the test and host strain, the virulence of ϕ PRS-1 against the test strain was classified as highly virulent (EOP 0.1-1.00), moderately virulent (EOP 0.001-0.099), avirulent but active (EOP < 0.001), or avirulent (no plaques detected) (Gibson et al., 2019),

2.3.5 DNA extraction, Genome Sequencing, and Analysis of ϕ PRS-1

A high-titer phage stock was used to isolate DNA from ϕ PRS-1. PureLink Viral RNA/DNA Mini Kit (Thermo Fisher Scientific) was used to extract the DNA, following the manufacturer's protocol. Subsequently, a 16S rRNA gene amplification was done to ascertain that the extracted DNA was devoid of host DNA. The amplicons were evaluated on 0.8% agarose gel electrophoresis together with Gram-positive and Gram-negative bacterial DNA as positive controls and standard molecular weight markers (1Kb). The gel was visualized using the BioEra Gel Documentation System.

The concentration of the phage DNA was measured on a Qubit 4 Fluorometer (Invitrogen) using a dsDNA HS (High Sensitivity) assay kit fluorometer (Invitrogen). For the whole genome library, the samples were prepared using the Ligation Sequencing Kit (SQK-LSK114) and Native Barcoding Kit (SQK-NBD 114.24) as per the manufacturer's instructions and with certain modifications. The library was loaded onto flow cell R10.4.1 (Oxford Nanopore Technologies), and the sequencing run was carried out for ~56 hours using the MinION Mk1C sequencing platform.

The reads were processed from the sequencing data for quality control and adapter trimming using FastQC (Galaxy Version 0.25.1+Galaxy0) and Porechop (Galaxy Version 0.2.4+galaxy0). Flye, a *de novo* genome assembler v 2.9.1, was used for genome assembly. The sequence homology for the generated assembly was validated with known phage sequences using NCBI nucleotide BLAST (BLASTN). Further, the annotation of the putative proteins in the phage genome annotation was predicted with Rapid Annotation using the Subsystem Technology (RAST) annotation server (<https://rast.nmpdr.org>). The GenBank file generated from RAST was used in Proksee for in-depth Analysis and visualization of the phage genome (Grant et al., 2023).

The protein-coding sequences in the phage genome were identified with an open reading frame (ORF) finder from NCBI (<https://www.ncbi.nlm.nih.gov/orffinder/>). Additionally, the genome-wide similarities between ϕ PRS-1 and other reference viral genomes were analyzed using VipTree (Nishimura et al., 2017). The Average nucleotide identity (ANI) of the whole genome between ϕ PRS-1 and other closely related phages was computed with OAT v0.93.1 (Orthologous Average Identity Tool) software (Lee et al., 2016). The intergenomic similarities at nucleotide levels of ϕ PRS-1 and other related viruses were determined with the VIRIDIC tool (Moraru et al., 2020). Moreover, an integrated analysis of the identification and taxonomic classification of the phage, its predictive lifestyle, and the host was performed using PhaBOX, a comprehensive web tool (Shang et al., 2023). Furthermore, to ascertain that the genome of ϕ PRS-1 is devoid of any potential ARGs, the CARD database function in Proksee was used.

2.4 Time-dependent evaluation of antibacterial efficacy of ϕ PRS-1 on abiotic surface

The antibacterial efficacy of ϕ PRS-1 was evaluated against PAO1 (ATCC 15692) using sterile disposable square petri plates (120×120 mm, Hi-Media). In this experiment, inhibition of bacterial growth by ϕ PRS-1 was assessed as a function of time. A total of 1 mL of overnight grown culture of *P. aeruginosa* (1×10^8) was spread and air-dried for 1 hour on the plates. Subsequently, the plates were placed in an incubator at 37°C for 24 hours to ascertain a thick

layer of bacterial growth. One set of three replicates was used to determine the CFU of PAO1 at this time point. Subsequently, 100 μL of ϕ PRS-1 (4.4×10^{10}) was spread on the Petri plates coated with PAO1 and allowed to dry for 1 hour. The plates placed in an incubator at 37°C for 48 hours, and the CFU of each treated set was determined at 12, 24, and 48 hours by adding 1 mL of saline to the plate and mixing the bacterial-phage complex, followed by plating for CFU determination. Additionally, 100 μL of the sample was subjected to assess the metabolic cell viability using resazurin and measuring the relative fluorescence intensity at 550/590 nm.

2.5 Time-dependent Visualization and Quantification Antibiofilm Potentials of ϕ PRS-1

In-vitro efficacy of ϕ PRS-1 was evaluated against PAO1 (ATCC 15692) in inhibition and disruption of biofilm using Film Tracer™ LIVE/DEAD® Biofilm Viability kit (Invitrogen). The experiment was divided into two sets as follows:

Set I: Biofilm Inhibition: Herein, the overnight grown host culture cells were diluted in SCDB (1×10^8) and inoculated in a tissue culture dish, followed by the addition of ϕ PRS-1 at 0.1 MOI. CFU was determined after each time point of 6, 12, and 24 hours. Additionally, live-dead cell imaging of the replicates was performed at 4, 8, 12, and 24 hrs.)

Set II: Biofilm Disruption: In this set, the overnight grown host culture cells were diluted in SCDB (1×10^8), inoculated in a tissue culture dish, and allowed to form biofilm for 24 hours. The planktonic cells were washed, followed by the addition of ϕ PRS-1 at 0.1 MOI. CFU was determined after each time point of 6, 12, and 24 hours. Besides, the replicates were also subjected to live dead cell imaging at 6, 12, and 24 hrs.). The untreated cells of PAO1 served as culture control in both sets.

For each time point, three replicates were used for confocal imaging, two for FE-SEM sample preparation, and three for CFU determination. While imaging with confocal microscopy, the cells were washed with 1X-PBS (Hi-Media) and stained with a mixture of SYTO® 9 and propidium iodide (PI) stains from Film Tracer™ LIVE/DEAD® Biofilm Viability kit following manufacturer protocol. The stained dishes were visualized with an inverted confocal laser scanning microscope (CLSM) (Leica Stellaris 5, DMI8) using a $20\times$ objective. The fluorescence from live and dead bacteria was visualized using excitation wavelengths of 488 nm (SYTO® 9) and 588 nm (PI), respectively. Additionally, the ϕ PRS-1 treated and non-treated sets after 24 hours were subjected to Field emission scanning electron microscopy (FESEM, Nova Nano SEM 450) imaging to understand the effect of phage treatment on the biofilm morphology. Statistical test of RM-one-way ANOVA with multiple comparisons and a p-value of 0.05 was used in GraphPad Prism v9.5.1 to analyze the results.

2.6 Bacteriophage-based biocontrol for reduction of bacterial counts from raw sewage

2.6.1 Sample collection and details of the sampling site

Phytorid sewage treatment plant (STP) (18° 32.8246' N, 73° 48.8338' E) situated at CSIR-National Chemical Laboratory, India, with a capacity of 0.15 million liters per day (MLD) was chosen for sampling of untreated wastewater. A total of 1 L (n=3) of untreated wastewater samples were collected in a sterile bottle by grab-sampling method. The samples were brought immediately to the lab and processed for experimental setup.

2.6.2 Time-dependent evaluation of *P. aeruginosa* biocontrol by ϕ PRS-1 in untreated raw sewage

The raw sewage (untreated wastewater) samples from Phytorid STP were divided into test and control groups. For the test group (100 mL aliquot of the raw sewage was challenged with 1 mL of high titer phage stock of ϕ PRS-1 (10^{10} PFU/mL), while the control group was devoid of any treatment. The experiment was carried out in triplicates, and the flasks from each group were incubated at 37°C for 24 hours. A time-kill assay was conducted to assess the potential of ϕ PRS-1 in improving the reusability of wastewater and associated environmental health. The results were recorded as CFU/mL, while the reduction in the bacterial counts was calculated in the form of log reduction and percent reduction, as described previously (Bashir et al., 2022).

2.7 In-vitro cytotoxicity evaluation of ϕ PRS-1 on L929, Vero, and HUVEC cell lines

A purified stock of ϕ PRS-1 preparations, i.e., the enriched filtrate ($\sim 2 \times 10^8$ PFU/mL), high titer stock (3.8×10^{10} PFU/mL), PEG precipitated stock (2.9×10^{10} PFU/mL), endotoxin removed (ETR) preparation (2.5×10^9 PFU/mL) and vehicle control (only SM buffer), and growth control (non/untreated cells) were evaluated for cytotoxicity on L929 and Vero mammalian cell lines, and Primary Human Umbilical Vein Endothelial Cells (HUVEC).

Initially, the cells were propagated in T-flasks (Tarsons) using Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% Fetal bovine serum (FBS) (Gibco). The confluency of the cells was ascertained through microscopic observation (Olympus CKX53 inverted microscope) of the cells. After achieving more than 80% confluency, the spent media was decanted from the cell culture flask. Subsequently, 200 μ L of 1X Trypsin-EDTA solution (Hi-Media) and an equal volume of DMEM were added to the flask and incubated for 2-3 mins in a CO₂ incubator (Thermo Fischer Scientific). The contents were then transferred into a sterile 1.5 mL microfuge tube (Tarsons) and centrifuged at 6000 rpm for 2 mins. The supernatant was

poured off, and the cells were suspended back in 1 mL of fresh DMEM medium. From the resulting suspension, the cells were taken as 10 μ L cells + 10 μ L trypan blue + 80 μ L PBS in separate sterile 200 μ L microfuge tubes, and the number of cells was numbered using a Neubauer counting chamber. The seeding of the 96-well microtiter plate was done using 10^4 cells in each well, followed by incubation at 37°C for 24 hours in a 5% CO₂ incubator. Subsequently, phage lysates were added to the wells (>80% confluency) and incubated for 24-48 hours to assess the cytotoxicity. After 24 hours, the plate was visualized microscopically for morphological changes in the cells and incubated further for up to 48 hours.

Cytotoxicity was evaluated employing the (2,5 diphenyl tetrazolium bromide) (MTT) method (Khan et al., 2023). A total of 1mg/mL of MTT stock was prepared, and ~30 μ L of MTT was added in all the wells such that the final concentration was 0.5 mg/mL/well. The plate was incubated in the dark for 4 hours at 37°C. Following that, the contents of the wells were poured off, and the resulting formazan crystals were dissolved using 100 μ L DMSO. The resulting suspension was incubated for 5 mins, after which the absorbance was measured at 570 nm with a microplate reader (Biotek synergy H1). Cell viability percentage was determined using the standard formula:

$$\% \text{ Cell viability} = \text{Average O. D. of the Sample} \div \text{Average O. D. of the Control} \times 100$$

2.8 Data Availability

The genome sequence data of phage PRS-1 has been submitted to the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/WebSub/>) under accession number PP337210.

3. Results and Discussion

3.1 Isolation, identification, and growth characteristics of phage (ϕ) PRS-1 and whole genome analysis, annotation of host strain-PAO1

A recent study by Xuan et al. 2022, suggested that the quorum-sensing (QS) machinery in PAO1 promotes phage infection wherein the bacterial lipopolysaccharide receptors required for the binding of the phages are upregulated. This phenomenon of *las*QS-mediated phage susceptibility, depending on the host cell density, increases the phage adsorption and the consequent infection of PAO1. The knowledge derived from the above study was employed to isolate virulent phages targeting PAO1 (Xuan et al., 2022). This enrichment method resulted in the isolation of 12 phages targeting PAO1 (ATCC 15692). Amongst all the isolated phages, ϕ PRS-1 showed significant inhibitory activity against PAO1 for 48 hours (**Fig 1A**). Consequently,

ϕ PRS-1 was chosen further for detailed characterization in this study. The host range of ϕ PRS-1 has been tabulated in **Table 1**.

Table 1: A tabulated comprehension detailing the lytic spectra of ϕ PRS-1

Name of the bacterial strain	ATCC accession number	Source of isolation (as provided by ATCC)	Lysis by ϕ PRS-1
<i>Pseudomonas aeruginosa</i> PAO1	15692	Infected wound	Clear lysis zone
<i>P. aeruginosa</i> NCIM 2200	9027	Outer ear infection	Clear lysis zone
<i>P. aeruginosa</i> NCIM 5029	27853	Blood Culture	Clear lysis zone
<i>P. aeruginosa</i> KI113	NA	Sewage Treatment Plant	Clear lysis zone
<i>P. aeruginosa</i> ER103	NA	Sewage Treatment Plant	Clear lysis zone
<i>P. aeruginosa</i> BH113	NA	Sewage Treatment Plant	Clear lysis zone
<i>P. aeruginosa</i> PR113	NA	Sewage Treatment Plant	Clear lysis zone
<i>Escherichia coli</i>	8739	feces	No lysis
<i>E. coli</i>	25922	Clinical isolate	No lysis
<i>E. coli</i> O157; H7	43888	feces	No lysis
<i>Acinetobacter baumannii</i>	19606	Urine	No lysis
<i>Klebsiella pneumoniae</i>	700603	Urine	No lysis
<i>Yersinia enterocolitica</i>	27729	Blood	No lysis
<i>Listeria monocytogenes</i>	19112	Spinal Fluid	No lysis
<i>Staphylococcus aureus</i>	6538	Lesion	No lysis
<i>Salmonella enterica</i>	12011	NA	No lysis
<i>Shigella flexneri</i>	12022	NA	No lysis
<i>S. boydii</i>	8700	NA	No lysis
<i>S. boydii</i>	9207	NA	No lysis
<i>Enterococcus faecalis</i>	19443	NA	No lysis

The antimicrobial resistant profile of PAO1 (ATCC 15692) through VITEK-2 indicated its resistance toward more than three antimicrobial classes, namely the members of second and third-generation cephalosporins (2GC, 3GC), beta-lactams, quinolones, second and third-generation tetracyclines, diaminopyrimidine, phosphonic and nitrofurans class of antibiotics. These observations suggest that PAO1 is multidrug-resistant. Besides, the genome annotation results revealed that PAO1 (ATCC 15692) has an arsenal for MDR, which includes efflux pumps (Multidrug and toxin extrusion (MATE type), Resistance-nodulation-division (RND type), tripartite systems, and enzymes that are crucial for the intrinsic and acquired multidrug resistance in Gram-negative bacteria (Pang et al., 2019). PAO1 also possesses the MexEF-OprN efflux pump, which regulates virulence and imparts resistance to clinically significant antibiotics like quinolones, chloramphenicol, and trimethoprim. Furthermore, the presence of proteins and enzymes conferring resistance to vancomycin, fosfomycin, beta-lactam and extended-spectrum beta-lactams, tetracyclines, acriflavine, glycylicycline, and nitrofurans were also observed.

Additionally, this bacterium also possesses Metallo-beta-lactamases (MBLs), which are class B carbapenemases as per the Ambler classification. MBLs are among the clinically most significant groups of carbapenemases, as per the list of priority pathogens published by the WHO in 2017, which suggests the carbapenem-resistant *P. aeruginosa* group is of critical importance (Yang et al., 2023). Moreover, this aspect is of critical importance because the resistance profile of PAO1 maps to the list of highest priority and critically important antimicrobials for human medicine by the World Health Organization (WHO). A detailed MDR profile of PAO1 obtained through whole genome annotation has been tabulated in **Table 2**.

Table 2: MDR profile of PAO1 host strain.

Sr. no	MDR profile
1	Vancomycin B-type resistance protein VanW
2	Transcriptional regulator, TetR family
3	Transcriptional regulator, TetR family
4	PqsE, quinolone signal response protein
5	Metal-dependent hydrolases of the beta-lactamase superfamily I
6	COG1680: Beta-lactamase class C and other penicillin-binding proteins
7	Multidrug resistance protein D
8	Fosfomycin resistance protein FosA
9	Transcriptional regulator, TetR family

10	Inner membrane component of tripartite multidrug resistance system
11	Membrane fusion component of tripartite multidrug resistance system
12	Outer membrane component of tripartite multidrug resistance system
13	Transcriptional regulator, TetR family
14	Type I secretion outer membrane protein, TolC precursor
15	Transcriptional regulator, TetR family
16	Multidrug and toxin extrusion (MATE) family efflux pump YdhE/NorM
17	Transcriptional regulator, TetR family
18	Transcriptional regulator RutR of pyrimidine catabolism (TetR family)
19	Beta-lactamase class C and other penicillin-binding proteins
20	Transcriptional regulator, TetR family
21	Transcriptional regulator, TetR family
22	RND efflux system, inner membrane transporter CmeB
23	RND efflux system, membrane fusion protein CmeA
24	Transcription repressor of multidrug efflux pump acrAB operon, TetR (AcrR) family
25	Translation elongation factor G
26	Translation elongation factor G
27	Lactam utilization protein LamB
28	Transcriptional regulator, TetR family
29	Beta-lactamase class C and other penicillin-binding proteins
30	Transcriptional regulator, TetR family
31	Beta-lactamase class C and other penicillin-binding proteins
32	Membrane fusion component of tripartite multidrug resistance system
33	Transcriptional regulator, TetR family
34	Transcriptional regulator MexT
35	Transcriptional regulator MexT
36	Multidrug efflux membrane fusion protein MexE
37	Multidrug efflux transporter MexF
38	Outer membrane protein OprN
39	Nitroreductase family protein
40	Transcriptional regulator, TetR family

41	ABC-type multidrug transport system, permease component
42	ABC-type multidrug transport system, ATPase component
43	Inner membrane component of tripartite multidrug resistance system
44	Membrane fusion component of tripartite multidrug resistance system
45	Outer membrane component of tripartite multidrug resistance system
46	Acyclic terpenes utilization regulator AtuR, TetR family
47	Multidrug and toxin extrusion (MATE) family efflux pump YdhE/NorM, homolog
48	Transcriptional repressor CifR, TetR family
49	Transcriptional regulator, TetR family
50	Predicted transcriptional regulator for fatty acid degradation FadQ, TetR family
51	Predicted transcriptional regulator for fatty acid degradation FadQ, TetR family
52	Predicted transcriptional regulator for fatty acid degradation FadQ, TetR family
53	Transcriptional regulator, TetR family
54	Transcriptional regulator, TetR family
55	Membrane fusion component of tripartite multidrug resistance system
56	Inner membrane component of tripartite multidrug resistance system
57	DNA gyrase subunit A (EC 5.99.1.3)
58	DNA gyrase subunit A (EC 5.99.1.3)
59	Membrane fusion component of tripartite multidrug resistance system
60	Metal-dependent hydrolases of the beta-lactamase superfamily I; PhnP protein
61	ABC-type multidrug transport system, permease component
62	ABC-type multidrug transport system, permease component
63	FolM Alternative dihydrofolate reductase 1
64	Dihydrofolate reductase (EC 1.5.1.3)
65	RND efflux system, outer membrane lipoprotein CmeC
66	RND efflux system, outer membrane lipoprotein CmeC
67	RND efflux system, inner membrane transporter CmeB
68	RND efflux system, inner membrane transporter CmeB

69	RND efflux system, membrane fusion protein CmeA
70	Multidrug resistance transporter, Bcr/CflA family
71	Transcription repressor of multidrug efflux pump <i>acrAB</i> operon, TetR (AcrR) family
72	Metallo-beta-lactamase family protein, RNA-specific
73	ABC-type multidrug transport system, ATPase component
74	Transcriptional regulator, TetR family
75	Transcriptional regulator, TetR family
76	ABC-type multidrug transport system, ATPase, and permease components
77	Transcriptional regulator, TetR family
78	Transcriptional regulator, TetR family
79	Membrane fusion component of tripartite multidrug resistance system
80	Membrane fusion component of tripartite multidrug resistance system
81	Inner membrane component of tripartite multidrug resistance system
82	Outer membrane component of tripartite multidrug resistance system
83	Transcriptional regulator, TetR family
84	ABC-type multidrug transport system, ATPase component
85	Beta-lactamase (EC 3.5.2.6)
86	RND multidrug efflux transporter; Acriflavin resistance protein
87	Translation elongation factor G
88	Lactam utilization protein LamB
89	Transcriptional regulator, TetR family
90	Transcriptional regulator RutR of pyrimidine catabolism (TetR family)
91	RND efflux system, outer membrane lipoprotein CmeC
92	RND efflux system, outer membrane lipoprotein CmeC
93	RND efflux system, inner membrane transporter CmeB
94	RND efflux system, membrane fusion protein CmeA
95	Transcriptional regulator, TetR family
96	Cation/multidrug efflux pump
97	Dihydrofolate reductase (EC 1.5.1.3)
98	Transcriptional regulator, TetR family
99	Transcriptional regulator, TetR family

100	Metallo-beta-lactamase superfamily protein PA0057
101	DNA gyrase subunit B (EC 5.99.1.3)
102	Beta-lactamase class C and other penicillin-binding proteins
103	Beta-lactamase (EC 3.5.2.6)
104	Beta-lactamase (EC 3.5.2.6)
105	Multidrug and toxin extrusion (MATE) family efflux pump YdhE/NorM, homolog
106	ABC-type multidrug transport system, permease component
107	ABC-type multidrug transport system, permease component
108	Inner membrane component of tripartite multidrug resistance system
109	Membrane fusion component of tripartite multidrug resistance system
110	Outer membrane component of tripartite multidrug resistance system
111	Transcriptional regulator, TetR family
112	Type I secretion outer membrane protein, TolC precursor
113	Topoisomerase IV subunit B (EC 5.99.1.-)
114	Topoisomerase IV subunit A (EC 5.99.1.-)
115	Unsaturated fatty acid biosynthesis repressor FabR, TetR family
116	Transcriptional regulator, TetR family
117	Transcriptional regulator NfxB
118	Multidrug efflux RND membrane fusion protein MexC
119	Multidrug efflux RND transporter MexD
120	RND efflux system, outer membrane lipoprotein, NodT family
121	Transcriptional regulator NfxB
122	Transcriptional regulator NfxB
123	Lactam utilization protein LamB
124	RND multidrug efflux transporter; Acriflavine resistance protein
125	RND multidrug efflux transporter; Acriflavine resistance protein
126	Beta-lactamase class C and other penicillin-binding proteins
127	Beta-lactamase class C and other penicillin-binding proteins

Additionally, we also tested the multiple antibiotic resistance index (MARI) of PAO1 with other MDR isolates of *P. aeruginosa* from sewage treatment plants (STPs). Results revealed that 6 out of 7 isolates showed a MAR index above 0.2, indicating that these strains have been

exposed to several antibiotics or disinfectants. Furthermore, their antibiotic resistance profiling showed that these six isolates are resistant to more than three classes of antibiotics, indicating their MDR nature. Therefore, the use of a novel bacteriophage to tackle the MDR *P. aeruginosa* from wastewater is an effective alternative strategy amidst the AMR crisis. Details of *P. aeruginosa*-specific host range, their MAR index, EOP, and virulence pattern have been tabulated in **Table 3**.

Table 3: MAR index of *P. aeruginosa* strains and evaluation of the efficiency of plating (EOP) of ϕ PRS-1.

<i>Pseudomonas aeruginosa</i> bacterial strain	ATCC Accession number	Source of isolation	MAR Index	Phage Titre (PFU/mL)	EOP	Virulence pattern Interpretation of ϕ PRS-1 on host
PAO1	15692	Infected wound	0.36	4.4×10^{10}	1 ± 0.23	Highly Virulent
NCIM 2200	9027	Outer ear infection	0.29	3.6×10^{10}	0.8 ± 0.1	Highly Virulent
NCIM 5029	27853	Blood culture	0.22	4.1×10^{10}	0.93 ± 0.18	Highly Virulent
KI113	NA	Sewage Treatment Plant	0.18	2.5×10^{10}	0.56 ± 0.15	Highly Virulent
BH113	NA	Sewage Treatment Plant	0.93	5.2×10^9	0.08 ± 0.28	Moderately virulent
PR113	NA	Sewage Treatment Plant	0.93	1.8×10^9	0.04 ± 0.09	Moderately virulent
ER103	NA	Sewage Treatment Plant	0.93	2.3×10^7	0.0005 ± 0.11	Avirulent but active

The plaque morphology of ϕ PRS-1 with this host comprised of large clear plaques surrounded by a halo (**Fig 1B**). This type of plaque morphology could be attributed to the probable presence of polysaccharide depolymerase, either virion-associated (the structural proteins or tail fibers) of ϕ PRS-1 or of the soluble type, which diffuses in the surrounding medium. These enzymes are non-lytic and cleave only the extracellular polysaccharide (EPS) of the bacteria in their planktonic or biofilm form so as to provide access to the phage for its entry into the cell wall of the bacteria (Samson et al., 2024). Thus, the virulence of the bacteria is reduced, rendering them susceptible to the action of phage.

Morphological characterization of ϕ PRS-1 was carried out with HR-TEM and STEM, which revealed typical myovirus-like features. The structure of ϕ PRS-1 comprised of a prolate head (length, 99 ± 2 nm; width, 52 ± 3 nm) showing an icosahedral symmetry, a collar with whiskers, a long contractile tail (length, 105 ± 5 nm; width, 11 ± 2 nm), a small baseplate (24 ± 2 nm) with short spikes, and around six terminal (tail) fibers (**Fig 1B**). Given the described morphological characteristics, ϕ PRS-1 was classified within the class *Caudoviricetes* based on the recent taxonomic updates by the International Committee on Taxonomy of Viruses (ICTV) (Turner et al., 2023).

The titer of ϕ PRS-1 was determined as 4.1×10^{10} PFU/mL. The MOI for ϕ PRS-1 was 6.30, effective in achieving a bacterial clearance of 99.81%, with a probability of infected cells being $P_0=0.9981$. An MOI of five or higher suggests a simultaneous infection of the host cell by more than one phage particle, indicating the virulent ability of the phage to establish a productive infection within the susceptible host (Koonjan et al., 2020). The results substantiate the virulent nature of ϕ PRS-1 and its ability to establish a productive infection in PAO1 (ATCC 15692) cells.

The applicability of the phage relies on its adsorption, i.e., binding of phage to specific receptors on bacterial cells, followed by initiating infection in susceptible hosts. The adsorption studies with ϕ PRS-1 indicated >50% of its adsorption within 10 mins, which increased to >90% by 15 mins. Additionally, the percentage of free phages in the unadsorbed fraction decreased rapidly at 15 mins (**Fig 1C**). Further, the replication cycle of ϕ PRS-1 was understood through a one-step growth curve experiment, which showed a latent period of ~25 mins and a burst size of $37 (\pm 5)$ phage particles per infected cell of PAO1 (host) (**Fig 1D**).

The persistence of phages is affected by various external factors. In view of this, the thermal and acid-base stability of ϕ PRS-1 was assessed in this study. The temperature stability of ϕ PRS-1 was determined from 4°C to 75°C. Interestingly, optimal temperatures for phage proliferation were observed from 4°C to 37°C with no significant change in the phage titer. This phenomenon

may be attributed to the origin of the phage, which is from the upper stretch of the Ganges River at the Maneri location, where temperatures are relatively colder. However, a notable decrease in the phage titer was observed when the temperature increased from 37°C to 75°C (ANOVA, $p < 0.001$). (**Fig 1E**).

The pH stability of ϕ PRS-1 was assessed from a range of pH 3 to 11. Based on the phage titration, pH 7 was identified as the optimal pH for maintaining phage stability. On either side of the pH scale, it was observed that the phage titer was reduced (**Fig 1F**). However, a phage titer of 10^9 PFU/mL is considered a high titer (Bonilla and Barr, 2018). This suggests that ϕ PRS-1 continues to have an infectious nature for PAO1 even at a diverse pH range. The distribution of *P. aeruginosa* is ubiquitous, and it persists on the surfaces in hospital environments with varied temperatures and pH (Lušić et al., 2021; Schulster and Chinn, 2003; Wood et al., 2023). The experimental evidence suggests that ϕ PRS-1 can infect its target host across different temperatures and pH, making it an ideal candidate for anti-pseudomonal applications to tackle the emerging MDR concern of *P. aeruginosa* under one health.

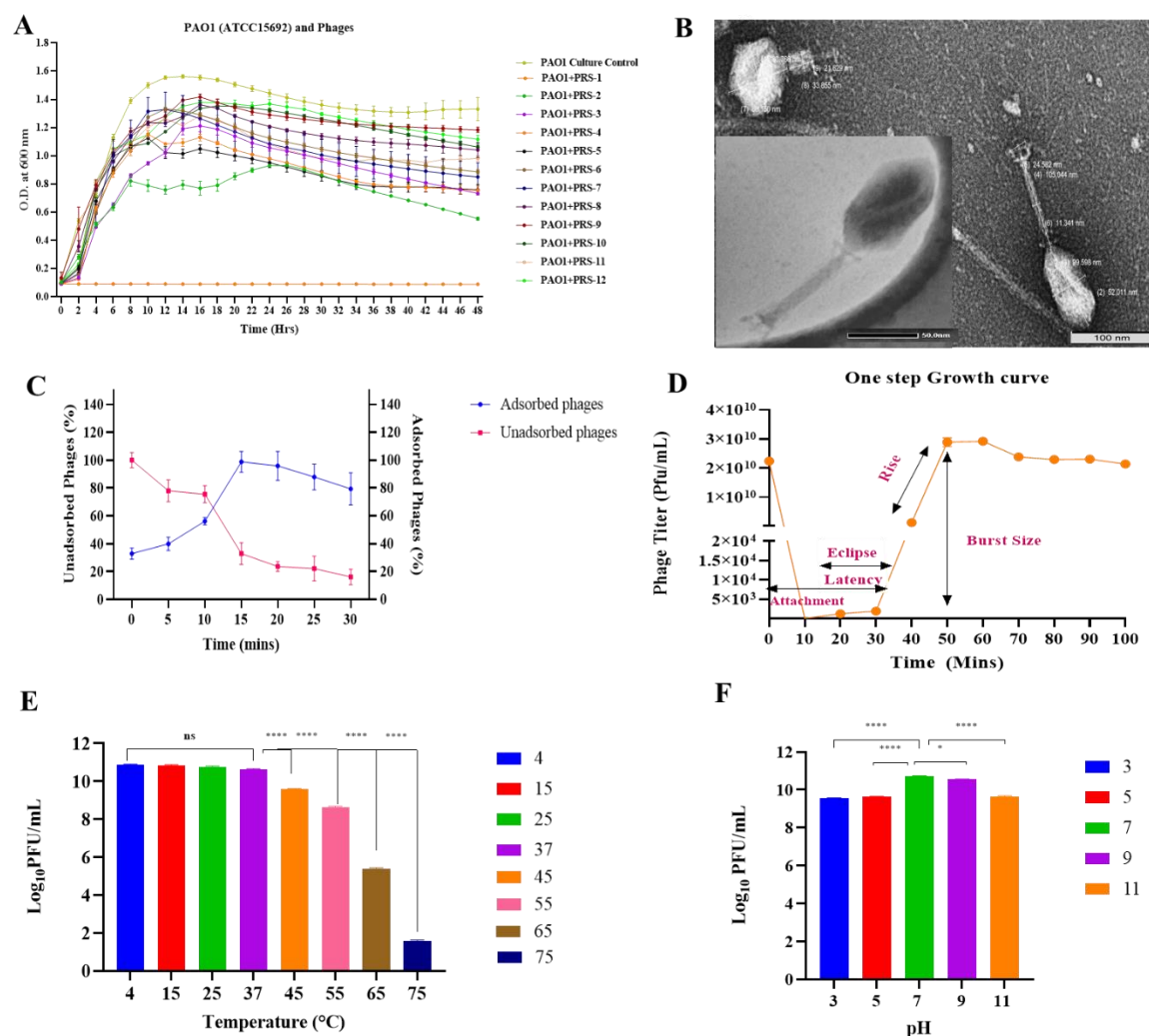


Figure 1: Morphology and growth parameters of ϕ PRS-1. (A) Screening of potent phages against PAO1. (B) HR-TEM micrographs of ϕ PRS-1 in its extended and contracted state at 80000× and scalebar of 100 nm. The insets feature- top right: plaque morphology, and bottom left: a STEM image of ϕ PRS-1 in its extended state. (C) A graphical illustration of the variation in the percentage of adsorbed and unadsorbed phage particles of ϕ PRS-1 as a function of time. (D) One-step growth curve of ϕ PRS-1 with the phases of its lifecycle marked (pink). (E) A graphical illustration depicting the influence of temperature on the stability of ϕ PRS-1 (F) A graphical illustration demonstrating the impact of various pH ranges on the stability of ϕ PRS-1. The data depicted in graph panels C through F are the average values of experimental replicates and their standard deviations (SD). The symbols (*, **, ***, ****) are indications of statistical significance determined using repeated measures of one-way ANOVA (RM-ANOVA). No significant difference between the compared test groups is designated with the abbreviation 'ns.'

3.2 Genome analysis, annotation, and proteome of ϕ PRS-1

The genome of ϕ PRS-1 spans 65,628 bp with a 57.6% G+C content. Analysis of open reading frames (ORFs) identified 799 ORFs, primarily consisting of hypothetical proteins, undefined phage proteins, and proteins that are responsible for defining phage structure, DNA replication, repair, lytic cassette, and packaging of the virion. The specific positions and details of various proteins within the genome of ϕ PRS-1 have been depicted in **Fig 2A**. From genome annotation using RAST and Prokka v1314.6 tool, endolysin was identified as the key enzyme responsible for the host lysis by ϕ PRS-1.

Analysis of amino-acid sequences in the whole genome of ϕ PRS-1 and closely related phages was performed using VipTree. A total of 1997 of the genomes related to ϕ PRS-1 were used to construct the phylogenetic tree (**Fig 2B**). The initial observation of the circular phylogenetic tree of ϕ PRS-1 was consistent with the morphological features of myovirus. Furthermore, based on the genomic similarity (SG) scores from tblastx, a subset comprising twenty closely related phages and four distantly related phages was selected to construct a rectangular phylogenetic tree. (**Fig 2C**). The genome of ϕ PRS-1 clustered with the phages infecting *P. aeruginosa*, belonging to *Pbunavirus*, a genus within the *Caudoviricetes* class and unclassified family according to ICTV.

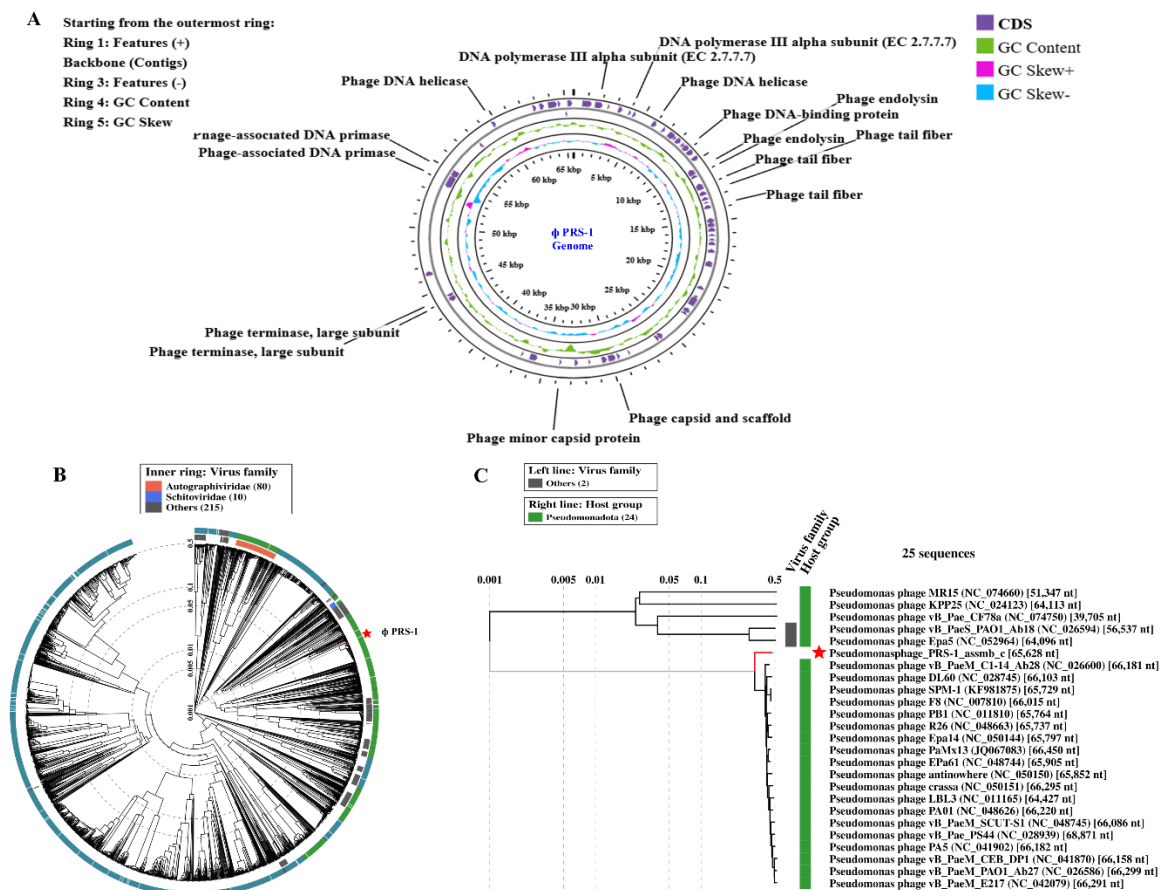


Fig. 2: Genomic Features and Amino Acid-Based Comparative Assessment of ϕ PRS-1. (A) Circular genome map of ϕ PRS-1. The outer rings in purple arrows represent the RAST-annotated protein-coding genes of the positive and negative strands of the DNA. The inner circle in green indicates the total GC content, while the pink and blue colors depict GC skew information for the positive strand and negative strands, respectively. (B) A circular phylogenetic tree, created from VipTree, with 1997 reference genomes of phages related to ϕ PRS-1. (C) A rectangular phylogenetic tree of ϕ PRS-1, along with its 20 closely related and 4 distantly related phage genomes, was constructed based on S_G scores. The red star denotes *Pseudomonas* phage ϕ PRS-1.

Further, from the orthoANI comparison of the genome of ϕ PRS-1 and closely related *Pbunavirus* members, it was observed that ϕ PRS-1 is 90.02% related with *Pseudomonas* phage vB_Pae_Mat and 89.72% with *Pseudomonas* phage 109, indicating the close relatedness of its genome as evident in the unweighted pair group method with arithmetic mean (UPGMA) tree (Fig 3A). As per ICTV, the isolated phages are classified within the same genus if the identities of their nucleotide sequences exceed 70% (Bin Jang et al., 2019). This indicates that ϕ PRS-1 could be classified as a new member within the unclassified family of the *Caudovirces* class

and *Pbunavirus* genus. Additionally, the intergenomic similarities analysis with the VIRDIC tool revealed only 89% similarity of this phage with its closest relatives, indicating distant relatedness of the genome of phage ERS-1 (**Fig 2C**).

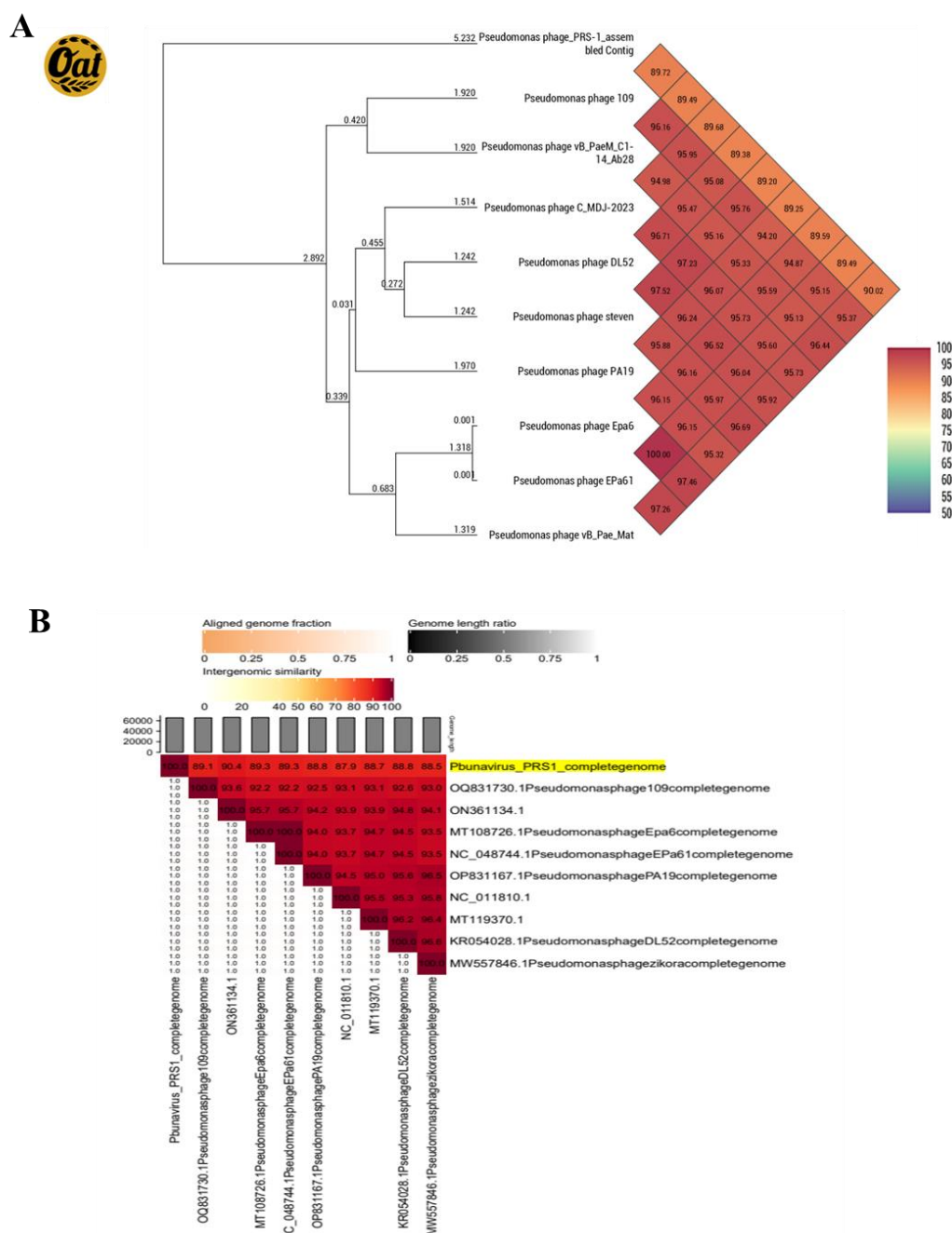


Fig. 3: Whole-genome-based and intergenomic similarities of ϕ PRS-1 (A). UPGMA tree illustrating OrthoANI values between ϕ PRS-1 and closely related *Pbunaviruses* generated using OAT software. The color scale (lower right) represents the similarity percentage between the genomes. The numerical values on the branches indicate branch length as determined by the OAT software. **(B).** The heat map shows alignment indicators (left half) and intergenomic similarity values (right half) generated through VIRDIC.

Additionally, a whole genome comparison was done by aligning the genome of ϕ PRS-1 and eight closely related phages based on the genome similarity (S_G) scores (**Fig 4**). The findings revealed differences in the synteny of ϕ PRS-1 compared to the related phages, indicating its separation as a distinct member within this group of phages. Furthermore, PhaBOX Analysis of the phage lifecycle and CARD analysis of the phage genome revealed that the ϕ PRS-1 is an absolute lytic phage (devoid of lysogeny-related genes) and shows no apparent presence of any antimicrobial resistance genes in its genome.

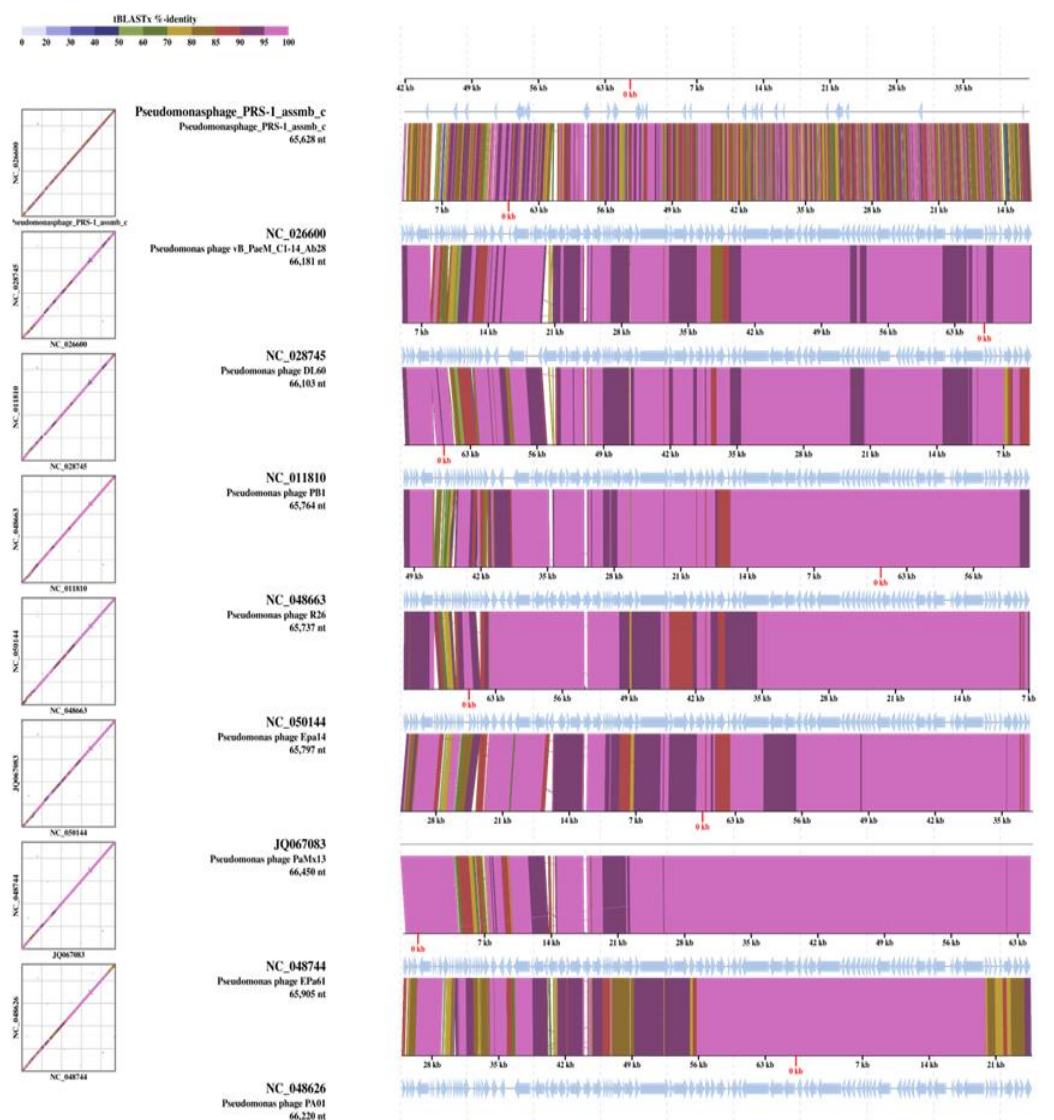


Figure 4: A synteny plot generated by TBLASTX using ViPTree server illustrates whole genome sequence alignment ϕ PRS-1 with eight closely related phages. The light blue arrows represent the open reading frames (ORFs). The color of the bands between the genetic maps indicates the % identity from TBLASTX, as specified in the legend at the top of the figure.

The members of *Pbunavirus* are known to infect *Pseudomonas* species exclusively. (<https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi>). However, it is important to note that there are no reports in the NCBI database of the phages from the *Pbunavirus* genus isolated from the Ganges River and that which infects *P. aeruginosa*. Therefore, to the best of our knowledge, this study represents the first comprehensive report providing a detailed analysis of the complete genome, as well as nucleotide and amino acid-based phylogenetic understanding, of a *Pbunavirus* member isolated from an unexplored source of the Ganges River, having the capability to infect MDR *P. aeruginosa* strains (ATCC 15692, 9027). The GenBank accession number for ϕ PRS-1 is PP337210.

3.3 Antibacterial Potential of ϕ PRS-1

Evaluation of the antibacterial potentials of ϕ PRS-1 at its various dilutions for inhibition of PAO1 bacterial growth revealed that the lowest dilution of ϕ PRS-1, which could inhibit the growth of PAO1, was 10^{-7} (0.0000001 MOI) (**Fig 5A**). The assessment of cell viability after phage treatment indicated a remarkable reduction of 8.14 $\log_{10}$ with a percent reduction of >99.99% in CFU/mL of PAO1 at 10^{-8} dilution (**Fig 5B**). Similarly, the antibacterial efficacy of ϕ PRS-1 on the abiotic surface revealed a reduction of 8.67 $\log_{10}$ counts of PAO1 cells within 48 hours. Several studies on the treatment of MDR *P. aeruginosa* in a diverse set of environments with phage(s) have shown a 1.5-5 $\log_{10}$ reduction (Chegini et al., 2020; Nawaz et al., 2024; Stachler et al., 2021). The commercially available *Pseudomonas* phage JG003 shows a 1.2 $\log_{10}$ reduction in the cell count of PAO1 (Kauppinen et al., 2021). However, to our knowledge, this is the first report showing experimental evidence of >8 $\log_{10}$ reduction in the viable count of MDR pathogen PAO1 from 0.0000001 MOI of a single, non-engineered phage. Interestingly, in the phage-treated sets, no bacterial growth was recorded up to the 10^{-3} dilution (0.001 MOI) of ϕ PRS-1, even after 30 days of incubation. The above observations underscore the potential of this novel phage, isolated from the Ganges River, as a promising candidate for inhibiting the growth of planktonic cells of MDR- *P. aeruginosa* PAO1 in environments.

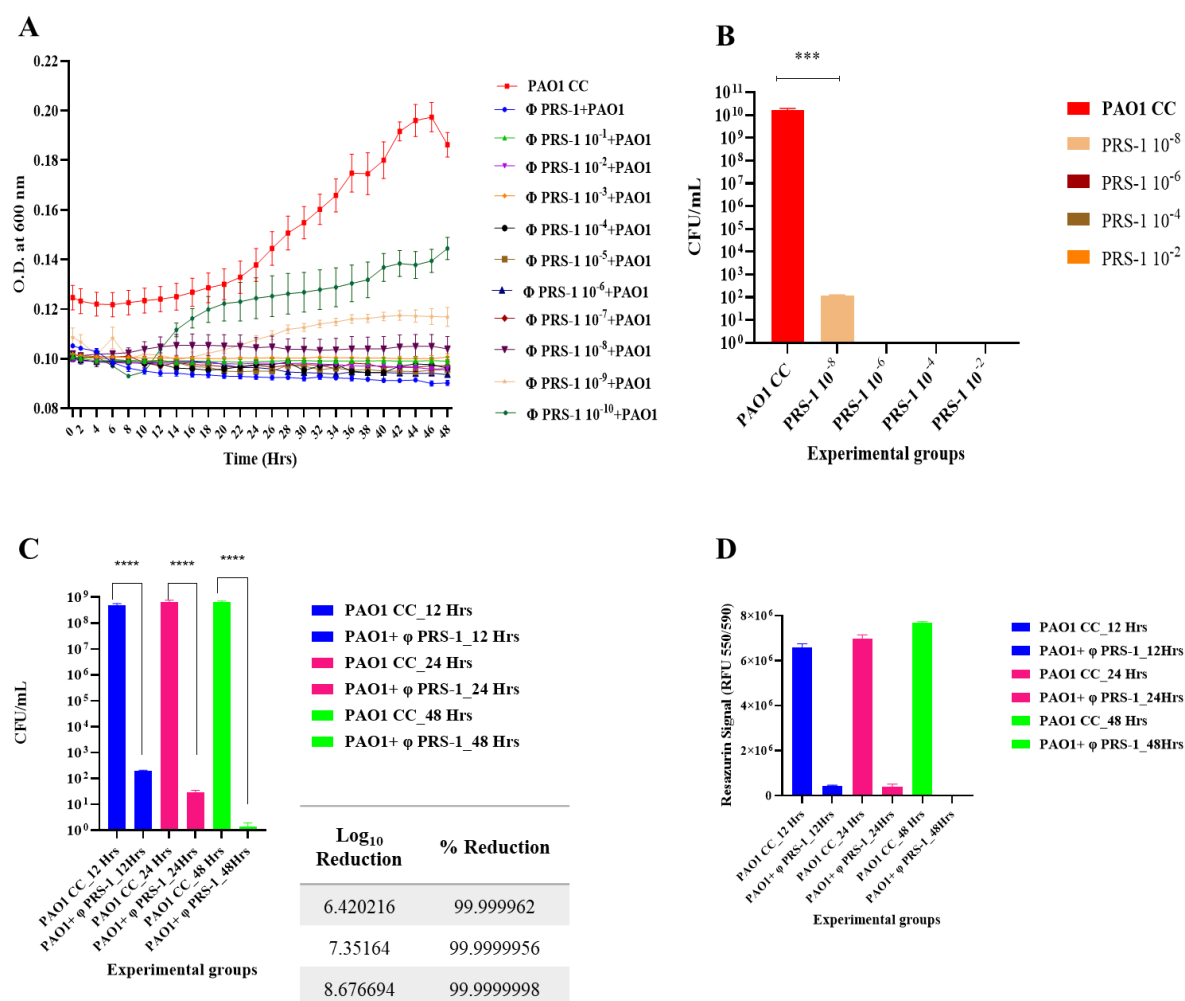


Fig. 5: Antibacterial Efficacy of ϕ PRS-1. (A) OD₆₀₀-based evaluation of the inhibitory effect of various dilutions of ϕ PRS-1 against *P. aeruginosa* PAO1. **(B)** Quantitative estimation of cell viability (CFU/mL) of PAO1 cells after treatment with various dilutions of ϕ PRS-1.

3.4 Time-dependent evaluation of *P. aeruginosa* PAO1 biofilm inhibition

MDR *P. aeruginosa* is a significant bacterial pathogen and resistant to chemical disinfectants, resulting in a wide range of severe life-threatening infections due to its resistance to various antibiotics in its biofilm form (Otter et al., 2015). Therefore, it is important to have alternate approaches to tackle the biofilm of *P. aeruginosa*. The genome annotation of PAO1 revealed its biofilm composition to be of Psl and Pel polysaccharide type, primarily composed of a dimeric repeat of α -1,4 linked galactosamine and N-acetylgalactosamine. Both Pel and Psl serve as structural scaffolds, contributing to the maintenance of biofilm integrity in pathogenic strains of *P. aeruginosa* PAO1, and PA14 (Le Mauff et al., 2022). Hence, in this study, we evaluated the potentials of ϕ PRS-1 in inhibiting and dispersing biofilms formed by PAO1 ATCC 15692 in a time-dependent manner.

The CLSM analysis of ϕ PRS-1 treated *P. aeruginosa* PAO1 showed red fluorescence at 4, 8, 12, and 24 hours, indicating the dead biomass of *P. aeruginosa* PAO1 cells (**Fig 6 B-E**), as compared to the control (devoid of ϕ PRS-1 treatment) (**Fig 6A**). The control and ϕ PRS-1 treated sets were also subjected to FE-SEM analysis after 24 hours, which revealed distinct morphological differences. The biofilm matrix of the control set was found to be intact (**Fig 6F**) with a typical coccobacillus morphology of *P. aeruginosa* cells (**Fig 6G**). Visualization of the phage-treated set of PAO1 cells after 24 hours revealed aggregates of dead cells with ruptured cell membranes, indicating cell lysis by ϕ PRS-1 (**Fig 6H-I**).

Furthermore, the quantification of the live biomass was ascertained through CFU counts. Results indicated a significant inhibitory effect on biofilm formation at 4 hrs. with a remarkable reduction of 7.8 log₁₀ corresponding to 99.99999846% reduction of bacterial growth reduction. This inhibitory effect persisted up to 24 hours with a 9.83 log₁₀ reduction equivalent to 99.99999999% reduction in the bacterial growth (ANOVA, $p < 0.0001$) (**Fig 6J**). Several studies have implemented the use of bacteriophages in biocontrol of MDR *P. aeruginosa* biofilms on various fomites, medical implants, and tubings, *in-vivo* studies demonstrating the effect of phages in controlling *P. aeruginosa* biofilms in various organs with a reduction of 2.5-5 log₁₀ within 24 hours (Adnan et al., 2020; Chegini et al., 2020; Połaska and Sokołowska, 2019). However, this is the first report with phage signifying a remarkable reduction of 9.83 log₁₀ of MDR *P. aeruginosa* in its biofilm form within 24 hours.

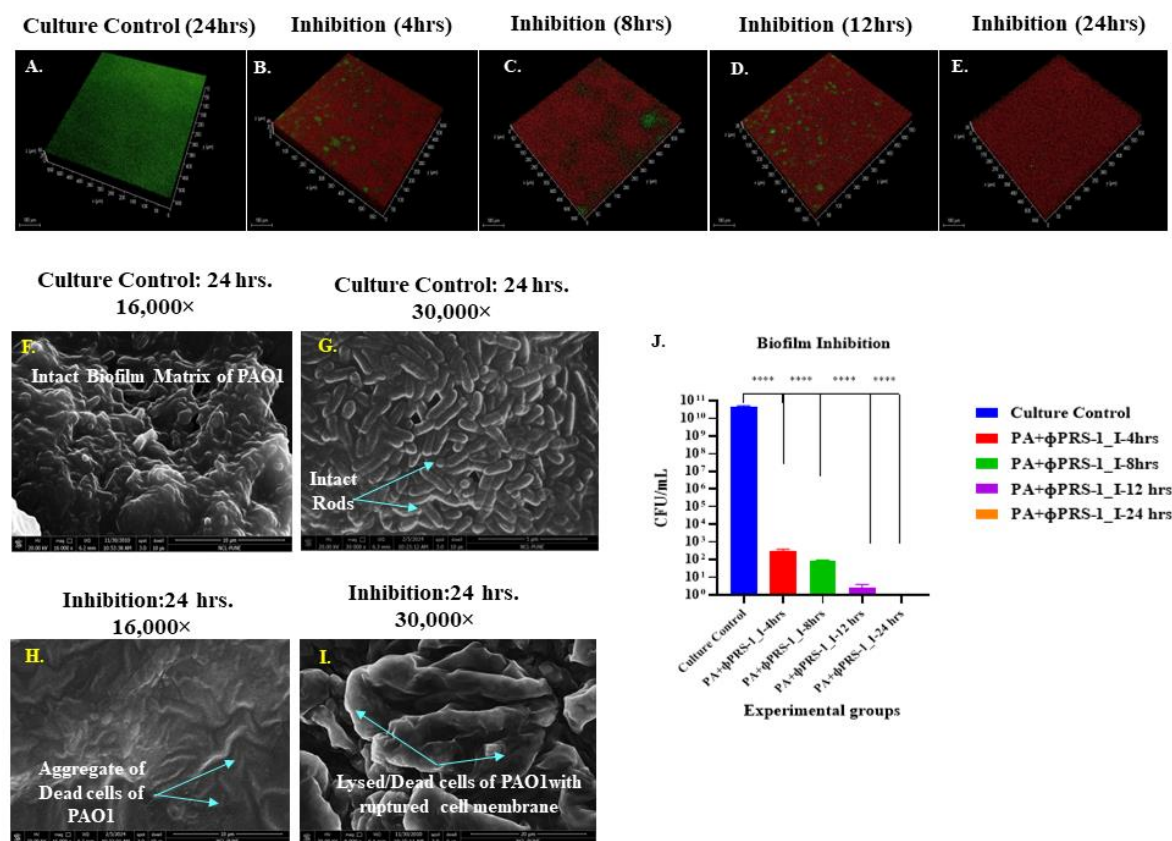


Fig. 6: Inhibitory effect of ϕ PRS-1 on biofilm formation by *P. aeruginosa* PAO1. Samples were observed under a Leica Stellaris 5, DMI8 microscope with a 20 $\times$ objective. Dead *P. aeruginosa* PAO1 cells are indicated by red fluorescence, while viable cells are indicated by green fluorescence at various time intervals. (A) CLSM image of untreated *P. aeruginosa* PAO1 cells capable of forming biofilm after 24 hours. (B) CLSM images of ϕ PRS-1 treated group were captured after 4 hours (B), 8 hours (C), 12 hours (D), and 24 hours (E). (F-I) FE-SEM images of biofilm. Culture control showing intact rods embedded within the EPS matrix of the biofilm 16,000 $\times$ (F) 30,000 $\times$ (G). (H-I) Biofilm Inhibition by ϕ PRS-1 showing dead and lysed cells of PAO1. (H) Aggregate of dead cell biomass at 16,000 $\times$. (I) A magnified view of dead PAO1 cells with ruptured cell membranes. (J) Quantification of the viable cells (CFU/mL) in both control (non-treated) and ϕ PRS-1 treated sets.

3.5 Time-dependent evaluation of *P. aeruginosa* PAO1 biofilm disruption

Biofilms of various microbes exist in their mature state on the surfaces of the hospital environments. Thus, to detach the biofilm, it is crucial to break down its EPS so as to release the planktonic cells (Stachler et al., 2021). Therefore, the antibiofilm efficacy of ϕ PRS-1 in disintegrating matured or preformed biofilms was assessed using CLSM, FE-SEM, and

quantification of viable cells via CFU/mL counts. The results indicated a gradual decrease in the viable cells of the ϕ PRS-1 treated group from 6 hours, which continued up to 24 hours (**Fig 7 A-D**). This indicates a gradual disruption of *P. aeruginosa* PAO1 biofilm by ϕ PRS-1.

Additionally, the FESEM imaging of the control, ϕ PRS-1 treated samples after 24 hours was done. The cells of the control set showed a well-defined, rod-shaped morphology immersed in a matrix of EPS (**Fig 7 E, F**), while the ϕ PRS-1 treated set of biofilm disruption showed impaired EPS matrix with distorted morphology of the cells within the biofilm together with the release of the phage by creating a hole in the host cells (**Fig 7 G-J**). Furthermore, the total viable counts at each time point revealed that ϕ PRS-1 was capable of disrupting the preformed biofilm layer of *P. aeruginosa* PAO1 with a reduction of 2.4 log₁₀ CFU/mL at 6 hours, followed by 4.8 log₁₀ CFU/mL at 12 hours, and 7.01 log₁₀ CFU/mL at 24 hours. These values indicate that ϕ PRS-1 can disrupt biofilm with a percent reduction of viable cells up to 99.60735% with 6 hours of treatment. After 24 hours, the percent reduction of *P. aeruginosa* PAO1 cells observed in the biofilm was 99.99999%. (**Fig 7 K**). Therefore, this study is the first report to document a remarkable 7 log₁₀ reduction within 24 hours of the preformed biofilm by the PAO1 cells. The potential of ϕ PRS-1 to inhibit and disrupt the biofilm of PAO1 could be attributed to the presence of phage depolymerase enzyme, which possibly cleaves the EPS matrix of PAO1 and disarm the bacteria, thereby affecting its virulence. This property of biofilm inhibition and disruption by ϕ PRS-1 also substantiates the reason behind the clear plaque encircled by a halo, as evident through its plaque morphology depicted in the inset of (**Fig 1B**).

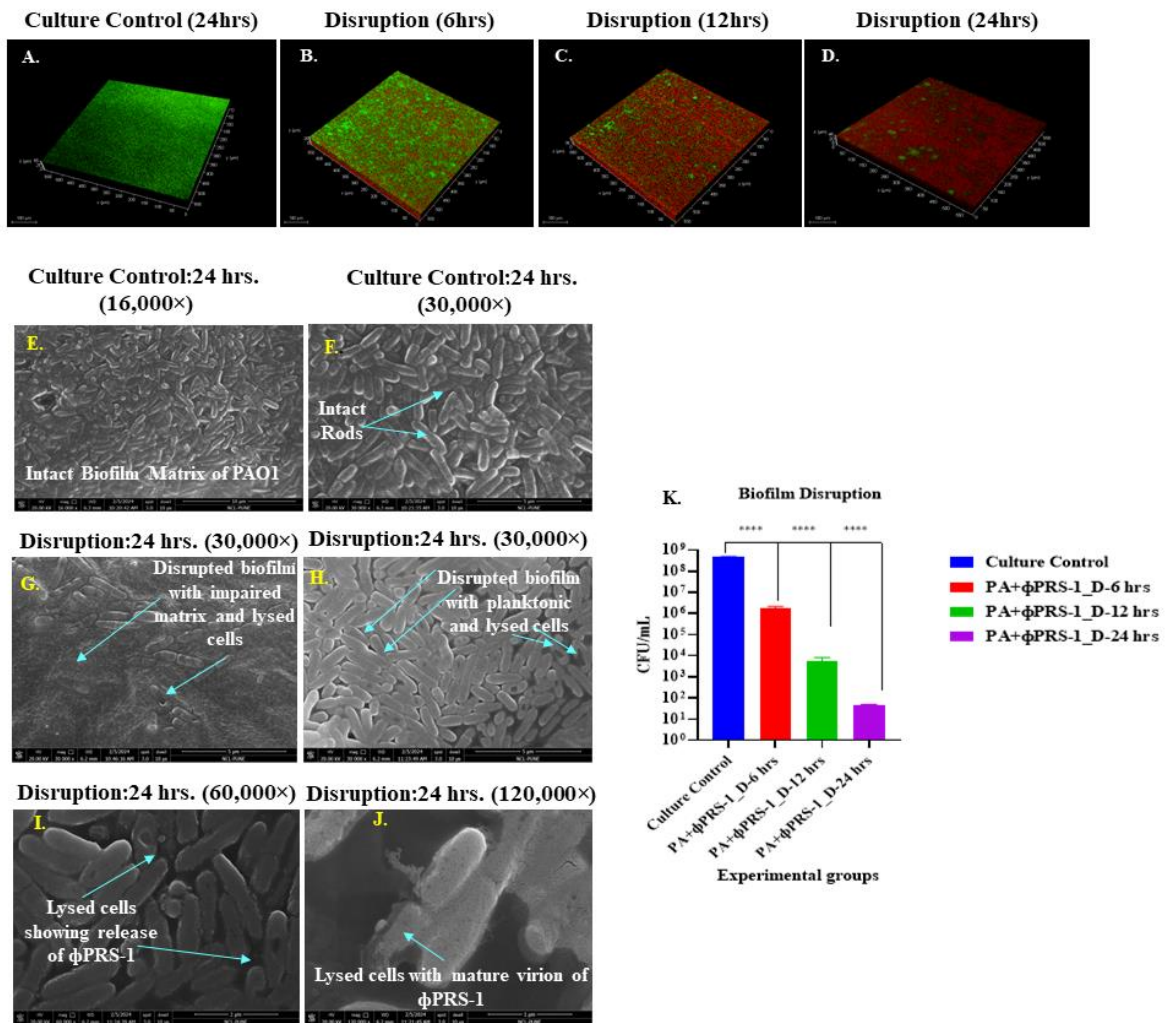


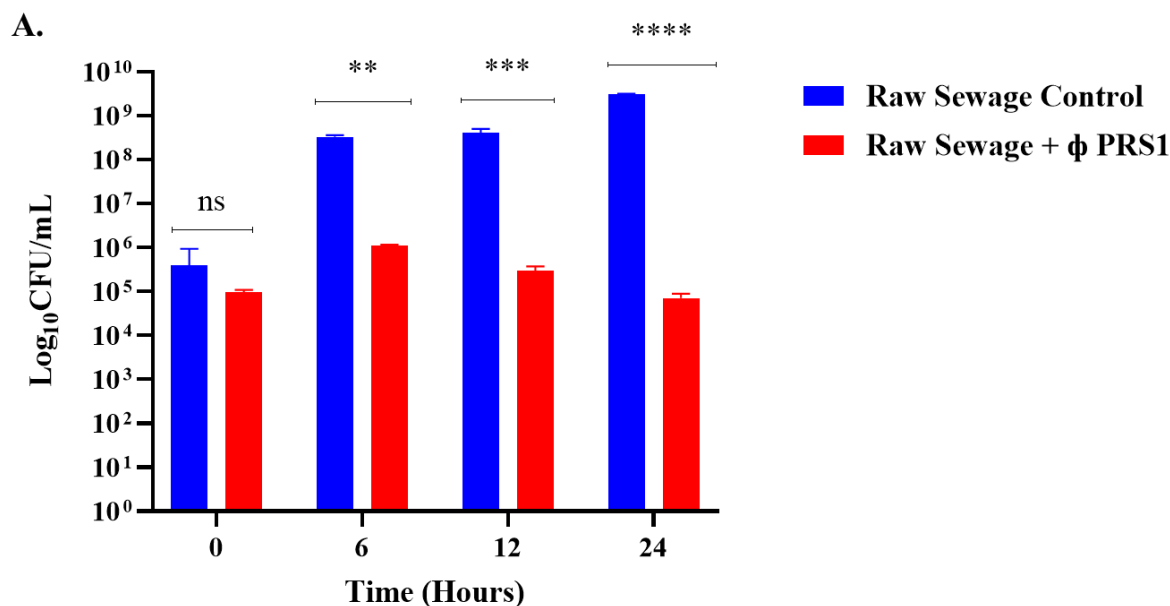
Fig. 7: Effect of ϕ PRS-1 on preformed biofilm of PAO1 (ATCC 15692). The control and treatment groups visualized through CLSM with 20 $\times$ objective at specified time intervals is shown in the panels as **(A)** CLSM image of culture control with preformed biofilm of PAO1 cells (lacking any treatment). **(B-D)** CLSM micrograph of ϕ PRS-1 treated preformed biofilm imaged after 6 hours, 12 hours **(C)**, and 24 hours **(D)**. **(E-G)** FE-SEM images of control biofilm at different magnifications 16000 $\times$ **(E)**, 30000 $\times$ **(F)**. Disrupted matrix of the PAO1 biofilm 30000 $\times$ **(G)**. **(H)** Disrupted biofilm with lysed planktonic cells. **(I-J)** Lysed cells of PAO1 show the release of the progeny of ϕ PRS-1. 60000 $\times$ **(I)**, and 120000 $\times$ **(J)**. **(K)** Quantification of the viable cells in disrupted biofilm as CFU/mL of the control and ϕ PRS-1 treated sets.

3.6 Time-dependent evaluation of reduction in *P. aeruginosa* counts from sewage water

A time-kill assay was performed to evaluate the magnitude of reduction in the viable counts of *P. aeruginosa* load in control and ϕ PRS-1-treated groups. The plating was carried out on cetrimide agar, which is a selective media for *P. aeruginosa*. Results after 24 hours of incubation

revealed a significantly lower number of viable bacteria in the ϕ PRS-1 treated group of raw sewage from 6 hours itself (ANOVA, $p < 0.001$). The $\log_{10}$ reduction accounted for 2.4 $\log_{10}$ with a percent reduction of 99.6%. Interestingly, after 24 hours, a 4.2 $\log_{10}$ CFU/mL reduction was observed in the ϕ PRS-1 treated group. **(Fig 8 A, B)**. This indicates that ϕ PRS-1 could be used as a green approach to reduce the counts of *P. aeruginosa* from raw sewage wastewater. VITEK-MS-based characterization of the colonies that grew on the ceftrimide agar plates of ϕ PRS-1 treated group revealed *Pseudomonas putida*, and *Serratia marcescens* to be amongst the surviving microbes amidst of ϕ PRS-1 treatment. However, at any given timepoint, strains of *P. aeruginosa* were not recovered from ϕ PRS-1 treated group, indicating the efficacy of ϕ PRS-1 in biocontrol of *P. aeruginosa*-specific strains from raw wastewater combined with the sludge.

Few studies have detailed the application of phages, especially phage cocktails, in wastewater treatment for reducing pathogenic and multidrug-resistant bacterial populations (Grami et al., 2022; Jassim et al., 2016; Periasamy and Sundaram, 2013). To our knowledge, this study is the first of its kind to detail an in-depth characterization of a novel phage ϕ PRS-1, highlighting its antibacterial and antibiofilm capabilities, along with the lab-scale demonstration of its effectiveness in reducing MDR *P. aeruginosa* bacterial counts in raw sewage. Nevertheless, achieving a thorough understanding of the optimized phage dosage and its effects on physicochemical and microbiological parameters pre-and post-treatment with ϕ PRS-1 is crucial for its practical application on-site. Furthermore, to combat bacterial resistance in the long term, combining various phages with ϕ PRS-1 or utilizing phage-derived enzymes from ϕ PRS-1 would enhance its effectiveness within the One Health framework, promoting the health of humans, animals, and the environment.



B.

Time (Hrs)	Log ₁₀ Reduction	Percent Reduction
6	2.491104	99.67723
12	3.13438	99.92661
24	4.638231	99.9977

Fig. 8: Evaluation of ϕ PRS-1 in reduction of *P. aeruginosa* specific counts. (A) Graphical representation of quantification of the viable cells in raw sewage as CFU/mL of the control (non-treated) and ϕ ERS-1 treated sets. **(B)** Log reduction and percentage reduction values indicate ϕ ERS-1 efficacy in reducing bacterial load in raw sewage after 24 hours.

3.7 In-vitro cytotoxicity evaluation of ϕ PRS-1 on L929, Vero, and HUVEC cell lines

As ϕ PRS-1 is a novel phage, the knowledge of its cytotoxic effects is of crucial importance. In view of this, we evaluated the cytotoxicity of ϕ PRS-1 preparations *in-vitro* on both L929 as well as Vero cell lines. The results showed no observable difference in the cellular morphology of the control cells versus those treated with ϕ PRS-1 preparation, i.e., either the enriched filtrate, high titer stock, PEG precipitated stock, endotoxin removed (ETR) preparation, vehicle control (only SM buffer) when imaged after 48 hours of incubation in both L929, Vero, and HUVEC cell lines. **(Fig 9 A-B).**

Further, to ascertain the cell viability, an MTT assay was performed with control and ϕ PRS-1-treated cells. Interestingly, it was observed that all of the phage preparations showed a cell viability between 95-99%, which was comparable with the growth control set (**Fig 9C-D**). This initial data serves as a foundational basis, implying that the phage PRS-1 could be a potent candidate for applications in various environments contaminated with MDR- *P. aeruginosa* as it shows no apparent cytotoxic effects on the cell lines under standard conditions.

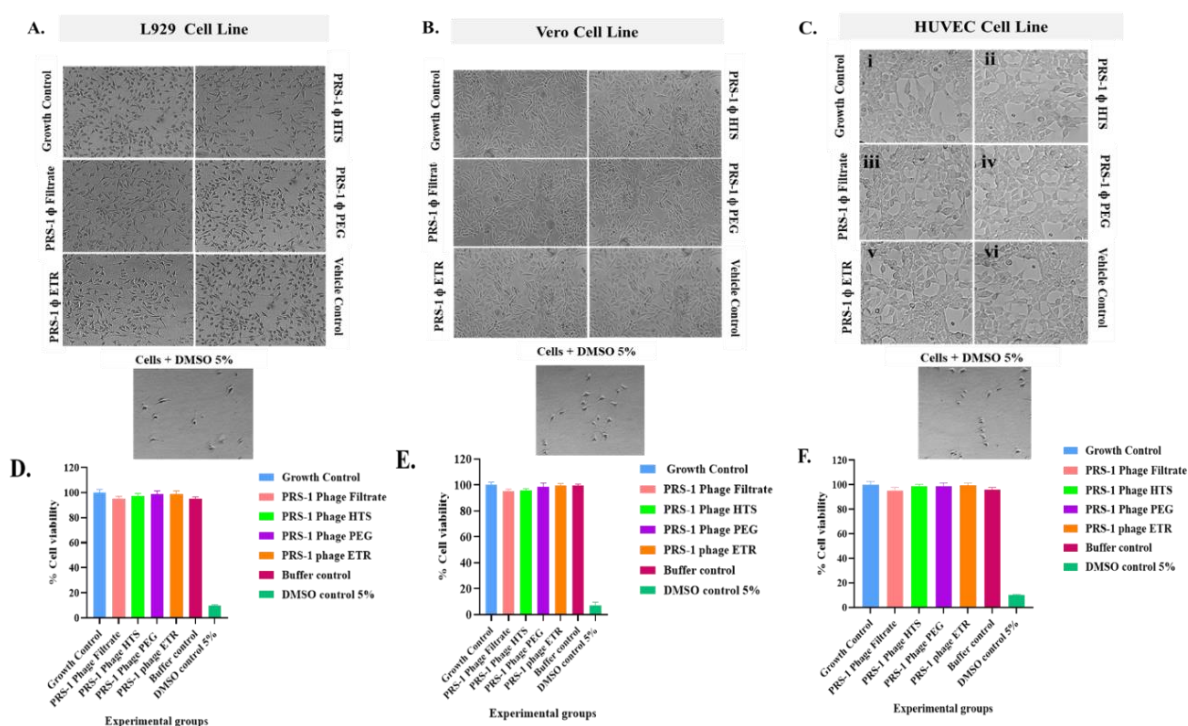


Fig. 9: In-vitro cytotoxicity evaluation of ϕ PRS-1 preparations. (A-C) Inverted microscopic imaging of (A) L929 cells, (B) Vero cells, and (C) HUVEC cells treated with various preparations of phage and observed under $20 \times$ objective. (D-F) Graphical representation showing quantification of the viable cells with MTT assay for the growth control (non-treated), Cytotoxic control (5% DMSO), and ϕ PRS-1 treated sets in (D) L929 cell lines (E) Vero cell lines (F) HUVEC cell lines.

4. Conclusion

This study demonstrates the isolation and characterization of a novel phage ϕ PRS-1 from an unexplored area of the Ganges River. The strength of our research lies in the uniqueness of the phage and its bacteriolytic and antibiofilm capabilities in reducing the abundance of environmentally significant MDR strains of *P. aeruginosa* in both their planktonic and biofilm states. The data also indicates the effectiveness of the newly discovered ϕ PRS-1 in regulating the MDR *P. aeruginosa* burden in sewage water and potentially enhancing the disposal quality of the wastewater effluent and sludge. Additionally, the non-cytotoxic nature exerted by this phage opens new avenues for various applications of ϕ PRS-1 under one health canopy. This approach can significantly contribute to achieving several Sustainable Development Goals: ensuring clean water and sanitation (Goal 6), promoting good health and well-being (Goal 3), fostering sustainable cities and communities (Goal 11), ensuring a healthy life on land (Goal 15), and ensuring a healthy life below water (Goal 14). Furthermore, using lytic phages to improve wastewater quality would reduce the strain on alternative treatment methods. This would lead to lower energy consumption, less use of harmful chemicals, and improved access to water reuse with reduced biological contamination, thereby fostering a circular economy.

5. References

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Chapter 5

Summary and Future Prospects

5.1 Overall Summary

Access to safe water is crucial for sustainable development and poverty alleviation. Rivers with good water quality support diverse microorganisms that are essential for their functioning. River health is intricately linked to human and animal well-being, reflecting the vitality of ecosystems. Yet, human activities pose significant threats to the rivers, perpetuating degradation and compromising their environmental integrity. Monitoring river health entails assessing physical, chemical, and biological components, but limited knowledge hinders our understanding of microbial communities and their functions in the rivers. Advanced sequencing technologies offer insights into microbial dynamics, aiding monitoring efforts. The Ganges, India's largest river, remains poorly understood in terms of its microbial communities and their functions. Leveraging metagenomics, we aim to map the total microbiome of the Ganges River, shedding light on its spatial-temporal dynamics, resistomes, and antimicrobial potentials. This endeavor promises to enhance our understanding of the health of the Ganges River for improved management strategies to preserve this riverine system and its unique microbial diversity.

Initial metagenomic analysis of the Ganges riverine system, particularly at its sacred confluence with the Yamuna River, underscored the first-ever report demonstrating transient impact of the Yamuna River on the taxonomic and functional diversity of the Ganges River. While changes were observed downstream, subsequent restoration of microbial communities occurred. Although sampling limitations existed, further investigations were warranted to understand the confluence microbiome dynamics comprehensively along the extended stretch. This study served as a proof-of-concept, prompting additional sampling efforts to unravel the total microbial diversity and resistome repertoire along the anthropogenically dynamic (impacted) segment of the Ganges River, including the confluence stretch, encompassing approximately 1500 km, over two years. The analysis identified the presence of antibiotics and metals as drivers for resistance gene emergence. Notably, important opportunistic pathogens like *Pseudomonas* and *Burkholderia* were identified as the key taxa carrying multiple antibiotic-resistant genes, emphasizing the severity of anthropogenic pollution on the riverine ecosystem. Just as every coin bears two facets, our investigation further delved into the intricate antimicrobial potential harbored within the microbiome of the Ganges River. The analysis identified the microbial diversity of the Ganges River with promising potential for bioprospecting and antibacterial properties, including the diversity of bacteriophages with lytic

activity against pathogens. These findings underscored the unique properties of the Ganges River, suggesting the potential of its restoration capabilities. The study sets a foundation for a comprehensive exploration of the bioactive potential inherent in the microbiota, notably the phages, in shaping the unique characteristics of the river.

Additionally, this study represents a significant milestone as the first-ever report to introduce an efficient extraction and amplification method for sequencing the microbiome in samples with low DNA concentration, paving the way for shotgun-based whole metagenomic analyses of glacier-fed streams and forelands. Further validation of the Long Amp-based targeted amplification across diverse low-input DNA samples is necessary to fully grasp its potential. This approach lays a foundation for exploring the pristine or untapped stretch of the Ganges River, providing valuable insights into its unique microbial diversity and potential applications in various fields.

Comprehensive metagenomic insights into the pristine stretch of the Ganges River revealed a compositional heterogeneity of microbial diversity along a spatiotemporal gradient. By dissecting the microbiome and exploring its functional potential, the study unveils the microbial diversity of this glacier-fed river as a source of secondary metabolite compounds with antimicrobial properties. Moreover, the identification of a diverse range of bacteriophages with lytic potential against putrefying and potentially pathogenic bacteria underscores their potential application in the context of one-health initiatives. Furthermore, a comparative analysis of the phage diversity between the pristine and anthropogenically impacted stretches of the Ganges River indicated a relatively higher abundance of lytic bacteriophages in the pristine stretch. These findings provide the first ever scientific insights into the special properties of the Ganges River. However, further studies elucidating the bioactive potentials of these microbes, including phages, are warranted for a comprehensive understanding of the untapped microbial diversity and its contribution to the special properties of the Ganges.

In parallel, this study showcased the isolation and characterization of two novel phages, ϕ ERS-1 and ϕ PRS-1, from previously unexplored areas of the pristine stretch of the Ganges River. Our research highlighted the unique bacteriolytic and antibiofilm properties of these phages, effectively reducing the bacterial load of environmentally relevant pathogens, including multidrug-resistant strains. Notably, the data demonstrated the efficacy of both phages in controlling bacterial abundance in sewage water, thereby potentially improving water quality. By employing absolutely lytic phages as a green alternative for bacterial biocontrol in sewage water, our approach emphasized the potential applications of these bacteriophages in enhancing effluent quality and sludge disposal. This strategy aligns with several Sustainable

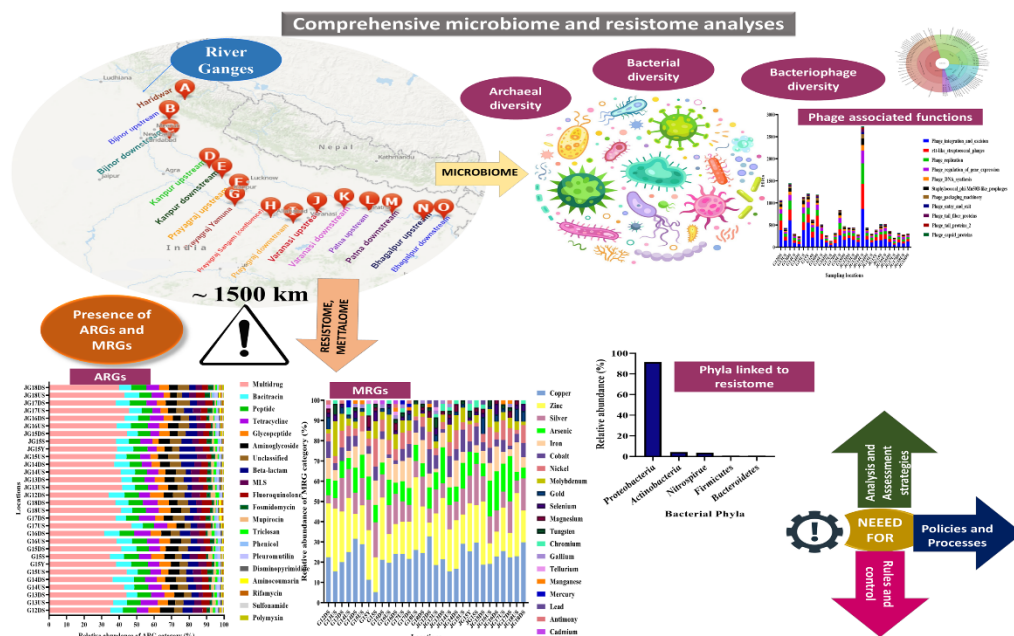
Chapter 5.1

Development Goals, including clean water and sanitation (Goal 6), good health and well-being (Goal 3), sustainable cities and communities (Goal 11), healthy life on land (Goal 15), and healthy life below water (Goal 14). Furthermore, utilizing lytic phages for wastewater treatment can alleviate the burden on traditional methods, leading to reduced energy consumption, minimized chemical usage, and improved water reuse with decreased biological contamination, thus fostering a circular economy while addressing environmental challenges.

Graphical Summary of Chapter 2

Chapter 2

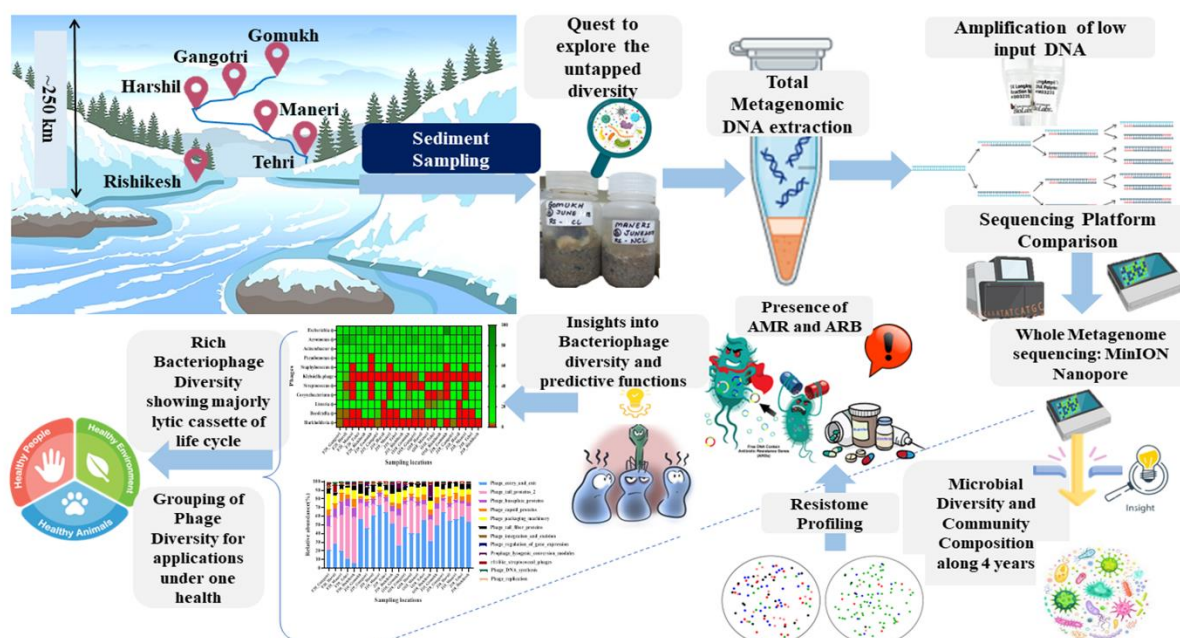
- First report on total microbial diversity and the resistome across its anthropogenically impacted stretch
- More diversity of Giant viruses (*Mimiviridae*) and phages
- First report to reveal the confluence microbiome of River Ganges and Yamuna
- Apparent restoration in microbial composition after confluence
- Transient influence of confluence another special property of River Ganges
- Microbiome (bacteria and phages) with bioactive potentials



Graphical Summary of Chapter 3

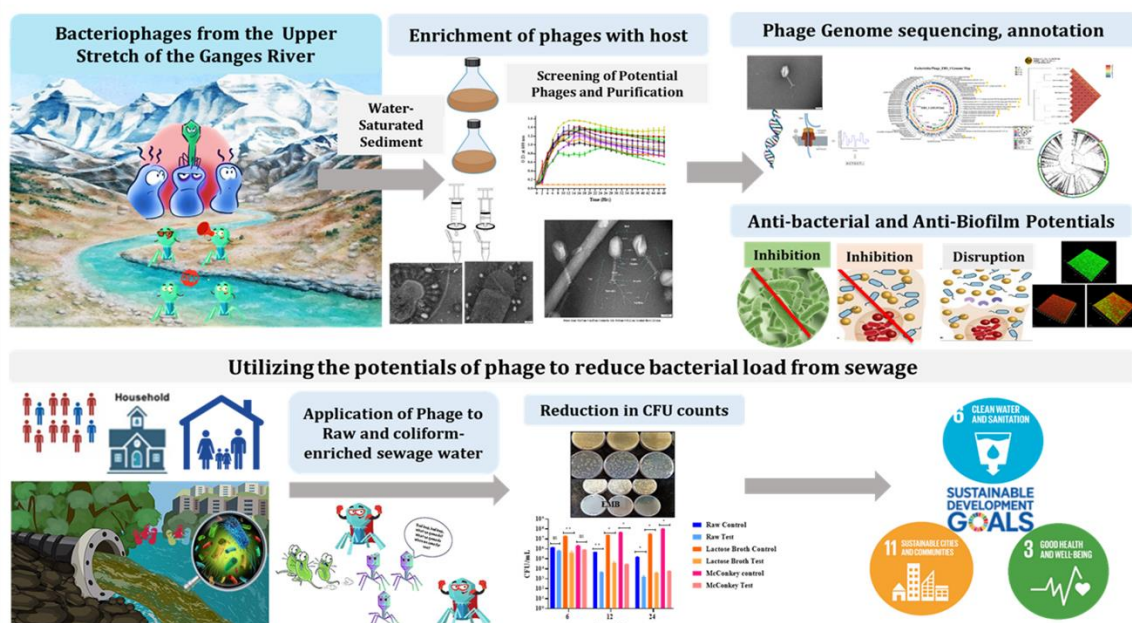
Chapter 3

- Upper stretch: Predominance of Oligotrophic bacterial diversity
- Microbiome with bioactive potentials-avenue for bioprospection
- More lytic bacteriophage diversity
- A rich bacteriophage diversity against putative pathogens: “non-putrefying” properties
- Metagenomic data: a blueprint to isolate potent phages



Graphical Summary of Chapter 4**Chapter 4**

- First report of isolation of two novel phages from pristine stretch
- First use of novel, lytic phage *Tequatrovirus* ERS-1 against multi-genera biofilm and reduction of coliform counts in wastewater
- A novel species of *Pbunavirus* PRS-1 could effectively inhibit and disrupt PAO1 cells in the biofilm by a remarkable 9 and 7 logs
- No apparent cytotoxic effects from *In-vitro* studies
- Absence of lysogenic cassette, paves way for potential applications under one health



5.2 Future Prospects

In the pursuit of comprehensively understanding the microbial ecology of the Ganges River, this study represents a significant stride by covering a substantial stretch of 1750 km from Gomukh to Bhagalpur. However, a notable gap persists in our understanding, particularly in the extended stretch from Bhagalpur to the Bay of Bengal, spanning approximately 750 km. This section remains largely unexplored in terms of its microbial diversity, emphasizing the necessity for further investigation. Furthermore, there is a need for longitudinal studies spanning multiple seasons to elucidate the spatio-temporal changes in microbial communities and resistome profiles along the lower stretch of the river. Understanding how microbial populations fluctuate in response to seasonal variations and anthropogenic pressures is essential for devising effective management strategies aimed at preserving the ecological integrity of the Ganges River.

One crucial aspect that warrants additional studies is the impact of mass bathing events on the microbial composition and functional profile of the confluence. These studies should encompass comprehensive sampling strategies that capture the dynamics of microbial communities in both water and sediment samples before, during, and after mass bathing events. Such investigations could unravel the intricate interplay between anthropogenic activities and microbial ecology, shedding light on potential implications for public health and ecosystem dynamics.

Looking ahead, future prospects for research on the microbial ecology of the Ganges River encompass a multifaceted approach integrating ecological, microbiological, and engineering perspectives. In addition to ecological studies, there is merit in exploring the application of innovative interventions for mitigating microbial contamination in environmental settings. Evaluation of water quality parameters such as Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), and Total Suspended Solids (TSS) following treatment with phage ERS-1 and phage PRS-1 holds promise for addressing pollution challenges associated with raw sewage.

Additionally, there are limited studies with this riverine system wherein novel virulent phages were isolated from its waters against MDR strains. Therefore, this unique aquatic ecosystem provides an opportunity for the bioprospection of its virulent phages. The cutting-edge potentials of these phages could be explored experimentally against certain critical and emerging pathogens of plant, aquatic animal, and human importance, as illustrated in **Fig. 1**.

APPLICATIONS OF PHAGES FROM THE GANGES: THE WAY FORWARD



Fig. 1: Comprehensive illustration of specific pathogens of agricultural, aquatic, zoonotic, and human importance, onto which the experimental insights of phages from the Ganges could be translated.