Comprehensive insights into microbial ecology and harnessing biomass conversion potential of culturable bacteria from riverine system

by

Yadav Rakeshkumar Jaynarayan

10BB18A26030

A thesis submitted to the
Academy of Scientific & Innovative Research,
for the award of the degree of
DOCTOR OF PHILOSOPHY
in
SCIENCE

Under the supervision of

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Ghaziabad, U.P.– 201 002, India
December 2023

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Signature of the Supervisor
Name : Dr. Mahesh S. Dharne
Date : 15/12/2023
Place : Pune

Dedicated to My Family and Friends

ACKNOWLEDGEMENT

Let me take a moment to give my deepest gratitude to all those who have been a constant support throughout my PhD life. The journey of a PhD is a challenging, multi-dimensional, and life-changing experience; thus, it requires the help and guidance of well-wishers. I am writing to express my deepest gratitude to everyone who has contributed to my work during my good and bad times and supported me throughout my academic journey.

First and foremost, I would like to express my heartfelt gratitude to my Family. My mother and Father, **Mrs Parvati Yadav** and **Mr Jaynarayan Yadav**. While writing this, I remember all the struggles they have gone through to make me reach here. My Sister and Brother, **Mrs Aarati** and **Mr Kamlesh** have constantly supported me. I would also like to be thankful for the blessings of my extended Family, my Nani, Nana, and Dada, my maternal aunts (Mrs Nirmala, Mrs Indira), Mrs. Poonam Yadav, and Mr. Manoj and Mr. Ashwani Yadav. Lastly, my nieces Anvika and Aashvi are my source of smiles.

I am immensely grateful to my PhD advisor, **Dr. Mahesh S. Dharne**, for providing valuable guidance and suggestions. He always gave me the freedom to do experiments, never hesitated in providing resources (without asking me why I required it), and supported me throughout. I am indebted to him for his encouragement and for being a driving force in my ultimate academic journey.

Thanks to my Doctoral Advisory Committee members, **Dr V.V. Bokade, Dr Syed G. Dastager** and **Dr Narendra Kadoo** for insightful feedback and constructive criticisms. Their encouragement, valuable suggestions, and rigorous evaluation have immensely enriched the quality of my thesis.

I am grateful to Prof. A.K. Nangia (former Director) and Dr. Ashish Lele, Director of CSIR-NCL, for allowing me to research at the institute. I would also like to thank the former Head of the Biochemical Sciences, Dr. Ashok Giri, and all the supporting staff of the department office. The research journey was made possible by the University Grant Commission's (UGC) financial support. A very special thanks to the organisation, considering the importance of money. I sincerely thank the Academy of Scientific and Innovative Research (AcSIR) for registration and the Government of India. I would also like to acknowledge Dr. Sunita Barve, Mr. Gati Krushna Nayak and other library staff members for all kinds of support and for giving access to the library. A special thanks to Dr Bhushan and Ms Sanjana for providing me with FTIR analysis.

I thank AcSIR coordinators Dr B. L. V. Prasad, Dr Mahesh Kulkarni, Dr M. Sasidhar, and Dr. Santosh Mhaske. A special thanks to the Student Academic Office staff: Mr P.K. Purushothaman, Mrs. Poornima Kohle, Purnima Ma'am, Mrs. Komal, Mrs. Vijaya, Vaishali Ma'am, and other members. They were responsible for the smooth operations of all my official work. I would also like to sincerely thank Dr. C.P. Vinod (my cricket team captain) and Mr. S.M. Mane for being a well-wisher.

Heartfelt thanks to all my NCIM staff members, who were always there for me and supported me every time. Mrs Shalaka G., Mrs Ratnamala V., Mrs Shivani, Mrs Sandhya, Mrs Monika, Mrs Meeta P., Mr Ambadas, Mr Shinde, Mr Kunal, Mr Sagar, Mrs Bhavana, Mrs Vrushali, Mrs Jini, Mr Digeshwar, Mrs Karthika, Mrs Sayali, Mr Raju, and Mr Irfan.

Extended thanks to all my labmates and seniors who have helped me on various occasions by providing and sharing knowledge: Dr Gajanan M., Dr Kumar Raja., Dr Kushal G., Dr Govinda N., Dr Meghana T, Dr Hariram, Dr Amit S., Dr Bharat K., Dr Vasudev W, Mr Tanmay, Mr Manan, Mrs Vidhyashri, Mr Anil, Mrs Priyanka, Kiran, Mr Vinod, Sai, Sejal, and Mr Pradeep.

A special thanks to Mrs Pranhita, Mr Ekramul, Mr Pranay, Mr Pushkar, Mr Hardik, and Ms Vaishnavi for supporting my thesis work and providing help whenever required.

There are few personalities whose memories will be life-long. These are the moments which we cherish throughout life. I want to thank all my special friends, without whom the PhD journey would have been tiresome. Thank you, Ms. Aishwarya, for being there always, you have been a constant. Life would have been dull without Mr Pranav Nair, Mr Vinay Rajput, Mrs Vibhavari, Mr Ashish, and Mr Junaid; I enjoyed a lot with you all. A special thanks to Dr Roopa. She has been a friend and a guide. I want to express my love for Pratiksha and Kush. My heartfelt gratitude to Dr. Aakash N., Mr Ravi, and Mr. Neeraj. A special thanks to my friends in NCL, Mrs Smita, Ms Rajnigandha, Ms Kritika, Mr Hari, and Mr Soumya; you guys were fabulous. I remember our first lecture at NCL, memories indeed. Thanks for being there.

Hostel life and friends, No one can replace them. I joined the hostel after two years; I wish I had joined the hostel earlier. Lots of moments, lots of gossip, and enjoyment. A special thanks to Dr Prashant, Dr Pratikshkumar (Pinka), Dr Mahendra W, Ravi Ranjan, Dr Pawan, Dr Gopal M, Dr Santosh, Dr Himanshu, Sonu, Kailash, Siddharth, Priyam, Dr Nirshad, Manish, Dhananjay, Ajithkumar, Dr Rohit Jr, Rohit Sr, Dr Rahul, Dr Mahendra P., Dr Arun, Dharmendra, Santosh (Sandy), Vishwambhar, and many more.

Special gratitude to Prof. Anuradha Nerurkar, Prof Devarshi Gajjar, Dr Vyas, Dr Yogesh Shouche, Rakhi Ma'am, Mrs Shailaja P and Dr Shrikant Pawar, who laid the foundation and interest of research in me.

Special gratitude to my outlier friends Mr Mandar, Dr Kunal, Dr Aniket, Mrs Amruta, Mrs Sayali, Anusha, Rucha, and Sonal. My Lab 3 friends Dr Diptaraj, Dr Siddharth, Dr Shreyas, Mr Vikas, Dr Akshay, Mr Deepak, Mr Abhijeet, Mr Rohan, and Mr Vikas.

Further, thanks to my M.Sc. friends Harpal, Ruchi, Monika, Dr Shailesh Lad, Merlin, Preeti, Reshma Suresh, Soumya, and Mayur. They all have contributed significantly to my life. A special thanks to Dr. Vihang Thite for always being there. Special gratitude to my special friends Abhishek, Swati, Lipsa, Dhwani, Divya, Jisna, Aakash Desai, Rahul Sharma, Yogesh, and Ramdas.

Above all, I sincerely thank the Almighty God for giving me wisdom, strength, health, and qualities to succeed.Thank you.

Yours sincerely,

Rakeshkumar Yadav.

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List of Abbreviations

SDGs	Sustainable Development Goals
CPCB	Central Pollution Control Board
NGSTs	Next Generation Sequencing technologies
TGS	Third Generation sequencing
WH	Water Hyacinths
VFDB	Virulence Factor Database
ARGs	Antibiotic resistance genes
AMR	Antimicrobial Resistance
CARD	The Comprehensive Antibiotic Resistance Database
PDEs	Pollutant Degrading enzymes
MGEs	Mobile Genetic Elements
MRGs	Metal Resistant genes
γ-PGA	Poly-gamma-glutamic acid
PGA	Poly-gamma-glutamic acid
2,3-BDO	2,3-butanediol
BDO	2,3-butanediol
TJ	Tomato Juice
NCIM	National Collection of Industrial Microorganisms
NCMR	National Center for Microbial Resource

IDA	International Depository Authority
LCB	Lignocellulosic Biomass
SCB	Sugarcane Bagasse

Synopsis Report

AcSIR	**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	Yadav Rakeshkumar Jaynarayan
Degree Enrollment No. &Date	10BB18A26030 and 13.08.2018
Laboratory	CSIR- National Chemical Laboratory
Title of the Thesis	Comprehensive insights into microbial ecology and harnessing biomass conversion potential of culturable bacteria from riverine system
Research Supervisor/ Co-supervisor	Dr. Mahesh S. Dharne

Introduction

Water is an absolute necessity for all the life forms on Earth. The irony is we have reached Mars in search of water; however, we have failed miserably to safeguard water resources on Earth. The rapidly increasing population, industrialization, and urbanization have severely impacted water bodies on the planet. Thus, most of the freshwater bodies are in a deteriorated state (Abraham et al., 2010). India has a vast riverine network; however, as per the latest reports, most rivers are in dismal conditions (Water Quality Database; Borthakur et al., 2016). Significantly, the rivers traversing through urban cities are influxed with wastewater from different industries and hospitals. Since rivers in India are worshipped, religious events such as Kumbh Mela, and Pandharpur Wari, where mass bathing is carried out along with dumping of flowers and garlands, also contribute to freshwater pollution. As per the report, in Maharashtra, out of 56 rivers, 55 rivers were found to be non-complying with the criteria used for assessment (Polluted River Stretches). Further, Pune city, one of the fastest-growing cities in India, has seven major rivers, and all of them are in a dreadful state (Marathe et al., 2017; Dhawde et al., 2018). Further, the Water Hyacinth from these urban rivers has a significant economic and environmental impact. These invasive plant species possess a prolific reproductive and dissemination rate, covering the entire water and affecting the overall water biogeochemistry.

The river microbiome is one of the major missing links in freshwater biomonitoring. Microbes possess higher growth rates and are sensitive to any physic-chemical changes or pollutants addition in rivers, thereby can act as excellent bioindicators for river health. Further, monitoring antimicrobial resistance can also indicate contamination of water bodies by wastewater from different pharmaceutical industries and hospitals. Apart from biomonitoring, these urban river microbiomes can also prove to be of biotechnological importance, as these are constantly exposed to various pollutants, changing environmental conditions, and adapting and developing specialized functions.

The overall of the present study was to understand the microbial ecology and functionality of the urban rivers, understand the link between water hyacinth and its associated microbes, and explore these unique microbes for potential biotechnological applications.

Statement of the problem

- Understudied Microbial Ecosystem: Urban river microbiome, despite their critical importance, have been largely ignored, particularly in relation to their impact on microbial dynamics and functionality in connected peri-urban rivers
- Anthropogenic influence: Human activities impact river health through urbanization, but the effects of sudden stress on microbial dynamics remain unexplored. Events like mass bathing provide insights into human-microbe interactions in rivers
- Anthropogenic influence: Human activities impact river health through urbanization, but the effects of sudden stress on microbial dynamics remain unexplored. Events like mass bathing provide insights into human-microbe interactions in rivers
- Need for Exploration of biotechnological potential: The urban rivers are constantly subjected to a complex interplay of environmental stressors, anthropogenic activities, and pollutants, thereby shaping the native microorganisms with specialized functions. Therefore it becomes essential to bioprospect the river microorganisms for sustainable production of valuable chemicals
- Need for Holistic Approach: An approach that necessitates the comprehensive investigation of the intricacies of urban river microbiome, with the end-goals of amalgamating and delineating dynamics of river-human health, environmental science, and provide sustainable biotechnological applications

Methodology

- **Pune riverine study:** Twenty-four river sediment samples were collected from different rivers (River Mula, Mutha, Ramnadi, Pavana, Mula-Mutha, Bhima, and Indrayani) in Pune. The sediments (400 mg) were subjected to DNA extraction using DNeasy PowerSoil Pro kit (Qiagen), followed by Library Preparation using SQK-LSK-109 and Native Barcoding for Nanopore sequencing. Following nanopore sequencing, the data was preprocessed using different tools, which included basecalling using Albacore (v2.3.4), Adapter trimming from the end and the middle of the reads using Porechop (v0.2.4). Data rarefactions were carried out using single_rarefaction.py and rarefy even depth function in QIIME and R, respectively. The taxonomic analysis was carried out using nr_euk database by Kaiju tool and functional analysis was perforemed using different tools such as Virulence gene mining using Virulence Factor Database, MG-RAST for functional annotations , RemeDB for remediation gene mining, NanoARG for Antibiotic Resistance Genes.
- **Wari Study:** The sampling was performed during the bathing and post-bathing events from two locations, i.e. in Indrayani River (Alandi) and Bhima River (Pandharpur) Water samples were collected in sterile containers. The water samples (n=14) subjected to DNA extraction using DNeasy PowerWater kit (Qiagen), followed by Library Preparation using SQK-LSK-109 and Native Barcoding for Nanopore sequencing. The taxonomy annotation was done in Kaiju and functional mining was carried out using Virulence gene mining using Virulence Factor Database, MG-RAST for functional annotations , RemeDB for remediation gene mining, NanoARG for Antibiotic Resistance Genes.
- **Water Hyacinth study:** The water hyacinth and corresponding water samples were collected from four sites (three river sites and one lake) from Pune City, India. The locations were named A1 (Mula River, 18.565264, 73.788792), A2 (Ramnadi River, 18.548429, 73.79844), A3 (Mula River 18.567607, 73.796593) and (Pashan Lake, 18.535551, 73.787922). The sampling was performed in the month of March 2020, and the follow-up sampling was carried out in 2022 in the same month from the same sites. In the first sampling, 11 WH samples were collected, triplicate from each site (A1, A2, A3, and WH), along with the four water samples (1 from each site). The WH was collected in sterile Hidispo bags (PW038, Himedia) and was immediately sealed and

transported in dry ice. Around 1 litre of water was collected in sterile polypropylene bottles. In the second sampling, we collected 8 WH samples (from three sites A1, A2, and A3) and three corresponding water samples. Around 250 mg of the crushed roots were processed for DNA extraction using DNeasy PowerSoil Pro Kit (Qiagen, 47014) as per the manufacturer's instructions. 1D Ligation sequencing kit SQK-LSK-109 was used for library preparation. Barcoding was carried out using EXP-NBD104 and NBD114. The library was prepared per the manufacturer's instructions with the following modifications. Qubit was used to quantify the resultant library, and 450 ng was loaded onto FLO-MIN-106D, R9 Version. The reads were basecalled and demultiplexed using Guppy software (V 5.0.11) (Wick et al., 2019). NanoFilt (v 2.7.1) was used for filtering and trimming of reads using a Q-score of 8 and read length of 500bp. The taxonomic classification was performed using the nr_euk database using the Kaiju tool with default Greedy mode using an in-house customized script. Further, functions were analyzed using the KEGG database in MG-RAST. The RemeDB was used for analyzing pollutant degrading enzymes (PDEs) using the DIAMOND alignment tool (v 0.9.26) using parameters for long reads (e-value of 10^{-5}, identity of 60, --long reads (-F 15, --range-culling, k 1), subject cover 40, --more-sensitive). Similarly, the BacMet database (v 2.0) was used for biocide and metal resistance genes (MRGs) using the same diamond parameters. For taxonomic classification of genes from BacMet and RemeDB, reads were extracted, and kaiju was used.

- **Production of 2,3-butanediol and Poly-gamma-glutamic acid:** EX5-6 bacterial isolate isolated from the sediment of Pawana River, Pune, Maharashtra (18.57305, 73.83121) on St. Nutrient Agar. The screening for BDO production was done using fermentation media with composition of Glucose 50g/L, K2HPO4 3g/L, Yeast Extract 10g/L, Peptone 10g/L, Sodium chloride 5g/L, MgSO4.7H2O 0.2 g/L, pH 7.0. The

culture were grown overnight in Nutrient Broth No. 3 (M1902, HIMEDIA) at 37°C for 12 hours and was inoculated in the fermentation media for 24hrs. After incubation period, the broth was analysed for the production of 2,3-butanediol by HPLC. The column used was Aminex HPX-87H (1250140, BioRad), 5mM H_2SO_4 was used as a solvent with column flow of 0.6ml/min, oven temperature of 60°C, and run time of 30 mins with detection using Refractive Index (RI) detector. Standard 2,3-butanediol was procured from Sigma. The 16S rRNA sequencing (27F and 1492R universal primers) was performed for molecular identification. The NCBI accession number for 16S rRNA is OR263461. The optimization for 2,3-BDO was performed using one factor at a time (OFAT) approach. The concentration of C-source, Yeast Extract, and Peptone was optimized. For all the optimization, seed medium consisted of Yeast Extract 5g/L, Peptone 5g/L, NaCl 5g/L, Beef extract 5g/L, Glucose 20g/l, and pH 7.0. A colony was inoculated in to seed medium at 37°C till O.D. 600 reached to 8.0. Further 10 % of inoculum was inoculated into fermentation medium to produce 2,3-BD. HPLC and Gas chromatography was employed for the detection of metabolites. HPLC conditions included: Column- Aminex HPX-87H (1250140, BioRad), Solvent- 5mM H_2SO_4, Flow rate- 0.6ml/min, Detector- RI, runtime- 30 minutes, HPLC (UHPLC, Ultimate 3000, ThermoScientific). The GC conditions were Column name: FFAP (30mx0.25mmx0.25µm), Detector and injector temperature of 300°C, sample volume 1 ul.

The bacteria WA11 isolated from Water Hyacinth was inoculated in nutrient broth and incubated for 12 hours at 37 °C. Briefly, a colony of WA11 isolate was inoculated in seed medium (Glucose 2%, Yeast extract, Peptone, Sodium Chloride, and Beef extract 0.5%, pH 7.0) at 37 °C overnight. 5% of the overnight grown isolates were inoculated in a fermentation medium (Glucose 5%, L-Glutamic acid monosodium salt

monohydrate 4%, Na2HPO4 0.1%, NaH2PO4 0.7%, MgSO4.7H2O 0.02%, Yeast extract 1%, pH 7.0). Following incubation for 24 hours at 28 ℃, the broth was centrifuged at 10,000 rpm for 20 minutes. The resultant supernatant was mixed with 4X chilled methanol volume and was observed for fibrous precipitate. The molecular characterization of the γ-PGA-producing isolate was carried out using 16S rRNA sequencing. The optimization was performed using one factor at a time (OFAT) approach. The produced crude γ-PGA was characterized using Thin Layer Chromatography using solvents n-Butanol: Acetic Acid: H2O in the ratio of 12:5:3, Fourier Transform Infrared (FTIR) Spectroscopy (ATR mode using a Bruker Tensor II spectrophotometer equipped with a diamond crystal probe detector. Absorbance spectra were recorded from 500-4000 cm–1 with a resolution of 4 cm–11), and Nuclear Magnetic Resonance (1H NMR, 10mg/mL sample was dissolved in D2O (deuterium oxide) and recorded 1H NMR spectra using Bruker AV 500 MHz).

Results and their interpretation

Pune riverine study:

Although not significant, we observed higher bacterial richness and diversity in urban waters (Fig. 1A.). On the contrary, peri-urban sites showed significantly higher archaeal richness (Welsch's t-test, $p<0.05$) (Fig. 1B.) than the urban waters. Beta diversity for understanding the overall community differences was estimated using the Bray-Curtis dissimilarity matrix. NMDS plot showed significant dissimilarity in the bacterial (ANOSIM, R=0.17, p-value <0.05, Stress value= 0.1) as well as archaebacterial (ANOSIM, R=0.18, p-value<0.05, Stress value= 0.1) composition of the urban and peri-urban waters (Fig. 1C and 1D.). the beta diversity analysis showed significant microbial dissimilarity between the riverine stretches; however, we observed lower ANOSIM R-values (0.17 and 0.18 for bacteria and archaebacteria), indicating significant differences with overlapping similarities. These similarities can be reasoned: Firstly, the rivers examined in this study are located in similar climatic conditions (with rivers as the variable factor we obtained PERMANOVA, $p>0.05$), and secondly, the riverine sites MM3, MM5, BMM1, BMM2, and BMM3 grouped into the urban waters

geographically are at the outskirts of the Pune city. However, unlike the other peri-urban sites in this study, MM3, MM5, BMM1, BMM2, and BMM3, being the downstream city sites, receive all the urban waters and thus are "urban-influenced". Upon excluding these samples from the urban water groups, the analysis revealed a significant dissimilarity in the microbial community between the riverine system's urban and peri-urban sites with a higher ANOSIM R-value of 0.55. Based on the diversity analysis, it can be concluded that the urbanisation and increased anthropogenic activities in the cities have not only a crucial impact on the microbial diversity of the urban surface water but also the downstream stretch of an interconnected riverine system.

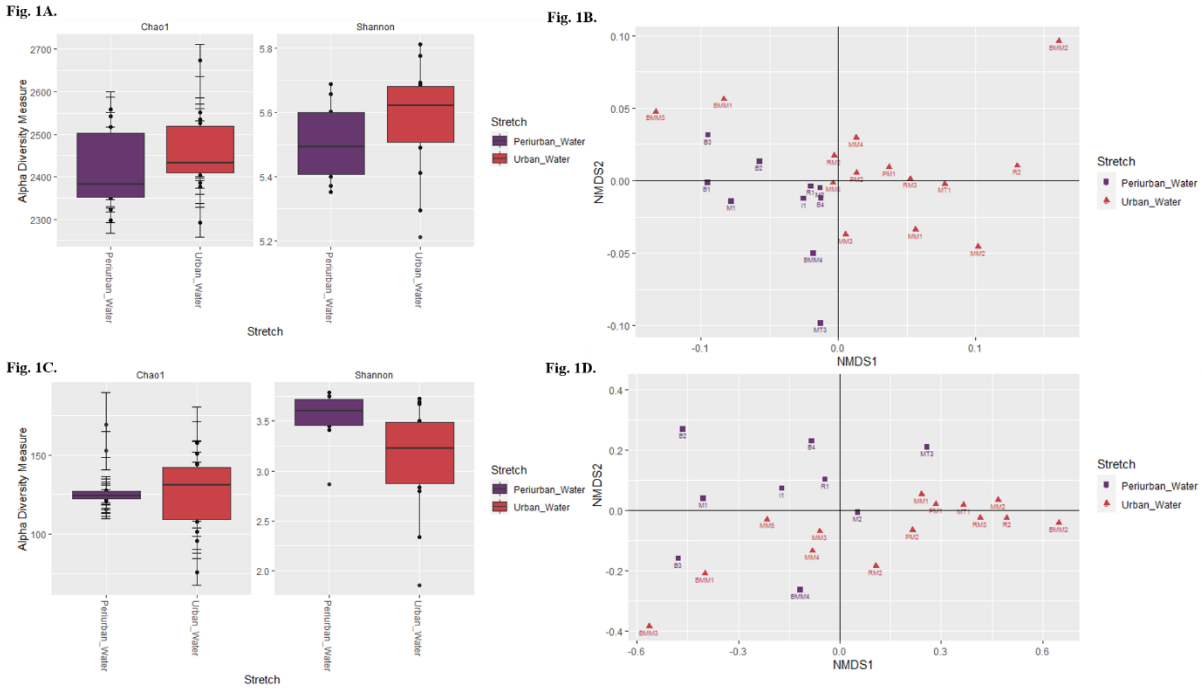

Fig. 1. Microbial diversity of the riverine system. 1A. and 1B. Bacterial alpha diversity (Welch Two Sample t-test, p-value >0.05) and beta diversity (ANOSIM, p-value<0.05, R=0.17), respectively with NMDS stress value= 0.1. **1C. And 1D.** Archaebacterial alpha diversity (Welch Two Sample t-test, p-value <.0.05) and beta diversity (ANOSIM, p-value<0.05, R=0.18), respectively.

The analysis revealed ARGs against 26 categories of antibiotics, including the last resort antibiotics such as polymyxin in the riverine system. Overall, 40% of the ARGs were classified to multidrug resistance (MDR) genes, followed by 10% conferring resistance to bacitracin, 7.5% to aminoglycoside, 7% to tetracycline, and 5% to glycopeptide (Fig. 2).

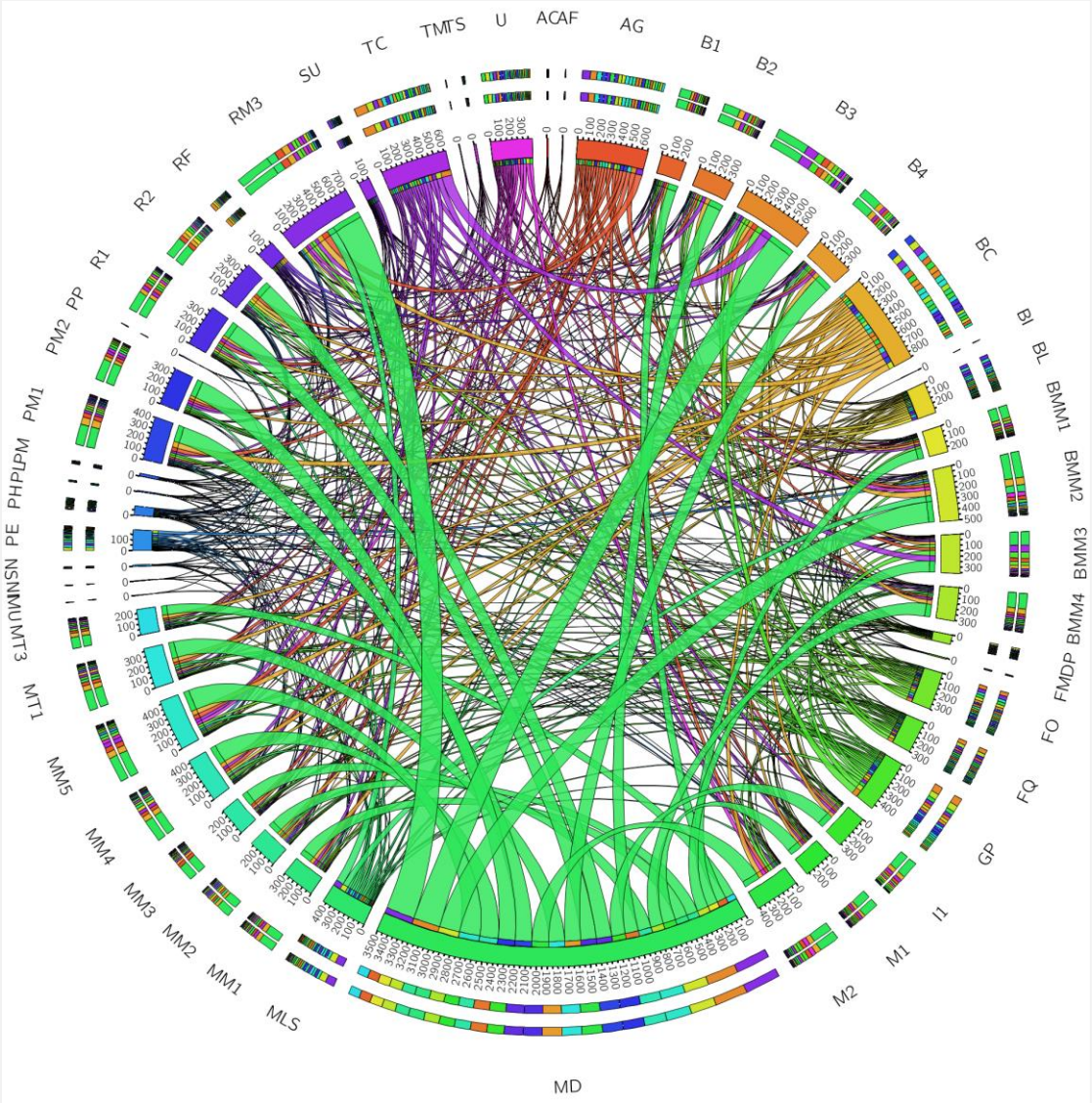

Fig. 2. Resistance against diferent categories of antibiotics.

Wari study:

We observed differences in the bacterial alpha diversity between the bathing and post-bathing samples for both the rivers, respectively. Similarly, lower bacterial diversity (Fig. 3C) was observed in Indrayani River. A significant differences in the microbial communities (Fig. 3B and D were observed. Further, functional gene analysis using MG-RAST tool indicated functional dissimilarity for the bathing and post-bathing samples of Indrayani (Permanova (Adonis test) p-value<0.05, R2 ¼ 0.45 and betadisper p-value>0.05) and Bhima (Permanova, p > 0.05). Overall we observed spatiotemporal variations in the overall microbial ecology collectively illustrated the impact of mass bathing.

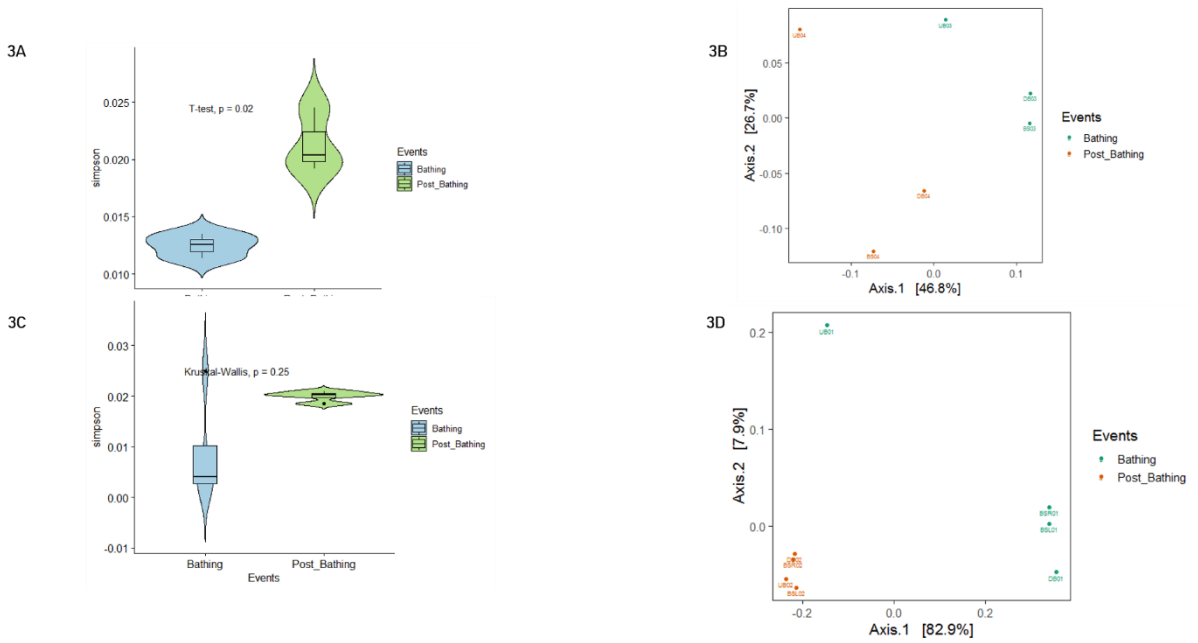

Fig. 3. Spatiotemporal variations of Bacterial Community. 3A and 3C.Violin plot depicting alpha diversity between bathing and post-bathing samples of Bhima River (t-test p-value 0.05) respectively. 3B and 3D. Beta diversity estimation (PCoA plot using Bray-Curtis dissimilarity matrix) of River Bhima (Adonis test and Permanova $p > 0.05$) and Indrayani (Adonis test and Permanova $p < 0.05$) respectively during Wari.

Water Hycinth study:

Water hyacinth (WH) is a widespread floating invasive aquatic plant having a prolific reproductive and dispersion rate. With the aid of its root-associated microbes, WH significantly modulates the ecosystem's functioning. Despite their irrevocable importance, the WH microbiome remains unexplored in detail. Here, we present a shotgun sequencing analysis of WH rhizobiome (from urban rivers and a lake) and their surrounding water to unveil the diversity drivers and functional relationship. PCoA analysis revealed that microbial diversity of the WH is significantly shaped by the type of the aquatic bodies (River Vs Lake) (ANOSIM-R of 0.94 to 0.98 and PERMANOVA ADONIS R2 of 0.36 to 0.54). Temporal variations (River WH_2020 vs WH_2022) (R of 0.8 to 1 and R2 of 0.17 to 0.41) were observed in river WH. Also, the WH microbiome significantly differed (R= 0.46 to 1.0 and R2 of 0.18 to 0.52) from its surrounding water (Fig. 4). Further, functional analysis predicted 140 pollutant-degrading enzymes (PDEs) known to be involved in the degradation of a wide variety of xenobiotic pollutants, including hydrocarbons, plastics, and aromatic dyes. The higher prevalence of metal and biocide resistance genes further highlighted the persistence of resistant microbes assisting WH in environmental remediation application.

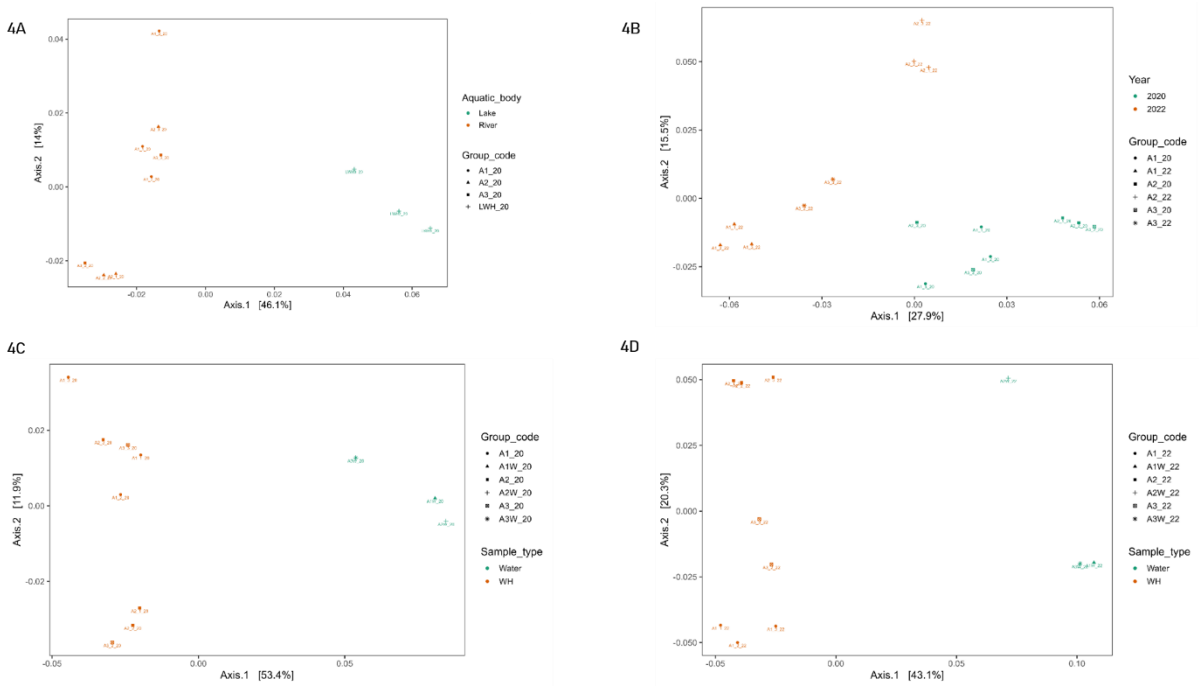

Fig. 4. Spatiotemporal variations of Bacterial Community in Water Hycinnth. 4A. Lake vs river, 4B. Temporal, 4C and 4D. Water vs WH.

2,3-Butanediol and Poly-gamma glutamic acid Production:

Identification and genomic characterization

The 16S rRNA sequence (1425bp) was analyzed and identified on EZtaxon. The top-hit strain was *Bacillus halotolerans* ATCC 25096 with 99.86% similarity and 95.2% completeness. Further, phylogenic investigation using Neighbour Joining method with bootstrap of 1000, suggested close phylogeny to Bacillus Halotolerans (Figure 5).

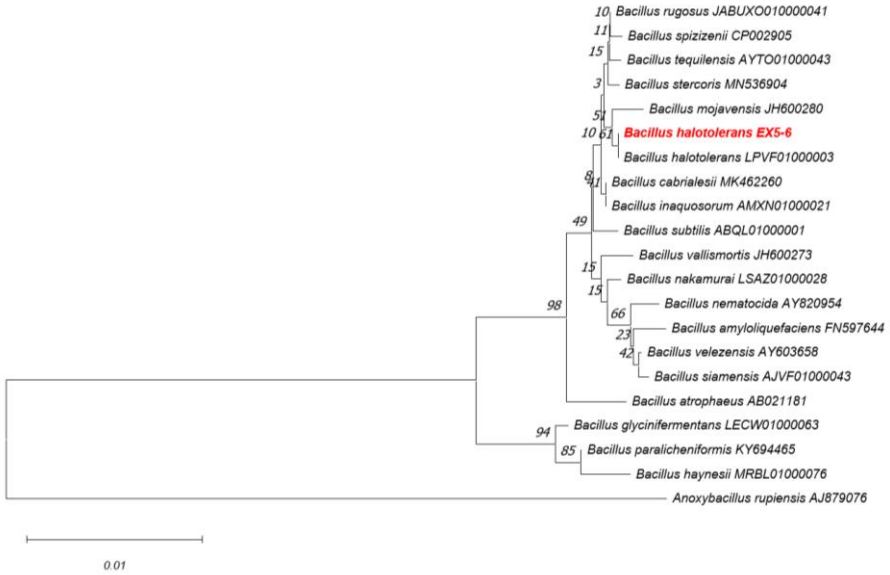

Fig.5. Molecular Phylogeny. 16S rRNA gene-based molecular phylogeny analysis using Neighbour Joining method. Red highlighted strain is the bacterial isolate used in the present study

3.2. Optimization of 2,3-BD production using Bacillus halotolerans EX5-6

We utilized OFAT strategy to optimize the media and physical parameters for BDO production. Higher production was obtained at 28°C and at pH 5. Glucose and yeast extract was found to be the favourable carbon and nitrogen source, respectively. Further, concentration optimization suggested 15% and 2.5% as the best glucose and yeast extract concentrations for 2,3-butanediol production by Bacillus halotolerans EX5-6. Upon final validation, we could obtain 73.19 g/L of BDO with yield and productivity of 0.5g/g glucose and 1.02 g/L/h from synthetic medium, respectively (Fig.2. A to H).

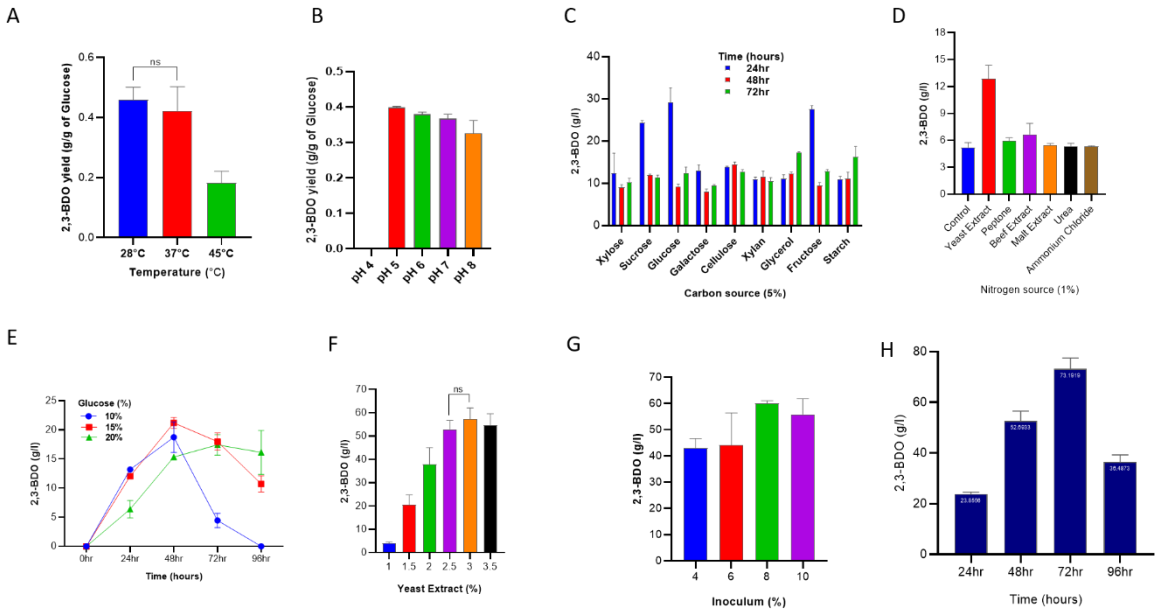

Fig.6. Optimization of media and conditions for 2,3-butanediol production from synthetic media. A and B. Optimization of temperature and pH; C and D. Optimization of different Carbon and Nitrogen sources; E. Effect of Glucose concentration; F and G. Effect of Yeast extract and Inoculum concentration on BDO production; H. Production from optimized medium. Statistical significance was assessed using One-way ANOVA with Tukey's multiple comparison test.

Bacillus halotolerans have never been reported for the production of BDO. The optimized synthetic medium consisted of Glucose 15%, K2HPO4 0.3%, Yeast Extract 2.5 %, Sodium chloride 0.5%, MgSO4.7H2O 0.02%, pH 5.0 with incubation condition: 180 rpm at 28°C. Overall, we observed 2.5 fold increase in the production of BDO from non-optimized

medium. We obtained 2,3-BDO concentration of 73.19 g/l with Yield and Productivity of 0.5 g/g and 1.02 g/l/h, respectively.

Lignocellulolytic activities, screening, and molecular characterization

We observed cellulolytic, ligninolytic, and xylanolytic activities in bacterial isolate WA11 and (Fig. 7.). Further, screening suggested WA11 positive for γ-PGA production. Therefore further optimization was carried out for γ-PGA using WA11 bacterial isolate.

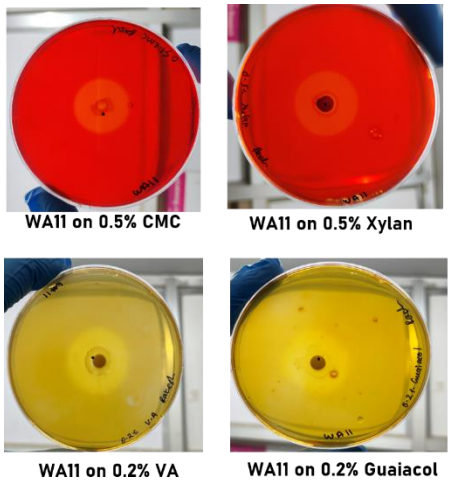

Fig. 7. Screening for ligninolytic activity (using 0.2% Veratryl Alcohol (VA) and Guaiacol), cellulolytic activity using 0.5% of Carboxymethyl cellulose (CMC), and xylanolytic activity using Beechwood xylan 0.5%.

The 16S rRNA sequence (1412 bp) was analyzed for closest type strain similarity using EZtaxon. The top-hit strain *was Bacillus velezensis* CR-502(T), with 100% similarity and 95.4% completeness. Further, phylogenic investigation using the Neighbour Joining method with 1000 bootstrap suggested close phylogeny to Bacillus velezensis (Fig. 8).

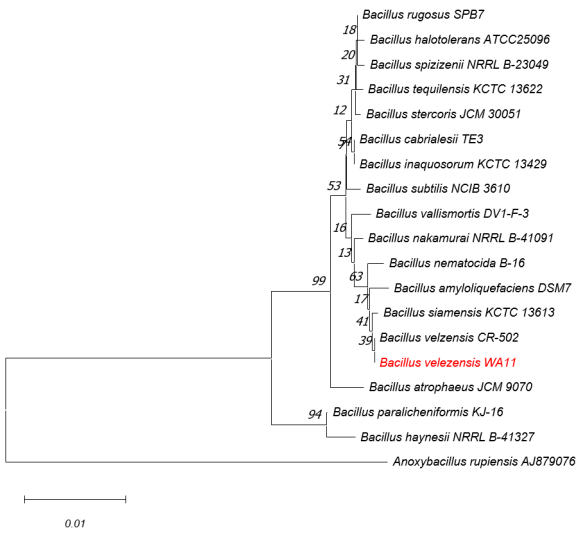

Fig 8. Molecular Phylogeny. 16S rRNA gene-based molecular phylogeny analysis using Neighbour Joining method. Red highlighted strain is the bacterial isolate used in the present study

Optimization for γ-PGA production from synthetic and bagasse containing medium

We utilized the OFAT strategy to optimize the media and physical parameters for γ-PGA production. Higher production was obtained at 28°C and pH 7.0. Maltose and yeast extract were the favorable carbon and nitrogen sources, respectively. Further, concentration optimization suggested 25%, 1%, and 6% as the optimum maltose, yeast extract, and L-glutamate concentrations for γ-PGA production by *Bacillus velezensis* WA11. Upon final validation, we could obtain 104.3 g/L of dried crude γ-PGA with productivity of 1.09 g/L/h from synthetic medium, respectively (Fig.9. A to J) from optimized synthetic medium.

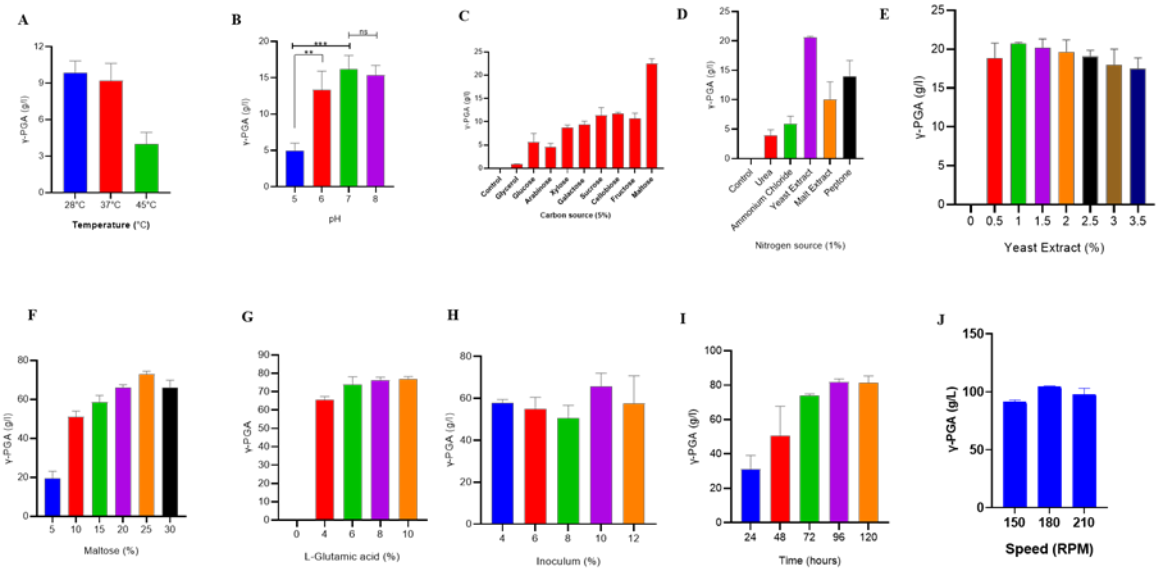

Fig. 9. Optimization of media and conditions for γ-PGA production from synthetic media. A and B. Temperature and pH optimization; C and D. Carbon and Nitrogen source optimization; E,F, and G. Yeast extract, Maltose, and L-Glutamic acid concentration optimization; H. Inoculum concentration optimization; I. Production using optimized conditions; and J. Shaking speed standardization (in Revolution per minute RPM). Statistical tests were performed using One-way Anova with Tukey's multiple comparison test.

The optimized synthetic media consisted of Maltose- 25%, L-Glutamic acid- 6%, Na2HPO4 - 0.1%, NaH2PO4- 0.7%, MgSO4.7H2O- 0.02%, Yeast Extract- 1%, pH- 7.0, RPM of 180. Further, the γ-PGA was characterized using different methods (Fig. 4). To the best of our knowledge, the highest γ-PGA production reported by using maltose as a carbon source from *Bacillus subtilis* was 35 g/l with a productivity of 0.36 g/L/h (Ogawa et al., 1997). In the present study, we report 104.3 g/L γ-PGA production with productivity of 1.09 g/L/h, 2.9-fold higher than the previous study using maltose as a carbon source.

Conclusions

The study of peri-urban, urban, and immediate city outskirts stretch revealed significant changes in microbial diversity and ARGs. Spatiotemporal variations in microbial ecology show mass bathing impact. Comparison with post-bathing samples reveals ecological changes during bathing. Diverse microbial communities found in water hyacinth roots. Higher ANOSIM R-value and Adonis R2 value imply water body types and time as key factors for microbial diversity. Methane and sulfur metabolism were major energy deriving process for the water hyacinths. First report of production of 2,3-BDO from Bacillus halotolerans, 2,3-BDO concentration of 73.19 g/l with Yield and Productivity of 0.5 g/g and 1.02 g/l/h, respectively. Through this study, we seek to not only advance scientific knowledge concerning microbial dynamics from riverine system, their resilience and adaptability by employing modern approach, but also contribute to the development of innovative solutions to pressing environmental and biotechnological challenges.

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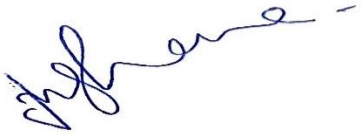

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Chapter 1. Introduction

Chapter 1. Introduction

1.1. State of the River ecosystem

Water is irreplaceable, and unarguably the most critical component for all the life forms on Earth. The irony is that we have reached Mars, questing for water; however, we have failed miserably to safeguard our water resources, resulting in a grim state of aquatic bodies. The ceaseless desire for socio-economic development has unappealing influences on river bodies, especially the urban rivers. These rivers traversing cities are often contaminated chemically and have become the nearest sink for wastewater from different sources such as hospitals and industries (Francis et al., 2014). The United Nations members drafted 17 different interlinked multidimensional goals in 2015, known as Sustainable Development Goals (SDGs) (SDGs. https://sdgs.un.org/goals). The primary objectives of these goals are to achieve the overall development of people and safeguard climate by establishing global partnerships across developed and developing countries. Two of these goals, SDG 6 (Clean Water and Sanitation) and 14 (Life below Water), concern providing clean water for humans and nature. As these goals are interlinked, achieving clean water is linked to other SDGs, such as SDG 2 (Sustainable agriculture) and SDG 11 (Sustainable cities), clean water is expected to play a pivotal role in overall development (Strokal & Kroeze 2020; Xu et al., 2019). However, as per the World Health Organization (WHO), 2 billion people consume contaminated water worldwide. Further, dreadful statistics include more than four lacs diarrhoea-related deaths yearly, and 80% of diseases are waterborne (Water Pollution). India has a vast riverine network; however, as per the latest reports, most rivers are in dismal conditions (Water Quality Database; Borthakur et al., 2016). The CPCB (Central Pollution Control Board) water quality report for 2019 and 2021 identified 311 polluted stretches on 279 rivers in 30 Indian States and Union Territories (Table 1.1). As per the report, in Maharashtra, out of 56 rivers, 55 rivers were found to be non-complying with the criteria used for assessment (Polluted River Stretches).

Table 1.1. The following table illustrates the number of polluted river stretches in India years 2019 and 2021 (Ref: Polluted River Stretches).

Sr. No.	State/Union Territory	Polluted stretch numbers
1	Andhra Pradesh	3
2	Assam	10
3	Bihar	18
4	Chhattisgarh	6
5	Daman and Diu, Dadra and Nagar Haveli	1
6	Delhi	1
7	Goa	6
8	Gujarat	13
9	Haryana	3
10	Himachal Pradesh	9
11	Jammu and Kashmir	8
12	Jharkhand	9
13	Karnataka	17
14	Kerala	18
15	Madhya Pradesh	19
16	Maharashtra	55
17	Manipur	13
18	Meghalaya	7
19	Mizoram	3
20	Nagaland	4
21	Odisha	7
22	Puducherry	3
23	Punjab	5
24	Rajasthan	14
25	Tamil Nadu	10
26	Telangana	9
27	Tripura	1
28	Uttar Pradesh	17
29	Uttarakhand	9
30	West Bengal	13
Total		311

1.2. River Microbiome: A Key Ecological Influence

Most human livelihoods and activities hugely depend on the availability of clean water. A study by Jones et al., 2021, estimated that 48% of global wastewater is released untreated into the environment. As per the World Health Organization and UNICEF report in 2020, only

56% of the world's domestic wastewater was treated (Wastewater). The standard criteria to interpret the water quality include assessment of pH, Dissolved Oxygen, Biochemical Oxygen Demand, and Coliforms (CPCB water quality criteria, Table 1.2.).

Table 1.2. Common water quality assessment criteria by CPCB (Reference: CPCB water quality criteria)

Designated-Best-Use	Class of Water	Criteria
Drinking Water Source without conventional treatment but after disinfection	A	• Total Coliforms Organism MPN/100ml shall be 50 or less • pH between 6.5 and 8.5 • Dissolved Oxygen 6mg/l or more • Biochemical Oxygen Demand 5 days 20C 2mg/l or less
Outdoor bathing (Organised)	B	• Total Coliforms Organism MPN/100ml shall be 500 or less pH between 6.5 and 8.5 Dissolved Oxygen 5mg/l or more • Biochemical Oxygen Demand 5 days 20C 3mg/l or less
Drinking water source after conventional treatment and disinfection	C	• Total Coliforms Organism MPN/100ml shall be 5000 or less pH between 6 to 9 Dissolved Oxygen 4mg/l or more • Biochemical Oxygen Demand 5 days 20C 3mg/l or less
Propagation of Wild life and Fisheries	D	• pH between 6.5 to 8.5 Dissolved Oxygen 4mg/l or more • Free Ammonia (as N) 1.2 mg/l or less
Irrigation, Industrial Cooling, Controlled Waste disposal	E	• pH between 6.0 to 8.5 • Electrical Conductivity at 25C micro mhos/cm Max.2250

		• Sodium absorption Ratio Max. 26 • Boron Max. 2mg/l
	Below E	• Not Meeting A, B, C, D & E Criteria

The microbial communities play a vital role in this environment, regulating the biogeochemical cycles forming the basis of food webs and responding to variability in local and global environmental conditions (Philippot et al., 2021; Gupta et al., 2017). However, the microbial community is often ignored when assessing the quality of rivers and other aquatic bodies. They have an essential role in aquatic ecosystems, primarily in accumulating, transforming, and migrating nutrients (Ren et al., 2019). Additionally, their metabolic capabilities can influence water biogeochemistry; thus, it becomes imperative to understand the roles of microbes in aquatic ecosystems. Owing to their high metabolic and growth rates, microbes are sensitive to physicochemical alterations and are influenced by changes in organic and inorganic compounds and other pollutants in the aquatic bodies. Thus, they can serve as bio-indicators to environmental stressors (Fig 1.1.).

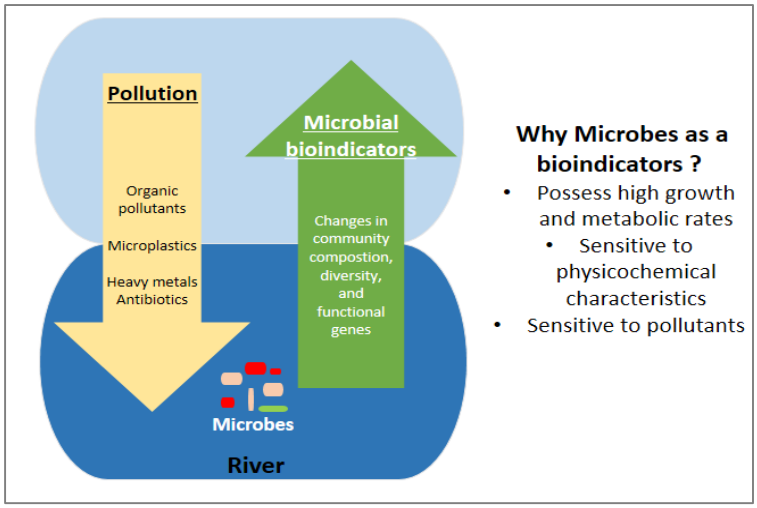

Fig. 1.1. Microbes as a Bioindicator. The figure depicts the significance of the microbes in routine freshwater biomontioring. The concept adapted from Sagova-Mareckova et al., 2021.

1.3. Application of metagenomics for river biomonitoring: A holistic approach

With more than 50% of the world's population residing in urban setups, the urban waterways, especially rivers, are subjected to immense stress by various anthropogenic activities. Rivers are revered in India; as a result, multiple religious events such as spiritual mass bathing during Kumbh and Pandharpur Wari and other anthropogenic activities such as dumping of garlands put additional pressure on these water bodies (Samson et al, 2019; Jani et al., 2018). The relationship between microbiology and water quality assessment goes hand in hand. It is estimated that more than 37 million people are annually affected by water-borne diseases in India alone, with more than 1.5 million child deaths due to diarrhoea (Kumar et al., 2022). Although effective, quantifying *Escherichia coli* or coliform-forming bacteria cannot represent the overall representation of pathogen levels in water. Owing to their highly responsive nature, the microbial communities can also indicate the type and levels of pollutants in the water bodies. However, culture-based methods fail to deliver the overall picture of microbial community structure due to various limitations. It is impossible to mimic the exact environment for the microbes in the laboratory; consequently, most of the cultures cannot be grown, and vast information is lost. Further, the culture-dependent approach is time-consuming, one of the most crucial factors in water quality assessment (Sagova-Mareckova et al., 2021; Datta et al., 2020).

1.3.1. Introduction to Next-Generation Sequencing Technologies

Metagenomics is an emerging science, a culture-independent approach that evaluates genome sequences directly from any environment or biome. It is a rapid and effective strategy to understand the microbial abundance pattern and their diversity, also evaluating the functions and marker genes associated with the microbiome of a particular biome (Sagova-Mareckova et al., 2021). Moreover, the development of various microbial taxonomy and functional databases

has further enhanced the potential of metagenomics in biomonitoring and bioprospecting studies. Modern sequencing technologies have evolved from Sanger sequencing technology to the Next Generation Sequencing technologies (NGSTs) and the Third Generation sequencing (TGS) (Zhang et al., 2021) (Fig. 1.2.).

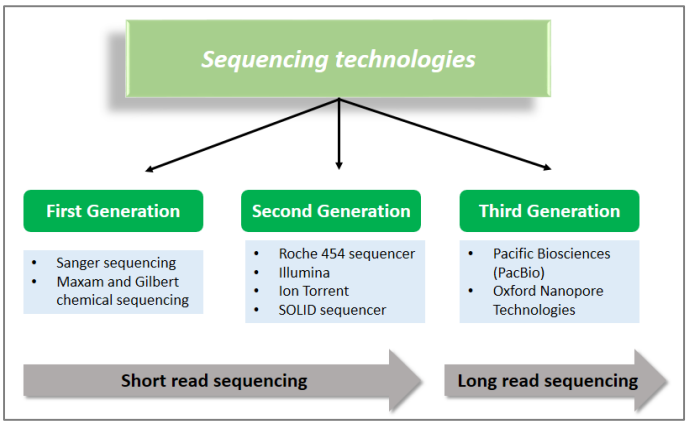

Fig. 1.2. Sequencing technologies (adapted from Akaçin et al., 2022)

Presently, three generations of sequencing technologies are extensively used. The first-generation sequencing technology, Sanger sequencing technology, also known as the gold standard of sequencing, utilizes the chain termination method, relying on di-deoxy nucleotides. The accuracy of Sanger is higher than other sequencing technologies and provides high-accuracy reads of 600 to 1000 bp. However, slow process and time consumption are its limitations. The second-generation sequencers are based on various principles, including sequencing by synthesis, pyrosequencing, and ligation-based sequencing. The read lengths vary between 50 bp to 500 bp. The second generation sequencer includes Roche, Illumina, and SOLID sequencers. The 3rd sequencing technologies are based on single-molecule sequencing technology (Zhang et al., 2021; Akaçin et al., 2022). In contrast to previous generation sequencers, the TGS provide long reads in rapid and real-time processes. Two commercially available TGS include PacBio sequencer from Pacific Biosciences, also known as a single molecule real-time (SMRT) sequencer that enables read lengths up to 50 kb; the other well-

known TGS includes Nanopore sequencer by Oxford Nanopore sequencing technologies. The Nanopore is a portable and real-time sequencer that relies on the characteristics and current changes of nucleotide bases for their detection (Zhang et al., 2021; Akaçin et al., 2022; Kerkhof, 2021). Table 1.3. provides the details of the Next generation sequencers.

Table 1.3. The details of various Next-Generation Sequencing tools

Manufacturer	Equipment	Principle	Pros	Cons
Illumina	Miniseq, Miseq, NextSeq 550, Hiseq 2000	Sequencing by synthesis (Bridge PCR)	High sequencing yield, Fast run time, Higher accuracy	Equipment cost is high
Thermo Fisher	Ion PGM Torrent, Ion 5 S	Sequencing by synthesis (Semiconductor-based)	Equipment cost is low compared to TGS platforms, Fast run time.	Short read length, homopolymer errors
Roche	454 GS and Junior	Pyrosequencing	Fast run time. Longer read length. Small data files	Reagent cost is high, Homopolymer errors.
Pacific Biosciences	Sequel, RSII	Single molecule real-time	Long-read Technology, High accuracy and sensitivity	Low yield at high accuracy, Equipment cost is high.
Oxford Nanopore Technologies	MinION, GridION, PromethION, VolTRAX	Sequencing by detection of current	Long reads, inexpensive, portable, real-time, Direct RNA sequencing	High error rates ,Sensitivity of nanopores changes depending on the sample and the Environment.

1.4. Integrating prokaryotes and their functions for River biomonitoring

With the advent of NGSTs, several metagenomic studies have been conducted in past decade to understand river microbial community and their functionality, indicating implementation of NGSTs to study river microbial ecology. Around 873 and 121 publications

with search term of "River Microbiome or River Metagenome" and "River Functional Metagenomics", respectively, were found on on Web of Science (Fig. 1.3).

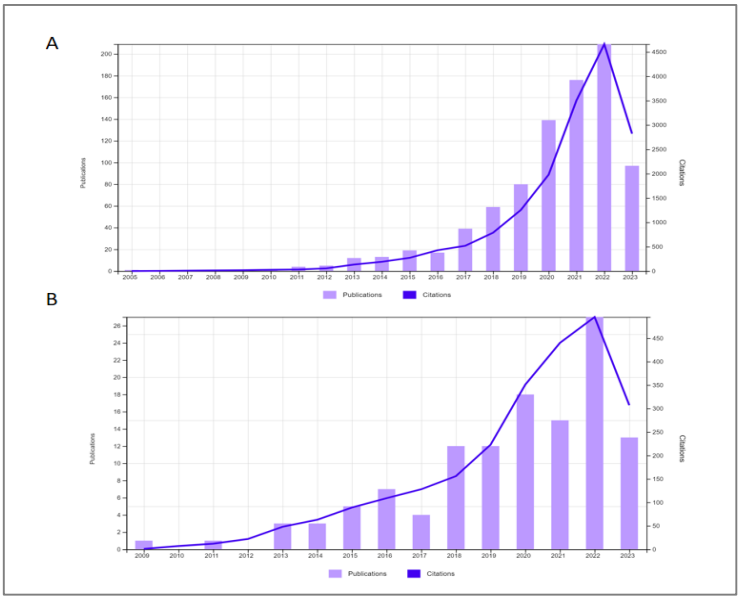

Fig. 1.3. The trend of NGS-based studies for River microbial ecology. A. Number of studies obtained with a search term of "River Microbiome or River Metagenome" in Web of Science, **B.** Number of studies observed with a search term of "River Functional Metagenomics" on Web of Science.

Despite their crucial role in environmental processes, microbes are rarely used as bioindicators for water quality assessment. Rivers are often exposed to environmental stressors, including inorganic nutrients from agriculture and domestic use, organic contaminants from urban areas, stormwater runoffs, effluents from wastewater treatment plants, and hospital discharges (Sagova-Mareckova et al., 2021, Ibekwe et al., 2016).

1.4.1. Microbial pattern and diversity shift upon exposure to pollutants

Microbes react to such changes, reflected by the differences in their abundance pattern and functional gene expression (Fig 1.1.). For instance, eutrophication leads to an increase in microbial richness due to higher levels of nutrients (Sagova-Mareckova et al., 2021, Ibekwe et al., 2016, Kiersztyn et al., 2019). Also, the studies have reported a proportional rise in cyanobacteria during eutrophication, while pelagic actinobacteria preferring oligotrophic

environment decrease with eutrophication. Similarly, the studies have also reported *Actinobacteria* as one of the indicators of organic pollution in the river sediments since they are adapted to higher organic matter concentrations (Sagova-Mareckova et al., 2021; Zeglin 2015). Such organic pollutants and compounds are generally associated with anthropogenic ctivities and matters from pharmaceuticals and cosmetics.

Further, organic fertilizers from agricultural fields result in increased nitrogen inputs to the water bodies. Many studies have reported significant shifts in the microbial communities in the sediments of freshwater bodies such as rivers and lakes due to increased levels of ammonium, nitrates, total nitrogen, and nitrites. Certain bacterial groups such as *Chloroflexi, Nitrospirae,* and members of *Betaproteobacteriales* are reported to increase with nitrogen proportionally (Sagova-Mareckova et al., 2021, Wan et al., 2017). Further studies have also evaluated the effect of wastewater treatment plant effluents on the lotic aquatic bodies, observing the increase in the abundance of *Bacteroidetes, Spirochaetes,* and *Nitrospirae* and a decrease in the *Actinobacterial* population (Sagova-Mareckova et al., 2021; Chonova et al., 2018).

Several studies evaluated the shifts in the microbial community pattern upon the exposure to different pollutants. Parabens, a growing organic contaminant, caused microbial enrichment in the Jinsha River in China, according to Liu et al., 2021. The *Flavobacterium, Prevotella,* and *Hydrogenophaga* were much more prevalent in riverine sites in industrial areas with higher pollutant levels. They also noted that certain pollutants enriching several taxa, including *Candidatus Protochlamydia,* which was positively correlated with various medications and personal care items, *Yonghaparkia,* associated with coffee, and Thiovirga, associated with methylparaben (MeP) and propylparaben (PrP). Furthermore, the parabens negatively correlated with the prevalence of Gram-positive bacteria while favouring the growth of bacteria that can withstand oxidative stress. In 2016, Guo and the team conducted another

investigation that showed the effects of a paper mill on the surrounding river. They claimed that the sediments impacted by paper mill waste included increased concentrations of *Fusibacter* and *Desulfobulbus*. Thiosulphate reduction, acidogenesis or syntrophic acid degradation, and polychlorinated biphenyl (PCB) degradation have all been attributed to these bacterial taxa (Shin et al., 2010). Similar findings were made by Yuan et al. in 2023, who found that heavy metals were crucial for altering variety and that *Pseudomonas* and *Ruminococcus* (faecal and sewage indicator bacteria) were connected with Copper and Zinc.

1.4.2. Monitoring of ecological and functional changes for biomonitoring

Two critical ecological processes, i.e. deterministic and stochastic processes, are involved in the microbial assemblage of the ecosystem. Deterministic is homogeneous and heterogeneous selection by similar environmental conditions and distinct selection pressure, respectively, while stochastic is random death, birth, and reproduction drift (Trego et al., 2021). Liu et al., 2021a observed that, as paraben concentrations increased along the river's rural-urban gradient, the stochastic process outperformed the deterministic approach and became the primary ecological driver of microbial assembly in residential and industrial regions. Furthermore, Liu et al., 2021b, demonstrated the functional impact of polycyclic aromatic hydrocarbons (PAHs) and phthalates (PAEs) on the microbial ecology of rivers. These pollutants negatively influenced phosphorous and nitrogen metabolism while positively impacting sulfur metabolism, finally leading to blackening.

A study by Gillings et al., 2015 documented marker genes to characterise the influence of human activities. The class 1 integron-integrase (*intl1)* gene is often linked to antibiotic and metal resistance genes. It is mainly assembled due to the selection pressure of anthropogenic activities and thus can be used as a proxy for anthropogenic pollution. Further, antimicrobial resistance (AMR) is one of the global threats that is one of the leading causes of death worldwide. The aquatic ecosystem plays a critical role in AMR transmission and provides an

environment for developing newer antibiotic-resistance genes (ARGs) (Liguori et al., 2022; Napier et al., 2020). The rivers are the ultimate sink for the active pharmaceutical ingredients (API), antibiotics, ARGs, antibiotic resistance bacteria, and mobile genetic elements released from pharmaceutical effluents, runoff from agricultural fields, and human and animal faeces (Liguori et al., 2022; Napier et al., 2020; Ram et al., 2020; Posada-Perlaza et al., 2019). Antibiotic resistance could be used as an indicator of pollution, which is essential not only in monitoring anthropogenic pollution of rivers but also can be employed to prevent potential future health crisis.

Thus, incorporating microbial communities and deciphering their diversity and functions with the help of modern sequencing technologies could provide a holistic approach to monitor freshwater ecosystems such as rivers. It enables policymakers and researchers to develop mitigating strategies for aquatic pollution and ensures ecosystem sustainability.

1.5. Water Hyacinths: An Ignored Green Invader of River

The Water Hyacinth (WH, ***Eichhornia crassipes***, Ponteneriaceae) is a rapid growing invasive gliding macrophyte that originated in the Amazon basin and has now been successfully introduced worldwide (Villamagna and Murphy et al., 2010, Ávila et al., 2019; Xu et al., 2022). Because of its fast reproductive rate and root structure, the WH produces dense mats on the surface of water bodies such as rivers, lakes, and wetlands (Ávila et al., 2019; Xu et al., 2022). They fully cover the water, preventing sunshine and oxygen from reaching the deeper levels making life difficult for other aquatic plants and fauna (Galgali et al., 2023). WH have critical effects on the overall biogeochemistry of water. The *Eichhornia crassipes* is widely used in phytoremediation, with its root system capable of accumulating different pollutants and metals (Galgali et al., 2023, De Laet et al., 2019, Jones et al., 2018) (Fig. 1.4.). Despite its prominence and ecological importance in urban waters, which are exposed to various pollutants, the studies of WH rhizobiome and its link with different aspects of rivers are largely missing.

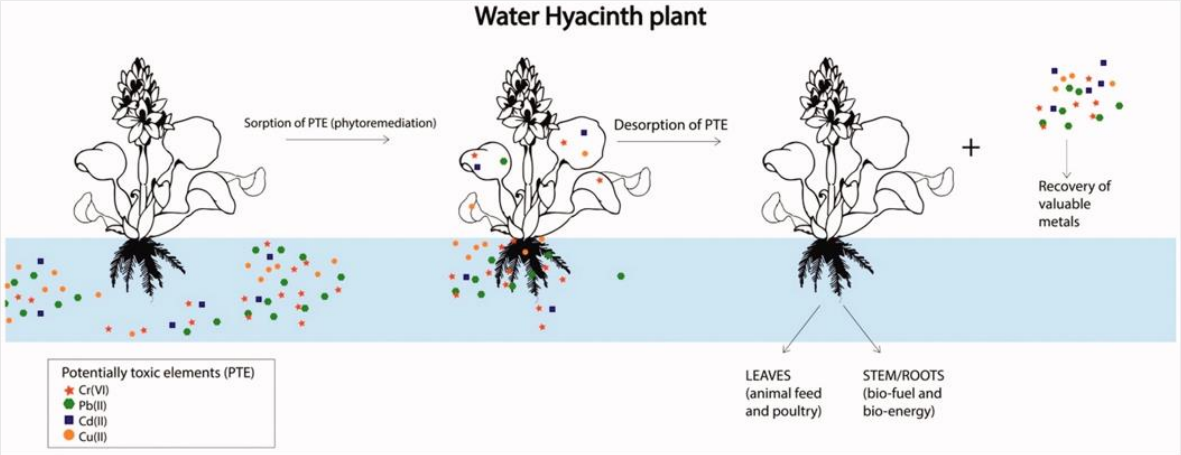

Fig 1.4. Role of Water Hyacinth in metal removal (Galgali et al., 2023)

1.6. Biotechnological potentials of River microbes

Unlike other environments, such as soil, river microbes are rarely explored for their biotechnological potential. The rivers, especially in urban settlements, are often subjected to various environmental stressors, pollutants, and anthropogenic activities (McLellan et al., 2015). These stressors could shape the indigenous microbial community in the rivers with specialized functions which can explored in biotechnology. Several metagenomic studies have already proved the functional repertoire of river microbiomes, indicating promising microbes in urban rivers and the need for bioprospecting of river microbes (Rajput et al., 2022; Breton-Deval et al., 2020).

1.7. Scope and objective of thesis

Urban rivers play a crucial role in the overall social, economic and environmental development. Despite their critical importance, these rivers are often contaminated by all kinds of wastewater and a plethora of anthropogenic activities. These rivers are connected to peri-urban rivers downstream, affecting the non-city riverine stretches. The impact of such urban rivers on the downstream peri-urban river stretch concerning the overall microbial diversity and functionality has yet to be studied. Further, during religious events such as mass bathing

in the rivers, there is a sudden stress on water bodies due to increased anthropogenic activities. The detailed investigation of the overall microbial ecological changes and functionality concerning antibiotic resistance and virulence still needs to be studied. Although undesired, the invasive water hyacinths have become an integral part of these rivers and affect the nutrient cycling and biogeochemistry of rivers. Water hyacinths are widely used in bioremediation applications; however, the rhizobiome of water hyacinths needs to be explored. Exploring water hyacinth-associated microbiomes would facilitate revealing their role in the propagation of water hyacinth environmental relations and provide bioprospecting opportunities for the production of industrially essential bioproducts. Apart from bioremediating abilities, it becomes imperative to bioprospect the urban rivers and the native water hyacinth for promising microbes, as they are subjected to multiple stressors and substrates ranging from hydrocarbons to lignocellulosic materials. The broad aim of the present work is to carry out a comprehensive and holistic study with an ultimate goal of delineating dynamics of river-human health, environmental science, and provide sustainable biotechnological applications.

The present study understands the influence of urban rivers on the connected downstream periurban rivers of the riverine system in the Pune district of Maharashtra, India. The comprehensive shotgun metagenomic study was performed using the latest MinION-based Nanopore sequencing to delineate the influence on microbial ecology and functionality. Moreover, to understand the microbial dynamics during immense anthropogenic stress, we studied the changes in the microbial community and functionality during mass bathing in Pandharpur Wari. Further, we examined the rhizobiome of water hyacinths to establish the several missing links concerning their predominance in urban waters. Additionally, we explored the bacteria from the river and water hyacinth for producing industrially important value-added chemicals and developed a sustainable process for their production. The following objectives were designed to achieve different goals of the present thesis.

- To study the microbial community structure and functions of Pune riverine system
- To understand the impact of mass bathing event (Wari) on the river microbial ecology
- To map the microbiome and functional aspects of water hyacinth
- To develop the microbial process for conversion of biomass-waste to 2,3-butanediol and Poly-γ-Glutamic Acid

1.8. References

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Chapter 2. To study the microbial community structure and functions of Pune riverine system

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Chapter 2.1. Microbial community and diversity

Abstract

The Megacities Rivers face a constant unrestricted inflow of severely polluted wastewater from diverse sources, and their impact on the downstream connected peri-urban river still needs to be studied in detail. The Rivers Mula, Ramnadi, Pawana, Mutha, and Mula-Mutha traverse through the urban settlements before reaching River Bhima in the peri-urban zone. Altogether, these rivers form the riverine system of Pune, Maharashtra. Here, we employed MinION-based shotgun metagenomic sequencing to comprehensively understand the microbial diversity differences between the urban and peri-urban zones, which has yet to be explored at the meta scale. The microbial taxonomic analysis revealed significant enrichment of pollution indicators taxa (Welsch's t-test, $p<0.05$, Benjamini-Hochberg FDR test) such as *Bacteriodetes*, *Spirochaetes, Synergistetes*, *Firmicutes,* and *Euryarcheota* in the urban traversing river stretch as compared to the peri-urban zone. Further, a considerably higher predominance of ammonium oxidising archaebacterial groups such as *Nitrososphaeraceae* (Student's t-test p-value <0.05, FDR correction) in the peri-urban river stretch, implying the influence of agricultural runoffs. The microbial community diversity assessment further substantiated the significant dissimilarity in the microbial communities of urban and peri-urban river stretches. Overall, the Virulence Factor Database (VFDB) analysis predicted 295 virulence genes in the riverine system. Moreover, the more comprehensive genome coverage (> 60%) for priority pathogens such as *Pseudomonas, Acinetobacter, Escherichia, Klebsiella,* and *Aeromonas* in the river sediment metagenome cement their predominance in the riverine system. In conclusion, our metagenomic investigation predicted that unrestrained anthropogenic and related activities could contribute to the deteriorating conditions and influence the microbial ecology of the downstream river stretches on the city's outskirts.

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2.1.1. Introduction

Discharging untreated wastewater, industrial effluents, and anthropogenic waste are undoubtedly disastrous for river health (Wang et al., 2018; Suthar et al., 2010; Fonseca et al., 2016). These ever-increasing pollutant levels in rivers lead to the emergence of waterborne pathogens and heighten infection rates, thereby degrading public health in fast-growing cities (Abraham, 2010). Global studies have implicated that urban rivers serve as an ecological niche for the accelerated dissemination and emergence of antibiotic resistance genes (ARGs) and pathogens (Samson et al., 2019; Almakki et al., 2019; Zhang et al., 2019; Marathe et al., 2019; Xu et al., 2016; Reddy et al., 2018; Zheng et al., 2017; Zhou et al., 2017). India has a vast network of rivers; however, various reports and studies suggest their dismal state. (Borthakur and Singh 2016, Water Quality Database). Untreated wastewater from hospitals, industries, and agricultural runoffs has exacerbated their quality.

Microbes can tackle diverse pollutants by utilizing them as their growth substrate and energy source due to their versatile metabolic adaptability (Chakraborty and Bhadury 2015). However, such multi-faceted environmental distress can result in functional shifts in microbial diversity and health-related factors (Chen et al., 2019a; Chakraborty and Bhadury, 2015). The peri-urban zones are symbiotic urban-rural interfaces characterized by hybrid features or urban and rural landscapes (Zheng et al., 2018; Chen et al., 2019b). The peri-urban rivers are resourceful in providing drinking water and are crucial for agricultural irrigation and recreational purposes (Zhang et al., 2020). Urbanization has significantly impacted peri-urban rivers, dumping massive waste from hospitals, wastewater treatment plants (WWTPs), industries, and other domestic sources. Several studies indicated peri-urban rivers as a reservoir of antimicrobial resistance (AMR) and related pathogens (Zheng et al., 2018; Chen et al., 2019a; Zhang et al., 2020; Chen et al., 2019b). Despite several studies on peri-urban rivers,

primarily focussing on AMR, the overall detailed assessment of the microbial diversity of the peri-urban rivers still needs to be improved.

Pune is the ninth-largest city in India, in the Pune district of Maharashtra, populated with 5.9 million people. It is also amongst the fastest-growing cities observing rapid industrialization and urbanization (Marathe et al., 2017). The riverine system of the Pune district can be categorized based on its overall course of flow. The Rivers Mula, Mutha, Ramnadi, Pawana, and Mula-Mutha flow through the industrialized urban settlements of Pune. In contrast, the River Bhima and its tributary Indrayani River, which primarily traverse peri-urban and agricultural dominant regions, converge River Mula-Mutha in the city outskirts. These rivers are reported to be degraded and plagued with diverse pollutants (Water Quality Database, Yewale et al., 2019; Nawani et al., 2016). Several recent studies (Marathe et al., 2019; Yewale et al., 2019; Razavi et al., 2017) examined the resistome and the pathogens prevalent in the city stretch of some of these rivers. Marathe et al. 2017 comprehensively profiled the antibiotic resistance genes (ARGs) in the city stretch of the Mutha River. Another study by Yewale et al. 2019 employed a conventional PCR-based strategy to delineate Mutha River's ARGs. Nevertheless, studies exploring microbial communities, outlining the community differences, and establishing the link between the urban and peri-urban river stretches are scarce.

The previous studies were limited to city stretches and focused only partially on the connected peri-urban and rural river stretches. A comprehensive investigation of the riverine system's overall microbial ecology and virulence trait still needs to be completed. The present study hypothesized that the microbial communities of the riverine system could differ in the connected urban and peri-urban riverine stretch. The primary goal of studying this riverine system was to i). Assess the influence of urban rivers on the connected peri-urban stretch by deciphering the dissimilarity in the microbial communities between the two stretches and ii).

Understanding the overall virulence potentials of the riverine system. We used a rapid, real-time long read MinION-based sediment shotgun sequencing to address these lacunas. The present study is one of the most comprehensive shotgun studies investigating the differences between the city stretch's microbial communities and the connected peri-urban stretch.

2.1.2. Experimental

2.1.2.1. Description of sampling sites

The Rivers Mula, Mutha, Mula-Mutha (formed after Mula and Mutha meet in the centre of Pune city), Pawana, and Ramnadi are the rivers flowing through Pune city. The River Pawana and Ramnadi, tributaries of the Mula River, meet Mula at the left and right banks, respectively. Wastewater from industries, domestic sources, and hospitals immensely affects these rivers traversing urban settlements. The River Mula dammed at Mulshi Lake, travels around 52 Km to meet the Mutha River. The Mutha River is dammed twice at Panshet Dam and Khadakwasla Dam and flows about 21 Km from its origin to join the Mula River. On the course of its flow, Mula merges with its tributaries, i.e., Pawana and Ramnadi, before its confluence with Mutha, following which it flows downstream as Mula-Mutha River. The River Mula-Mutha is a tributary to the Bhima River (originating from the Western Ghats), unloading all its urban contents in the River Bhima. The Bhima River meets the River Indrayani on the right bank at Tulapur. River Bhima travels more than 20 Km and joins River Mula-Mutha at the outskirts of Pune city near the village Ranjangaon Sandas in the peri-urban region. Following this, River Bhima merges with River Krishna, ultimately emptying itself into the Bay of Bengal. Based on geographical locations, population, population census (Census India 2011, Dhawde et al., 2018, Hui and Wescoat Jr. 2019), and urban influence, the riverine system was divided broadly into two groups, viz., urban river stretch/waters (sites in Pune city and downstream) (n=10) and peri-urban river stretch/waters (n=14). (where ‘n’ = number of sites or samples) (Fig. 2.1).

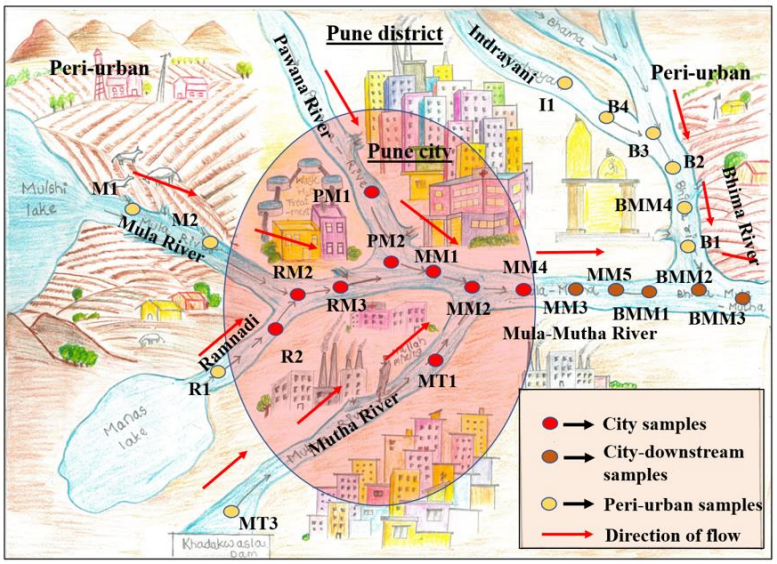

Fig. 2.1. Schematic illustration of the riverine system. The figure displays the sampling sites. The riverine system is broadly divided into urban (R2, RM2, RM3, PM1, PM2, MM1, MT1, MM2, MM4, MM3, MM5, BMM1, BMM3) and peri-urban stretch (M1, M2, R1, MT3, I1, B4, B3, B2, BMM4, B1) depending on the geographical location and population density. From MM2 site, River Mula flows as Mula-Mutha River and from point BMM2, the River Mula-Mutha flows as the Bhima River.

2.1.2.2. Sample Collection details

Maximum biomass responsible for the metabolic cycles is harboured by water-saturated river sediments (Gibbons et al., 2015). In total, 24 river sediment samples were collected in December 2018 in gamma-irradiated sterile containers from 5 to 10 cm and stored at 4°C until further processing. The sample sites' details and coordinates are stated in (Tables 2.1 and 2.2). The urban water includes R2, RM2, RM3, PM1, PM2, MM1, MM2, MM3 MM4, MM5, BMM1, BMM2, BMM3, MT1 sites, whereas the peri-urban water samples include M1, M2, R1, MT3, I1, B1, B2, B3, B4, and BMM4 sites.

Table 2.1. The table shows the taluka-wise sampling details along with population density (* A subdivision of a district).

Sr. No.	* Talukas or Tehsils in Pune district	Sample Codes	Population density/km^2	Classification
1.	Pune City	R2, RM2, RM3, PM1, PM2, MM1, MM2, MM3 MM4, MM5, BMM1, BMM2, BMM3, MT1	11539	Urban water
2.	Daund	I1, B1, B2, B3, B4, BMM4	291	Peri-urban water
3.	Mulshi	M1, M2, R1	166	Peri-urban water
4.	Velhe	MT3	96	Peri-urban water

Table 2.2. The table shows the map coordinates and descriptions of the sample sites.

Sr. No.	Sample codes	River	Latitude	Longitude	Riverine stretch
1	M1	Mula	18.48924	73.5045	Peri-urban water
2	M2	Mula	18.536114	73.667785	Peri-urban water
3	MT3	Mutha	18.441412	73.770296	Peri-urban water
4	MM3	Mula-Mutha	18.543683	74.12989	Urban water
5	MM5	Mula-Mutha	18.57612	74.27446	Urban water
6	BMM1	Bhima	18.559814	74.342655	Urban water
7	BMM2	Bhima	18.5609	74.343972	Urban water
8	BMM3	Bhima	18.558802	74.35015	Urban water
9	BMM4	Bhima	18.607848	74.155531	Peri-urban water
10	B1	Bhima	18.574517	74.340968	Peri-urban water
11	B2	Bhima	18.67011	73.99821	Peri-urban water

12	B3	Bhima	18.671205	73.995717	Peri-urban water
13	B4	Indrayani	18.668517	73.995562	Peri-urban water
14	I1	Indrayani	18.670319	73.982166	Peri-urban water
15	R1	Ramnadi	18.497735	73.736202	Peri-urban waters
16	R2	Ramnadi	18.54497	73.796735	Urban water
17	RM2	Mula	18.563009	73.796763	Urban water
18	RM3	Mula	18.568395	73.80793	Urban water
19	PM1	Pawana	18.575187	73.825639	Urban water
20	PM2	Mula	18.57305	73.83121	Urban water
21	MT1	Mutha	18.52998	73.86152	Urban water
22	MM1	Mula	18.549597	73.855576	Urban water
23	MM2	Mula-Mutha	18.533042	73.861156	Urban water
24	MM4	Mula-Mutha	18.542638	73.882804	Urban water

2.1.2.3. Library preparation and MinION-based sequencing

The DNeasy PowerSoil Pro Kit (Qiagen) was used for the extraction of community DNA from 400 mg of sediment samples, following the manufacturer's instructions. The initial quality and concentrations of DNA were assessed by Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific) followed by its qubit analysis on Qubit fluorometer (Thermo Fisher Scientific) using Qubit Broad range (BR) assay kit and Qubit High sensitivity (HS) assay kit (Thermo Fisher Scientific, Q32853 and Q32851 respectively). The DNA with a purity of 1.8 (OD 260/280) were further processed. The 1D Ligation sequencing kit SQK-LSK109 and Native barcoding EXP-NBD104 and NBD114 were used for the library preparation, per the manufacturer's instructions. The resultant library was quantified, and 500ng was loaded on the FLO-MIN 106D R9 Version.

2.1.2.4. Data processing and analysis

The base-calling and demultiplexing of the raw reads were carried out using Albacore (v2.3.4) and qcat (v1.0.1), respectively. Porechop (v0.2.4) was used for the adapter trimming from the end and the middle of the reads. Rarefactions were performed using single_rarefaction.py and rarefy even depth function in QIIME (Caporaso et al., 2010) and R, respectively. The taxonomic annotation was done using Kaiju (version 3) (Menzel et al., 2016) with default parameters. Kaiju performs a six-frame translation of each metagenomic read, classifying them using an annotated NCBI BLAST nr protein database (Menzel et al., 2016). The alpha diversity was calculated using the R package Phyloseq (Lahti et al., 2017). The virulence factors database (VFDB) (Chen et al., 2005) was used to map virulence genes. Alignment was done using DIAMOND (Buchfink et al., 2015) with parameters --min-score 60, --id 60 and --more-sensitive mode (Pearson et al., 2014). Furthermore, GraphMap was used for genome mapping and coverage estimation (Sović et al., 2016). Various R tools, such as the phyloseq package, microbiome, and MicrobiomeAnalyst, were used for illustrations (McMurdie and Homes, 2013; Lahti et al., 2017; Dhariwal et al., 2017).

2.1.2.5. Statistical analysis and Data availability

The variable sequencing depth was adjusted using the DESeq2 package. The beta diversity (Non-metric multidimensional scaling (NMDS)) was calculated using a counts-based Bray-Curtis dissimilarity measure, and statistical significance was estimated by ANOSIM and PERMANOVA (Adonis test, p-value threshold of 0.05). The compositional homogeneity among the groups was assessed by the Betadisper test (p-value threshold of 0.05). The Shapiro-Wilk normality test (p-value threshold of 0.05) and Q-Q plot were used to check the distribution of data. STAMP (Statistical Analysis of Metagenomic Profiles) and METAGENassist (Arndt et al., 2012) were used for analysing the differentially abundant significant features between city and non-city samples. The Welch's t-test (p-value threshold of 0.05) and Wilcoxon rank-

sum test, along with Benjamini-Hochberg FDR for multiple test corrections, were used. Fold-change analysis was carried out in METAGENaaist. The datasets supporting the conclusions of this article are publicly available in the MG-RAST with a project name of "PU_River_metagenomics_2019" and NCBI accession number PRJNA666546.

2.1.3. Results and Discussion

Over the last decade, the Pune Rivers have been exposed to immense anthropogenic activities (Water Quality Database; Yewale et al., 2019), resulting in a higher risk of antimicrobial resistance and pathogens. Altogether, 3.6 million reads yielding 19.5 Gbases with an average read length of ~5 Kb were obtained upon MinION sequencing. The taxonomic analysis using the Kaiju tool with the nr_euk database assigned 2.58×10^6 reads and 7.23×10^4 reads to bacteria and archaebacteria, respectively. The rarefaction analysis indicated the achievement of sufficient sequencing depth for all the samples.

2.1.3.1. Microbial community Composition

The analysis revealed 149 and 457 bacterial phyla and families in the riverine system, respectively. Among the bacterial phyla, the *Proteobacteria* (mean relative abundance %) (35.04%) was the most prevalent group, followed by *Actinobacteria* (12.51%), *Bacteroidetes* (8.43%), *Chloroflexi* (7.74%), and *Acidobacteria* (6.29%) in the riverine system (Fig. 2.2.A). The *Firmicutes* (4.9% vs. 2.8%) and *Bacteroidetes* (9.6 vs. 5.9%) were more enriched in the urban river stretch as compared to the peri-urban (Welsch's t-test, Benjamini-Hochberg FDR test, $p>0.05$). The other prevalent bacterial groups were *Verrucomicrobia*, *Cyanobacteria*, *Planctomycetes*, *Nitrospirae*, and *Gemmatimonadetes* in the riverine system (≥ 1%) (Fig. 2.2.A.). The fold-change analysis further revealed a higher abundance of *Spirochaetes*, *Tenericutes*, and *Synergistetes* in the urban stretch of the riverine system (Fig. 2.3.A, 2.3.B, 2.3.C).

Fig. 2.2.A.

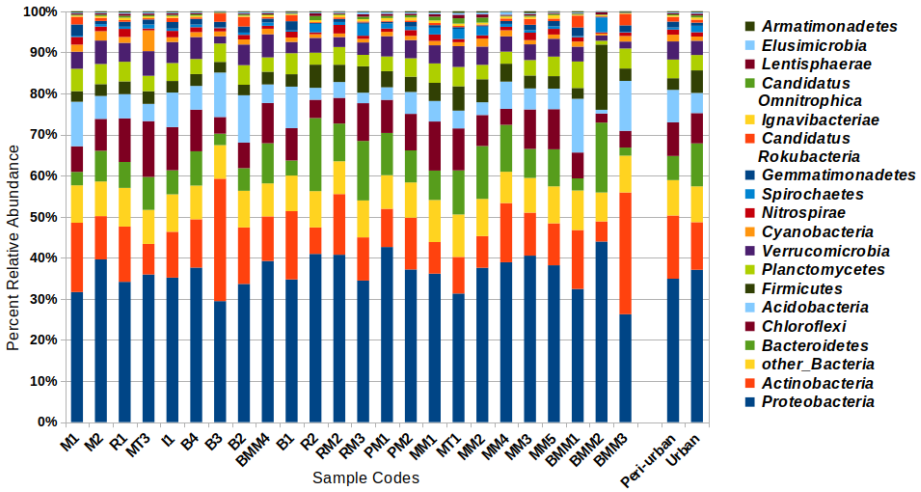

Fig. 2.2.B.

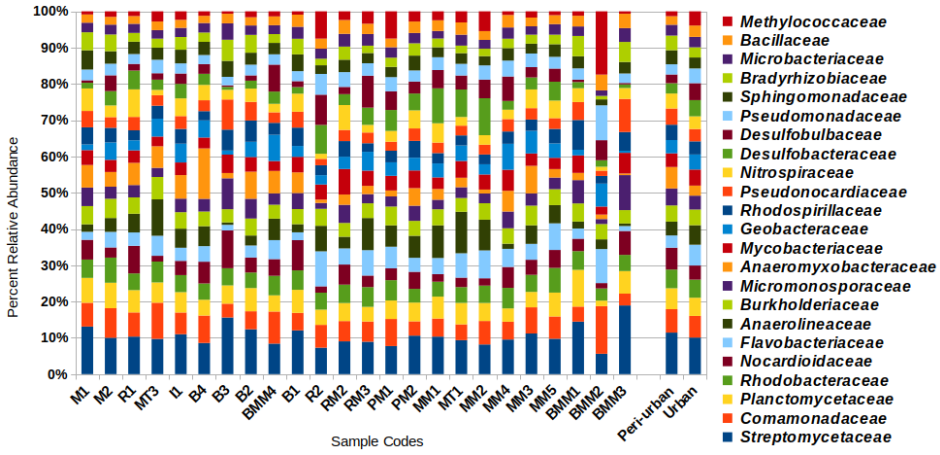

Fig. 2.2. Bacterial community pattern of the riverine system. The stacked bar plot indicate the percent relative abundance of Bacterial composition at Phylum (2.2.A.) and Family level (2.2.B.).

Among these prominent bacterial classes, *Bacteroidia, γ-Proteobacteria,* and *Clostridia* were significantly enriched in the urban stretch (Wilcoxon rank-sum test <0.05 with FDR correction). Moreover, the analysis at the family level (Fig. 2.2.B.) indicated a higher prevalence of *Streptomycetaceae, Planctomycetaceae,* and *Comamonadaceae* (≥1%) abundant bacterial groups. Notably, among the pre-dominant families (≥0.5%), significant enrichment

(Fig. 2.3.D.) of *Methylococcaceae* in the urban stretch, especially at the BMM2 site (a confluence site of River Bhima and Mula-Mutha) was observed.

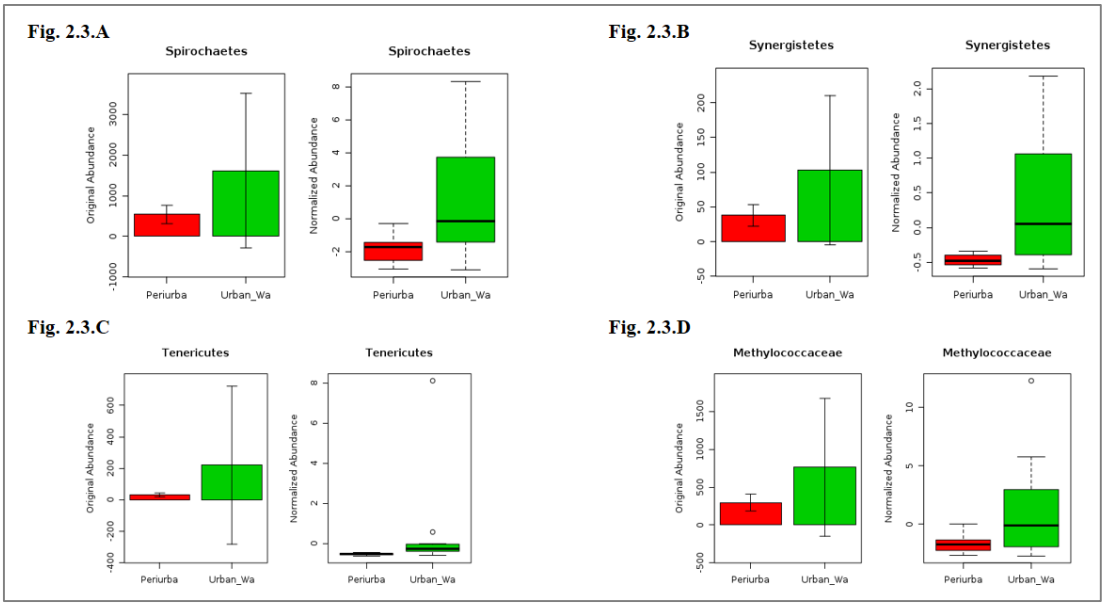

Fig. 2.3. Fold-change analysis. The figures show significant changes in the abundance of Spirochaetes (2.3.A.), Synergistetes (2.3.B.), Tenericutes (2.3.C.), and Methylococcaceae (2.3.D.)

Overall, the fold change analysis revealed the differential abundance of 37 bacterial families (Table 2.3.) in the urban stretch compared to four (*Conexibacteraceae, Gemmatimonadaceae, Myxococcaceae,* and *Solirubrobacteraceae*) in the peri-urban waters.

Table 2.3. Fold-change analysis showing differential abundant bacterial family (Peri-urban vs urban rivers).

Name	Fold Change	log2(FC)
Acholeplasmataceae	0.15987	-2.645
Acidaminococcaceae	0.47142	-1.0849
Bacteroidaceae	0.38151	-1.3902
Brachyspiraceae	0.46319	-1.1103
Campylobacteraceae	0.45704	-1.1296
Carnobacteriaceae	0.20033	-2.3196
Catabacteriaceae	0.17189	-2.5404
Chromatiaceae	0.36081	-1.4707

Clostridiaceae	0.43476	-1.2017
Clostridiales Family XI. Incertae Sedis	0.37103	-1.4304
Clostridiales Family XII. Incertae Sedis	0.29385	-1.7668
Clostridiales Family XIII. Incertae Sedis	0.22027	-2.1827
Conexibacteraceae	2.4511	1.2934
Desulfomicrobiaceae	0.26953	-1.8915
Enterococcaceae	0.47813	-1.0645
Erysipelotrichaceae	0.35947	-1.4761
Eubacteriaceae	0.28867	-1.7925
Fusobacteriaceae	0.49974	-1.0007
Gemmatimonadaceae	2.1891	1.1303
Helicobacteraceae	0.42444	-1.2364
Holophagaceae	0.19399	-2.366
Hydrogenophilaceae	0.36572	-1.4512
Lachnospiraceae	0.49861	-1.004
Marinilabiaceae	0.46513	-1.1043
Methylococcaceae	0.42441	-1.2365
Moraxellaceae	0.49023	-1.0285
Mycoplasmataceae	0.45913	-1.123
Myxococcaceae	2.1703	1.1179
Oscillospiraceae	0.41305	-1.2756
Peptostreptococcaceae	0.33168	-1.5922
Porphyromonadaceae	0.37744	-1.4057
Prevotellaceae	0.49583	-1.0121
Rhodocyclaceae	0.42423	-1.2371
Rikenellaceae	0.38381	-1.3815
Ruminococcaceae	0.46879	-1.093
Solirubrobacteraceae	2.3481	1.2315
Synergistaceae	0.41109	-1.2825
Syntrophaceae	0.30145	-1.73
Syntrophobacteraceae	0.4878	-1.0357
Syntrophomonadaceae	0.44664	-1.1628
Syntrophorhabdaceae	0.21656	-2.2071

The analysis revealed 18 and 45 archaebacterial phyla and families in the riverine system with higher enrichment of *Euryarchaeota* in the urban river stretch while

Thaumarchaeota in the peri-urban sites (Fig. 2.4.A and 2.4.B.). Notably, some urban sites (MM4, MM3, MM5, BMM1, and BMM3) were more populated with *Thaumarchaeota* members.

Fig. 2.4.A.

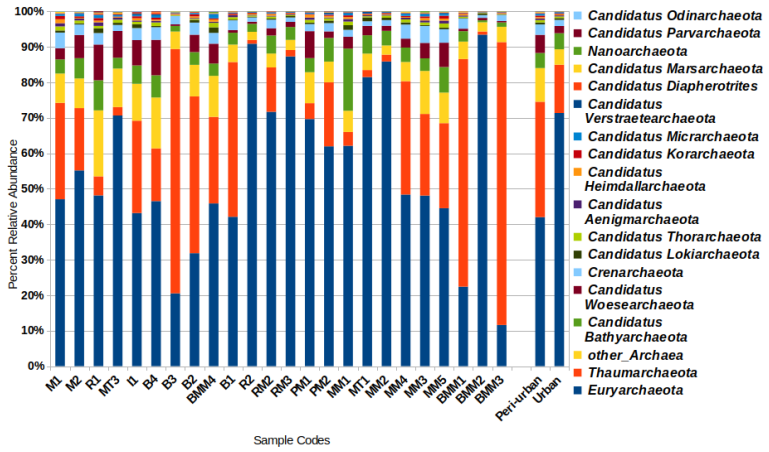

Fig. 2.4.B.

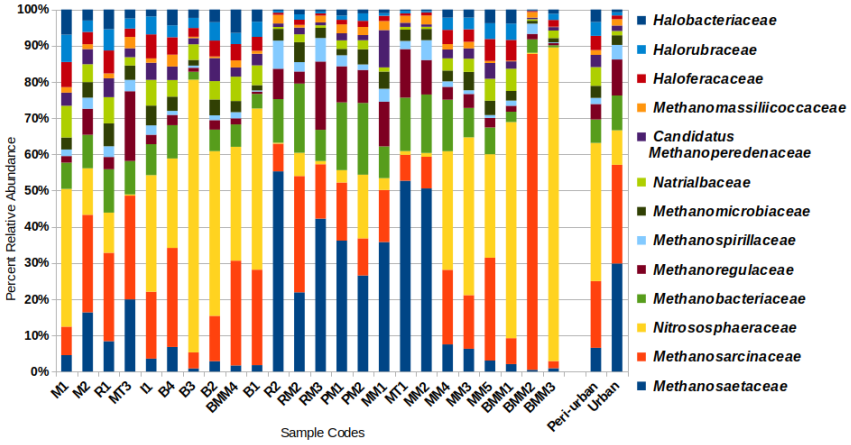

Fig. 2.4. Archaebacterial community pattern of the riverine system. Archaebacterial composition at Phylum and Family level (2.4.A and 2.4.B, respectively) in the riverine system

At the class level, the analysis revealed a significant increase in *Methanomicrobia* and *Methanobacteria* (especially at the BMM2 site) while a significant decrease in *Archaeoglobi*, *Halobacteria*, *Nanohaloarchaea*, and *Thermococci* in the urban-influenced waters (Student's t-test p-value <0.05 with FDR correction). The *Methanosetaceae* family was significantly

enriched (fold-change analysis) in the urban-influenced waters, while *Nitrososphaeraceae* was in peri-urban sites. Similar to the bacteria, the confluence site BMM2 showed significant variations in archaeal population with Thaumarchaeotal group *Nitrososphaeraceae* compared to its pre-confluence sites (BMM1 and B1) and post-confluence site (BMM3) (Fig. 2.4.A and 2.4.B.).

The rivers under study harboured a diverse microbial population (Fig. 2.2.A, 2.2.B, 2.4.A, and 2.4.B.). A significant increase of bacterial phyla such as *Firmicutes, Bacteriodetes, Spirochaetes, Tenericutes, Synergistetes,* and classes viz., *γ-Proteobacteria, Clostridia,* and *Bacteroidia,* in the urban-influenced sites probably implicated the possibility of faecal pollution in the riverine system (Paruch et al., 2019, Lin et al., 2019). The urban riverine system is infused continuously with treated and untreated wastewater from various industries containing recalcitrant pollutants. A higher abundance of bacterial groups such as *Sphingomonadaceae, Xanthomonadaceae, Pseudomonadaceae, Mycobacteriaceae,* and *Burkholderiaceae* were recorded, possibly utilising such pollutants as their nutrient source (Ghosal et al., 2016). The methanogenic *Euryarchaeota,* such as *Methanobacteria* and *Methanomicrobia* were significantly enriched in urban stretches compared to peri-urban waters. The presence of such anoxic methanogenic archaebacterial groups in polluted river water is consistent with the previous studies (Zhang et al., 2019, 2018, Wang et al., 2018). In contrast, the peri-urban stretches showed a significant predominance of *Thaumarchaeota* members, probably because the peri-urban stretches receive agricultural runoffs that are quite luxurious with ammonium due to the use of biosolids and fertilisers, thus supporting Ammonium Oxidising Archaea (AOA) group such as *Nitrososphaeraceae* (Pratscher et al., 2011). Nevertheless, AOA was also prevalent considerably in the urban water sites (MM4, MM3, MM5, BMM1, and BMM3), suggesting that they play a critical role in ammonia oxidation in polluted urban rivers (Tu et al., 2019). The BMM2 site, a confluence site of two

rivers, i.e., the Mula-Mutha and Bhima Rivers, which are differently impacted by anthropogenic activities, showed significant variations in microbial diversity. The BMM2 site observed an increased abundance of *Bacteriodetes* and *Firmicutes* and a corresponding increase of bacterial families such as *Flavobacteriaceae, Desulfobulbaceae,* and *Pseudomonadaceae.* Further, BMM2 also displayed an increase in the methanogenic microbial populations, which probably can be attributed to the excessive water hyacinth growth seen at BMM2 during the sampling (Avila et al., 2019). Such dynamic alterations in the microbial community at the confluence of two differently anthropogenically impacted rivers were previously noted by Samson et al. 2019 while studying River Ganga and Yamuna. Overall, these observations probably indicated the influence of urban rivers on the interconnected peri-urban rivers.

2.1.3.2. Microbial community diversity

The Chao1 and Shannon index were used to assess a riverine system's overall microbial richness and diversity. Although insignificant, a higher bacterial richness and diversity were observed in urban waters (Fig. 2.5.A). In comparison, peri-urban waters showed significantly higher richness in the archaebacterial population (Welsch's t-test, $p<0.05$) (Fig. 2.5.B.) than in urban waters. The beta diversity analysis showed significant dissimilarity in the bacterial (ANOSIM, R=0.17, p-value <0.05, Stress value= 0.1) as well as archaebacterial (ANOSIM, R=0.18, p-value<0.05, Stress value= 0.1) composition of the urban and peri-urban waters (Fig. 2.5.A and 2.5.B).

The significant differences in the microbial alpha diversity in the urban riverine sites relative to the peri-urban river stretches indicate the influence of different wastewater the riverine stretch receives. Although significant microbial dissimilarity was observed between the riverine sites upon beta diversity estimation, we observed lower ANOSIM R-values (0.17 and 0.18 for bacteria and archaebacteria), indicating overlapping similarities. These similarities

can be reasoned by two observations: Firstly, rivers examined in this study are in similar climatic conditions (so with rivers as the variable factor, we observed insignificant PERMANOVA, p>0.05), and secondly, the riverine sites MM3, MM5, BMM1, BMM2, and BMM3 grouped into the urban waters geographically are at the outskirts of the Pune city.

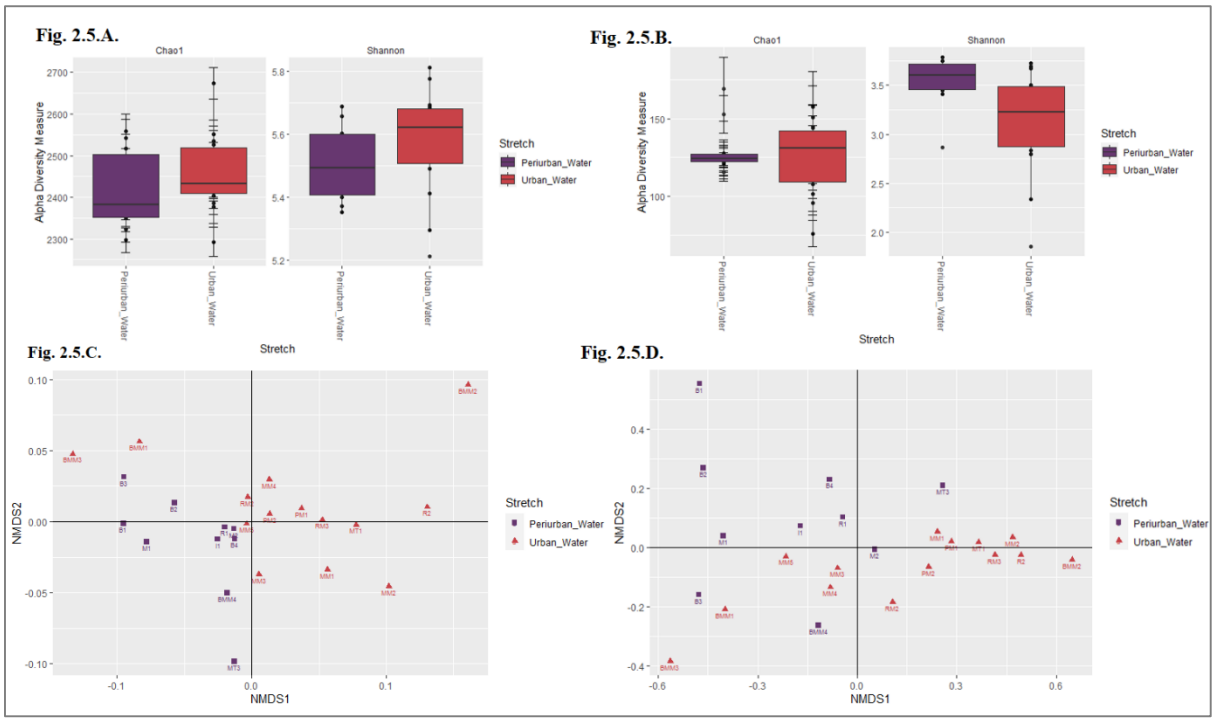

Fig. 2.5. Microbial community diversity. Bacterial alpha diversity **(2.5.A)** (Welch Two Sample t-test, p-value >0.05) and beta diversity (ANOSIM, p-value<0.05, R=0.17) **(2.5.C)**, respectively with NMDS stress value= 0.1. Archaebacterial alpha diversity (Welch Two Sample t-test, p-value <.0.05) **(2.5.B.)** and beta diversity (ANOSIM, p-value<0.05, R=0.18) **(2.5.D.)**, respectively.

However, unlike the other peri-urban sites in this study, these sites, being the downstream city sites, receive all the urban waters and thus are "urban-influenced". Upon excluding these samples from the urban water groups, the beta-diversity analysis revealed a significant dissimilarity in the microbial community between the riverine system's urban and peri-urban sites with a higher ANOSIM R-value of 0.55 (Fig. 2.6.). Based on these overall diversity analyses, it can be concluded that urbanisation and increased anthropogenic activities in the

cities have a crucial impact on the microbial diversity of the urban surface water and the downstream stretch of an interconnected riverine system.

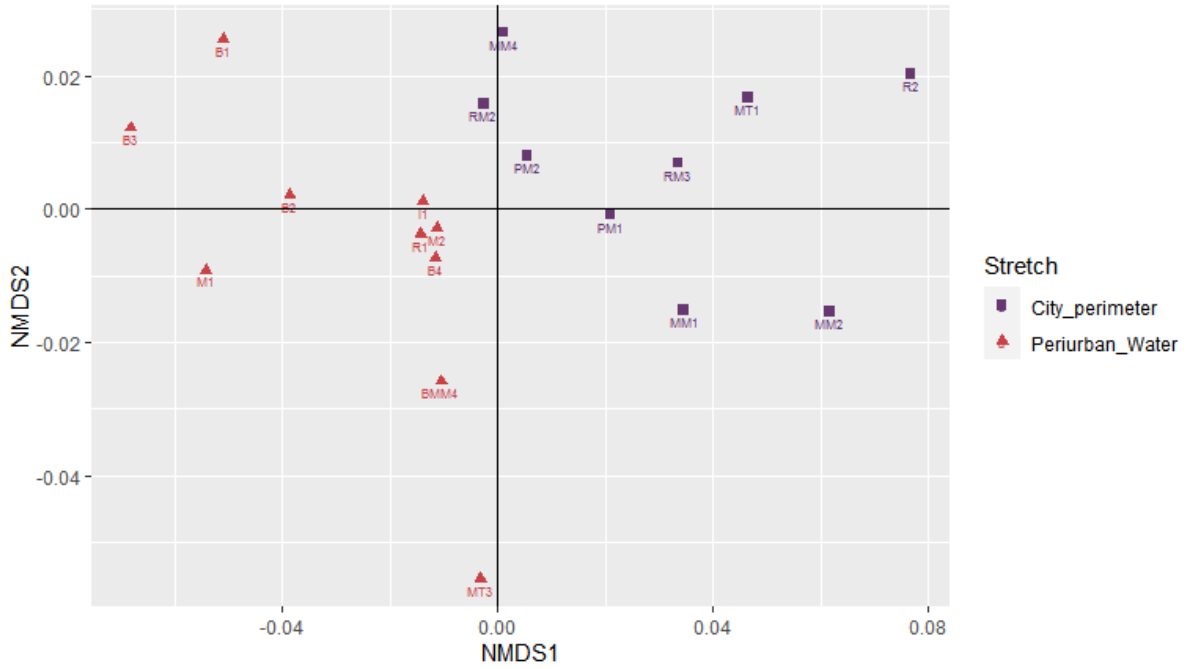

Fig. 2.6. Bacterial beta diversity between the sites in city perimeter and peri-urban sites (ANOSIM, $p<0.05$, $R=0.53$). The sites MM3, MM5, BMM1, BMM2, and BMM3 were excluded from the analysis.

2.1.3.3. Virulence factors in the riverine system

Here, we used the VFDB database to predict the virulence potential of the riverine system. Our analysis observed 295 virulence genes in the riverine system as a whole. The dominant virulence genes (≥2%) in the riverine system were *flrA, fleQ, tlpA, tlpC, tufA, fliP, flhA, cheV3, fliN, pilB,* and *pilT* (Fig. 2.7.). The genome mapping analysis using GraphMap for the global priority pathogens in the riverine system showed more than 60 % coverage (Table 1) with most of the reference genome of pathogenic strains except for the H.pylori (49%) with higher coverage depth. The World Health Organization (WHO) published a global priority pathogens list categorizing them into Critical, High, and Medium groups based on ten parameters: mortality, antibiotic resistance, and treatability (Asokan et al., 2019). Previous

studies utilized the PCR and culture-based approaches to characterize the widespread occurrences of the virulence genes encoding adhesins, toxins, inflammatory mediators, and environmental resistance (Søborg et al., 2013; Hamelin et al., 2007).

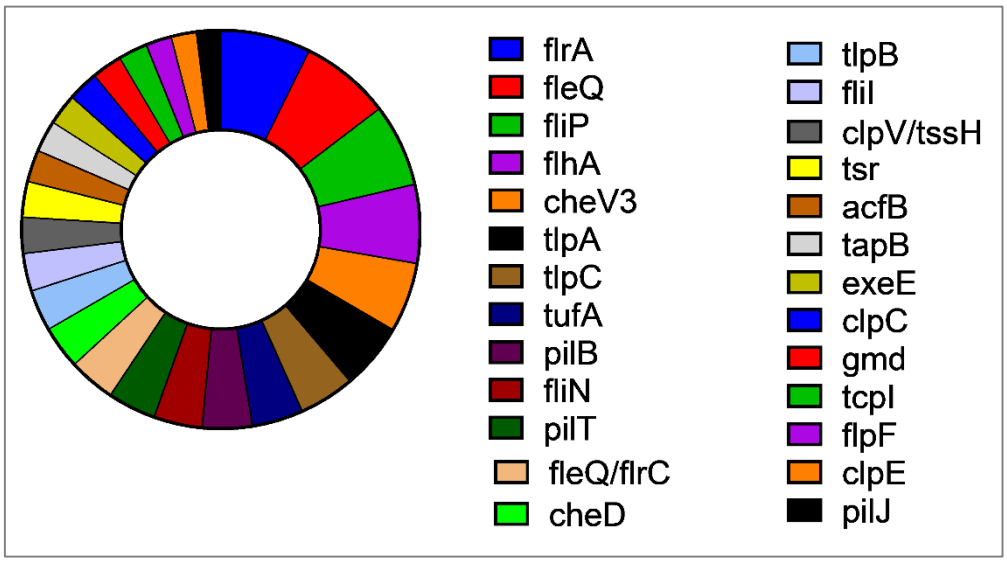

Fig. 2.7. Prevalent virulence genes (≥2%) in the riverine system

Table 2.4. Estimation of the Genomic Coverage. GraphMap was used for estimation of genome coverage of the pathogens (whose virulence genes were predicted) from the metagenomic sequences.

Pathogens	Number of Bases Covered	Reference Genome Length	Coverage for reference genome	Mean coverage depth (approximate X coverage)
Pseudomonas aeruginosa PAO1	~6.26×10^6	6.26×10^6	99.9%	700X
Aeromonas hydrophila subsp. hydrophila ATCC 7966	4.69×10^6	4.74×10^6	98.9%	356X
Legionella pneumophila subsp. pneumophila str. Philadelphia 1	2.14×10^6	3.4×10^6	62.9%	28X
Burkholderia pseudomallei K96243	7.12×10^6	7.25×10^6	98.2%	575X
Yersinia enterocolitica subsp. Enterocolitica	2.79×10^6	4.61×10^6	60.4%	92X

8081.				
Neisseria meningitidis MC58	2.09×10^6	2.27×10^6	91.9%	89X
Klebsiella pneumoniae subsp. pneumoniae NTUH-K2044	5.12×10^6	5.33×10^6	95.9%	385X
Haemophilus influenzae Rd KW20	1.58×10^6	1.83×10^6	86.1%	22X
Listeria monocytogenes EGD-e	2.11×10^6	2.94×10^6	71.5%	13X
Bordetella pertussis Tohama I	4.08×10^6	4.09×10^6	99.9%	914X
Mycobacterium tuberculosis H37Rv	$\sim4.41\times10^6$	$\sim4.41\times10^6$	99.9%	534X
Helicobacter pylori 26695	8.21×10^5	1.67×10^6	49.2%	46X
Acinetobacter baumannii ACICU	3.40×10^6	4.33×10^6	78.5%	25X
Shigella dysenteriae Sd197	3.33×10^6	4.37×10^6	76.2%	55X
Staphylococcus aureus subsp. aureus MW2	2.73×10^6	2.82×10^6	96.9%	40X

The present analysis utilized the shotgun metagenomic approach to predict virulence genes mapping to different priority bacterial pathogenic genera, including the ESKAPE pathogens (Asokan et al., 2019) in the riverine system. The *fleQ* gene, an essential regulator of flagellar biogenesis in *Pseudomonas aeruginosa* (Dasgupta et al., 2002) was the most dominant. It is an opportunistic pathogen in life-threatening infections such as cystic fibrosis (Savoia, 2014). The flr and laf encoded products facilitate *Aeromonas*, with the motility functions (Chen et al., 2005; Tomás et al., 2012; Rabaan et al., 2001) instrumental for its pathogenicity. The Environmental Protection Agency (EPA) has put the waterborne bacterium

Aeromonas on the Contaminant Candidate List due to its ability to cause human infections (Seshadri et al., 2006). The *pil* gene is pili-forming gene, helping in twitching motility and biofilm formation. The *clpC* and *clpE* encode ATPase, providing *Listeria* with multiple virulence potentials, such as escaping phagocytosis activity and high-temperature tolerability (Chen et al., 2005). Further, the higher coverage for the genome of priority pathogens in our metagenome validates their higher prevalence in this riverine system.

2.1.4. Conclusion

The present findings characterized the differences in the microbial community between the urban and peri-urban waters amid rapid urbanization and the related anthropogenic activities. It also identified the pathogenic potentials of the riverine system using high-throughput shotgun sequencing. Enrichment of faecal indicator microbial groups in the urban river stretch indicated the release of untreated sewage of faecal origin. Moreover, a predominance of pollutant degrading bacteria in urban waters indicated their adaptive potential towards recalcitrant pollutants. The peri-urban waters are most affected by agricultural runoffs as they showed a higher population of ammonium-oxidizing microbes. The beta diversity analysis of the urban, peri-urban, and directly influenced city outskirts sites indicated significant alterations in overall microbial diversity. Besides, it also shows the influence of urban-influenced waters on the connected downstream rivers, affecting the overall lotic ecosystems. Higher coverage of the priority pathogens and their virulence genes further implicates the ill effects of increasing anthropogenic stress on the riverine system. Overall, this detailed study revealed a clear distinction between the microbial communities on the pollution sources and the level of urbanization. It also signifies the need to properly treat wastewater to reduce environmental stress on urban and connected peri-urban rivers.

2.1.5. References

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Chapter 2.2. Antimicrobial resistance in the riverine system

Abstract

Antimicrobial resistance (AMR) in the water bodies of urban cities is an alarming concern worldwide. To understand the dynamics of antibiotic resistance in interconnected rivers, we evaluated the influence of urban rivers on the downstream peri-urban rivers. The comprehensive shotgun metagenomic analysis using a MinION nanopore sequencer revealed the resistance genes against 26 antibiotic classes, including the last line of antibiotics. Altogether, 278 antibiotic resistance genes (ARGs) subtypes conferring resistance against multiple drugs (40%), bacitracin (10%), aminoglycoside (7.5%), tetracycline (7%), and glycopeptide (5 %) were observed in the riverine system of Pune, India. A relatively higher ARGs diversity in the urban stretches than peri-urban stretches was observed in the riverine system. The beta diversity analysis revealed significant differences with overlapping similarities (Stress value=0.14, p-value= 0.004, ANOSIM statistic R: 0.2328). These similarities were reasoned by assessing the influence of downstream sites (sites at the outskirts of Pune city; however, directly impacted), which revealed significant differences in the ARGs contents of urban and peri-urban stretches (Stress value=0.14, p-value= 0.001, ANOSIM statistic R: 0.6137). Overall, we observed the dissemination of ARGs from the polluted urban rivers into the downstream peri-urban rivers, potentially driven by anthropogenic activities.

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2.2.1. Introduction

Unrestricted and widespread release of active pharmaceutical ingredients (antibiotics) is rapidly increasing in the natural environments. The contaminated wastewater from various hospitals, industries, agriculture, aquaculture, and animal production is released into the aquatic bodies, facilitating the evolution, emergence, and dissemination of antibiotic-resistant genes (ARGs) (Singh et al., 2019). Globally, India is the largest consumer of antibiotics; however, irrational usage in India is quite frequent (Marathe et al., 2017). As a result, there is a higher incidence of antimicrobial resistance (AMR), evidently reflected in the aquatic bodies of India, resulting in their detrimental conditions (Singh et al., 2019; Ahammad et al., 2014; Marathe et al., 2017; Yadav et al., 2021; Water Quality Database; Borthakur and Singh, 2016). Several studies have revealed the enormity of ARGs in Indian Rivers (Reddy & Dubey, 2019; Diwan et al., 2019; Yadav et al., 2020; Samson et al., 2019). Reddy and Dubey 2019 reported 23 different ARG subtypes in the River Ganga, whereas Yadav et al., 2020 reported 29 different ARG types in the River Bhima and River Indrayani (142 and 333 ARG subtypes, respectively).

The peri-urban rivers possess dual features of urban-rural traits. They are incredibly significant as they provide water for various purposes (Yadav et al., 2021; Zheng et al., 2018; Chen et al., 2019a; Chen et al., 2019b). Despite their irrevocable significance, rivers are subjected to uncontrolled wastewater discharges, resulting in diverse ARGs in these peri-urban river ecosystems (Zheng et al., 2018; Chen et al., 2019a; Zhang et al., 2020; Chen et al., 2019b). Pune rivers have been reported for harbouring diverse ARGs and Antibiotic resistance bacteria (ARBs), including the resistance to the last line of antibiotics in the last decade (Marathe et al., 2017; Yewale et al., 2019; Razavi et al., 2017). However, previous studies were limited to fewer sites of the riverine system and did not address urban and peri-urban aspects. Only some efforts have systematically explored the ARGs of the interconnected urban and peri-urban rivers (Zheng et al., 2018; Chen et al., 2019a; Zhang et al., 2020; Chen et al., 2019b).

In the present study, we hypothesized dissimilarity in the ARGs in the interconnected urban and peri-urban stretch amid the differential level of urbanization. The MinION-based shotgun metagenomic sequencing of river sediments was carried out to examine the differences of ARGs in the urban and peri-urban stretches and understand the overall ARGs structure in the riverine system.

2.2.2. Experimental

2.2.2.1. Prediction of ARGs in the riverine system

The ARGs and Mobile Genetic Elements were predicted using a long-read specific NanoARG tool (Arango-Argoty et al., 2019) with default parameters. The NanoARG tool uses a deep learning-based approach to predict ARGs. Further, stringency was achieved by selecting only those ARG reads with a minimum bit score of 60 and identity of 30 (Pearson et al., 2014). The alpha diversity and beta diversity analysis used R-packages Phyloseq and Microbiome (McMurdie and Homes, 2013; Lahti et al., 2017).

2.2.2.2. Statistical analysis

As only a 3X difference between the minimum and the maximum number of ARG reads per sample, alpha diversity was performed on the unfiltered data. The significance was assessed using the Welch Two Sample t-test. The sequence depth was adjusted using the DESEQ2 package (Love et al., 2014). ANOSIM (p-value threshold of 0.05, permutation of 999) was used to interpret the ordination analysis statistically. Beta-disper (p-value threshold of 0.05, permutation of 999) was used to assess the homogeneity. Fold-change analysis was performed using METAGENassist (Arndt et al., 2012).

2.2.3. Results and Discussion

ARGs are emerging environmental pollutants, disseminating and evolving independently via various transfer routes, such as mobile genetic elements (Ouyang et al.,

2015). Urbanization affects the movement of people in the urban surroundings, increasing population density and industrialization to facilitate economic activities (Peng et al., 2020; Neiderud, 2015). Unrestricted use of antibiotics, additives in animal and fish feedings, and release of untreated wastewater are the multiple factors of urbanization that affect the riverine systems (Ouyang et al., 2015). These direct impacts of increasing anthropogenic activities on the urban rivers have a chain effect, polluting the connected downstream rivers in the outskirts of cities. To understand such a phenomenon concerning antibiotic resistance in the rivers, we used a metagenomic approach to study the differences in the resistome of a riverine system composed of urban and periurban stretches. The nanopore sequencing generated 3.64 million reads, yielding around 18.9 Gbases with an average quality score and sequence length of 8.86 and 5.1 kb, respectively.

2.2.3.1. Occurrence and distribution of ARGs

The aquatic environment provides an optimum ecological niche for emerging and disseminating antibiotic-resistant microbes due to ARG transfer between autochthonous and allochthonous bacteria (Almakki et al., 2019). The analysis predicted ARGs against 26 antibiotic classes, including polymyxin in the riverine system. Most of these ARGs belonged to multidrug resistance (MDR) genes (46%), followed by resistance to bacitracin (10%), aminoglycoside (7.5%), tetracycline (7%), and glycopeptide (5%) (Fig. 2.8.). The other considerable predicted ARGs (0.1%) were against fosmidomycin, fluoroquinolone, Macrolides, Lincosamides, and Streptogramines (MLS), beta-lactam, sulfonamide, phenicol, diaminopyrimidine, peptide, rifamycin, polymyxin, triclosan, aminocoumarin, nucleoside, pleuromutilin, and fosfomycin (Fig. 2.8.). The fold change analysis increased resistance against nitroimidazole, fosfomycin, sulfonamide (t-test with FDR correction, $p<0.05$), and tetracenomycin C in the urban and urban-influenced sites (Fig. 2.9.). At the same time, the peri-urban region displayed increased resistance against polyamine and polypeptide groups. The

total ARGs (against antibiotic classes) were more extensive in this riverine system as compared to the other polluted rivers of India, such as the River Ganges and River Yamuna (Das et al., 2020; Reddy and Dubey et al., 2018) and were comparable to urban and peri-urban rivers across the world (Chen et al., 2019c; Chen et al., 2019d; Zheng et al., 2018; Singh et al., 2019). Relatively, the ARGs against six categories, including antibacterial free fatty acids, tetracenomycin, polyamine: polypeptide, nitroimidazole, mupiromycin, and bicyclomycin, were scarcely identified (Fig. 2.8.).

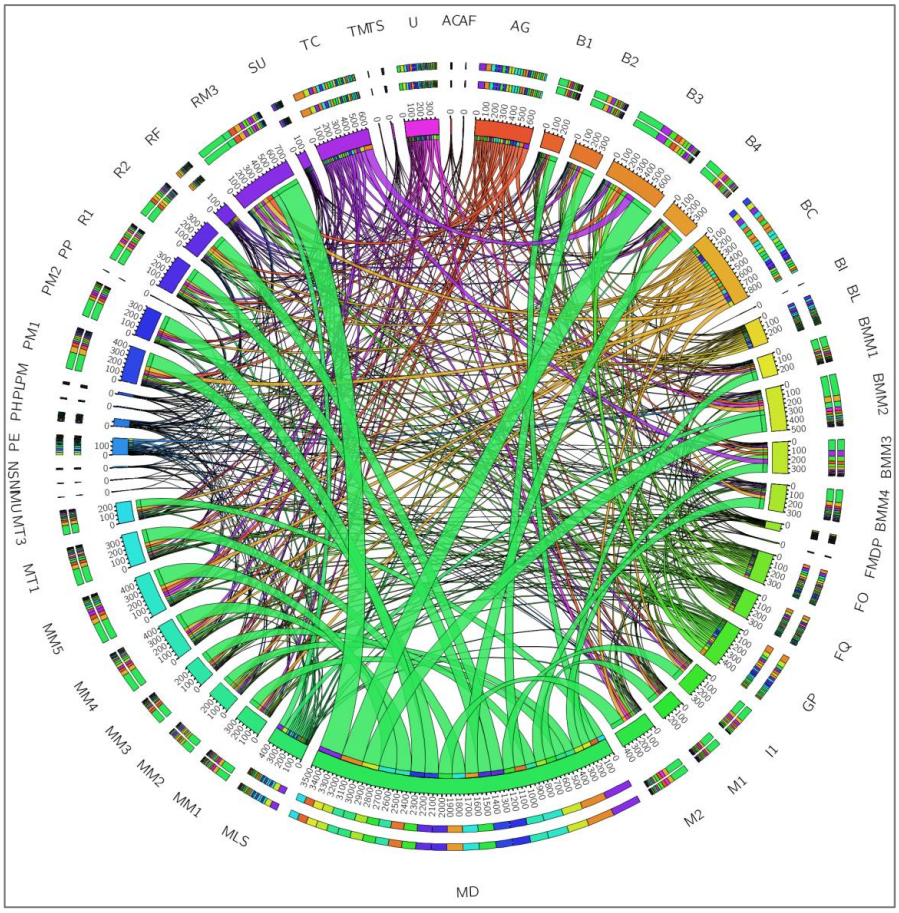

Fig. 2.8. Resistance against different categories of antibiotics. The circos plot illustrates the detection of different antibiotic resistance genes against 26 categories of antibiotics. GP- Glycopeptide; BL- Beta-lactam; PP- Polyamine and Polypeptide; MD- Multidrug; SU- Sulfonamide; AG- Aminoglycoside; FQ- Fluoroquinolone; BC- Bacitracin; PE- Peptide; TC- Tetracycline; FO- Fosmidomycin; U- Unclassified; MLS- Macrolides, Lincosamides, and Streptogramines; RF- Rifamycin; PH- Phenicol; DP- Diaminopyrimidine; AC-

Aminocoumarin; PM- Polymyxin; FM- Fosfomycin; TM- Tetracenomycin C; NS- Nucleoside; PL- Pleuromutilin; TS- Triclosan; NI- Nitroimidazole; M- Mupirocin; BI- Bicyclomycin; AF- Antibacterial free fatty acids

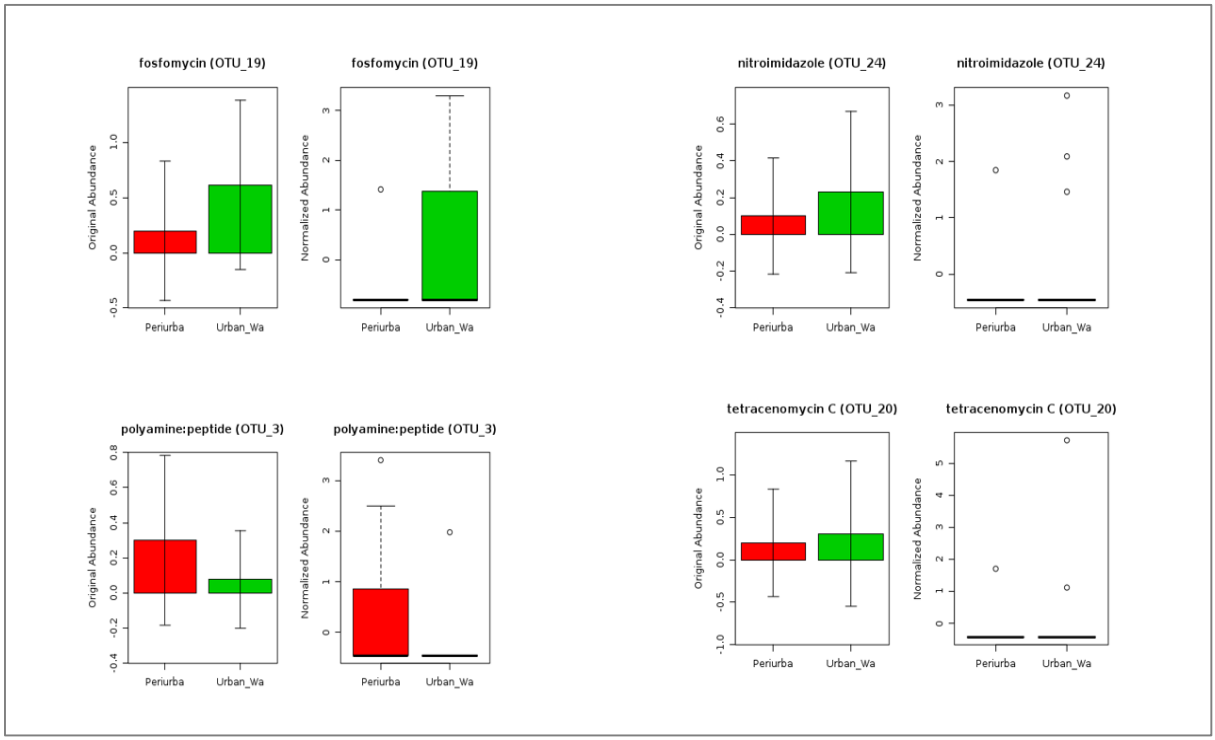

Fig 2.9. Fold change analysis reveals significant changes in fosfomycin, nitroimidazole, sulfonamide (t-test with FDR correction, $p<0.05$), tetracenomycin C, and polyamine:polypeptide groups between the two stretches

Altogether, the analysis revealed 278 different ARG subtypes in the riverine system. The previous study by Marathe et al. 2017, on the Mutha River identified 175 ARGs, most of which were mapped to city samples. The ARGs such as *ompR, rpoB2, bcrA, bacA, tetA(48), kdpE, vanR, multidrug ABC transporter, mtrA, rosB, oprM,* and *patA* were amongst the most dominant ARG (≥2%) subtypes in the riverine system (Fig. 2.10.). The *ompR*, a regulator gene involved in the significant up-regulation of numerous MDR drug exporter genes (Hirakawa et al., 2003), was the most dominant ARGs, followed by the rpoB2 RNA polymerase variant, which evades the action of a rifamycin (CARD database). Further, most of the detected ARGs such *as mtr, acr, ade, cme, mds, mdt, mex,* and *sme* (Fig. 2.10.), encoding resistance to multiple drugs, provide antibiotic efflux as the central mechanism of resistance (CARD ontology, and

CARD database). We observed numerous genes for various beta-lactamases that efficiently hydrolyze many third-generation antibiotics. Several members of VEB beta-lactamases (also known as Vietnamese extended-spectrum beta-lactamases) were observed, such as *VEB-1*, *VEB-2*, *VEB-3*, and *VEB-7*.

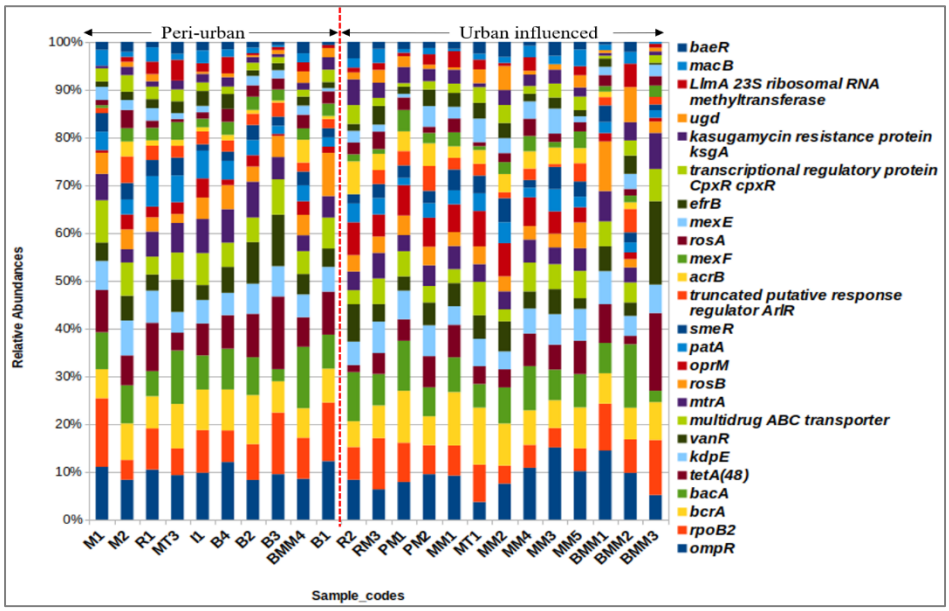

Fig. 2.10. Distribution of Antibiotic resistance genes. The relative abundance stacked bar plot illustrates the distribution of ARGs (≥ 1%) in the riverine system

These extended-spectrum beta-lactamases (ESBLs) are known to inactivate broad-spectrum cephalosporins and aztreonam (CARD database). The analysis also revealed several other important beta-lactamases, which include tem gene that confers resistance against cephalosporin, OXA beta-lactamases that are reported in pathogens such as *Acinetobacter baumannii* and *Enterobacter*, *NmcR beta-lactamase* commonly found in *Enterobacter* species (Palzkill et al., 2013; McArthur et al., 2013). Previously, the mobile colistin resistance gene (*mcr1*), conferring resistance to the last resort drug polymyxin, was reported in Mutha River by Marathe et al., 2017. These *mcr* genes confer resistance against drugs such as colistin belonging to polymyxin, often considered the last line of antibiotics. The analysis revealed the polymyxin inactivating *mcr5* and *arnA* gene in the riverine system. Overall, the results

indicated a higher prevalence of different ARGs subtypes conferred against almost all major clinically relevant antibiotics.

2.2.3.2. Spatial diversity of ARGs in the riverine system and Mobilome

Although insignificant, a relatively higher alpha diversity (Shannon and chao1 indices) of the antibiotic resistance genes in the urban stretch than in the peri-urban stretch was observed (Fig. 2.11.).

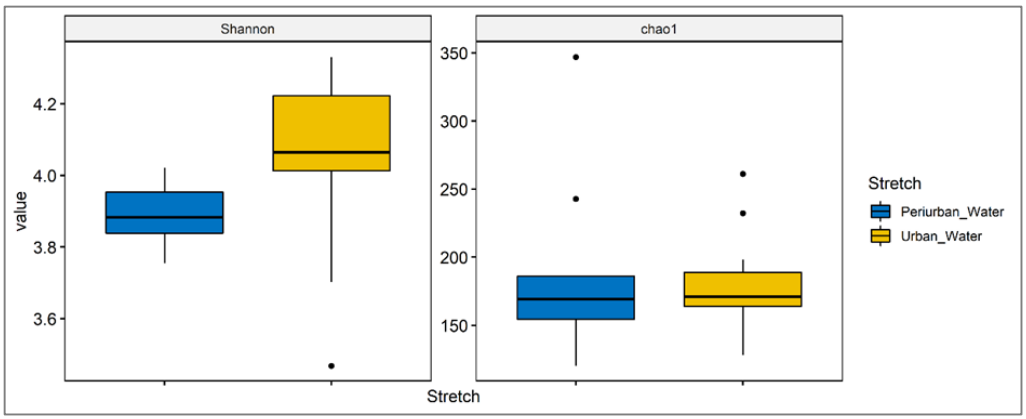

Fig. 2.11. Alpha diversity. Box plot showing alpha diversity (Shannon and Chao1 indices) analysis. The statistical significance was estimated using Welsch Two-sample t-test ($p>0.05$).

The beta diversity estimation with the Bray-Curtis dissimilarity method, along with the ANOSIM statistic, indicated significant diversity differences (p-value= 0.004, ANOSIM statistic R: 0.2328); however, the lower R-value indicated the overlapping similarities (Fig. 2.12). This could probably be attributed to the similar climate conditions and the downstream sites MM3, MM5, BMM1, BMM2, and BMM3. We confirmed this by taking "rivers" as a variable. We observed no significant influence of rivers on the observed diversity differences of ARGs (p-value= 0.301, ANOSIM statistic R: 0.05679). Although geographically, the downstream sites MM3, MM5, BMM1, BMM2, and BMM3 are on the outskirts of Pune city, these sites are directly influenced by the urban waters, unlike the other peri-urban sites, and thus these sites were grouped into urban waters. Further, comparing the urban waters (excluding the sites MM3, MM5, BMM1, BMM2, and BMM3) with the peri-urban sites

revealed significant differences (p-value= 0.001) with higher ANOSIM statistic R of 0.6137. These observations strongly indicate i). These sites are potentially responsible for the overlapping similarities observed due to their dual characteristics of urban and peri-urban, ii). We observed clear demarcation in the ARG diversity between the Pune city and peri-urban sites.

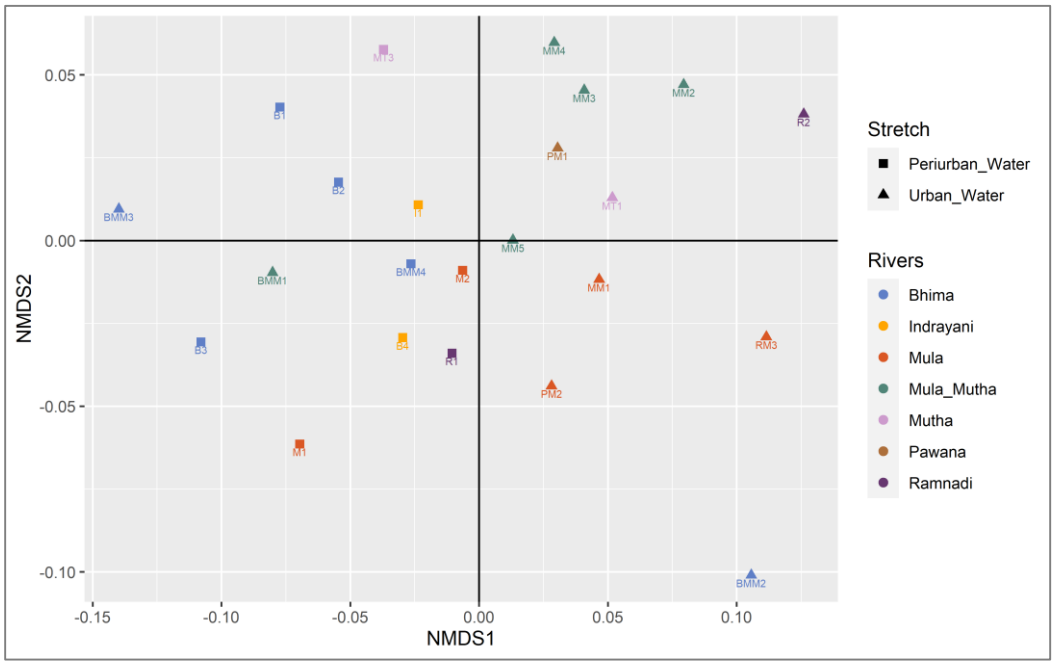

Fig. 2.12. Beta diversity of ARGs. The beta diversity was assessed using NMDS (Stress value= 0.14) with Bray-Curtis dissimilarity matrix. The statistical significance was estimated using ANOSIM (p-value= 0.004, ANOSIM statistic R: 0.2328).

The mobile genetic elements (MGEs) (Fig. 2.13.) are responsible for disseminating ARGs, increasing multidrug resistance. We observed the dominance of transposases, integrases, and recombinases at all the sites. The MGEs, such as Insertion sequences (IS) and transposons, are DNA segments carrying different ARGs that can move themselves to random locations with the help of enzymatic machinery that involves transposases and recombinases. The other elements, such as Integrons, with the help of integrases and various recombinases, carry out site-specific recombination to spread resistance (Frost et al., 2005). The class 1 integron (*intI1*) detected in all sediment samples is one of the significant MGEs present in

various environmental microorganisms (Chen et al., 2020). Together, these observations suggested the considerable impact of the urban waters on the connected downstream sites at the city's outskirts in the interconnected rivers.

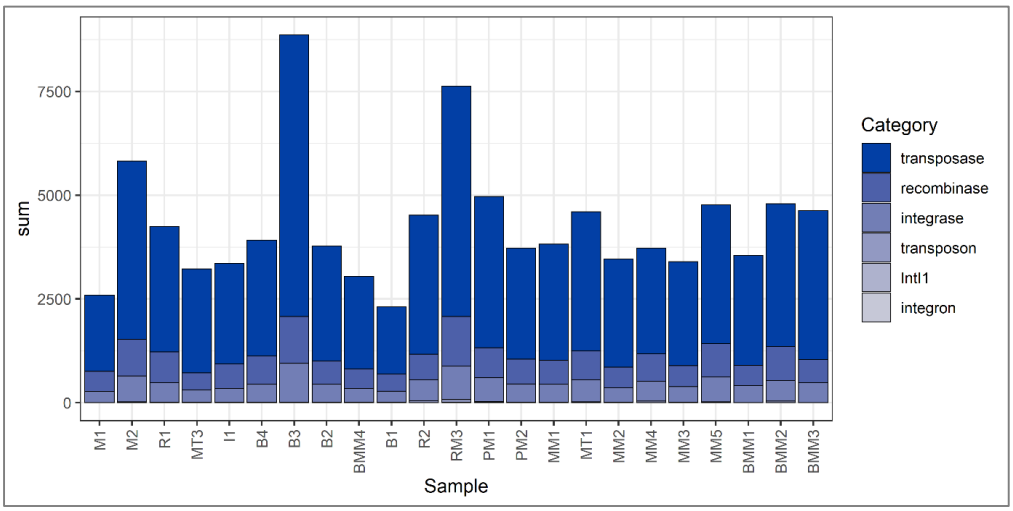

Fig. 2.13. Mobile Genetic Elements (MGE). The stacked bar plots indicate the total hits of MGE.

2.2.4. Conclusion

In conclusion, a relatively higher diversity of ARGs in the urban waters than in the peri-urban stretches indicated the differential influence of urbanization on the stretches of a riverine system. Nonetheless, the dissimilarity analysis also suggested the impact of the urban waters on the connected downstream stretch, implicating the cumulative impact of urbanization on the riverine stretch. Similar to the microbial diversity, the analysis here also reflected that the downstream periurban sites MM3, MM5, BMM1, BMM2, and BMM3 were directly impacted by the urban waters and thus exhibited overlapping similarities in the resistome. Further, we also observed ARGs against all the clinically significant antibiotics, including the last line of antibiotics, such as polymyxin. Overall, this study provides an overview of the ARGs prevalent in the riverine system and delineates the spatial differences of the antibiotic resistance genes. The results discussed in this study emphasize necessary strategies such as appropriate treatment

of the wastewater, removal of active pharmaceutical ingredients from the wastewater, adequate planning of city drains, and prevention of unrestricted release of untreated wastewater to curb the inflow of urban resistome entering into the downstream peri-urban river stretches.

2.2.5. References

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Chapter 2.3. Bioremediation potentials

Abstract

Rivers in metropolitan areas are subjected to a variety of xenobiotic contaminants. The majority of these pollutants are recalcitrant, necessitating the use of powerful technology to completely utilize or change them into non-toxic and bioavailable chemicals. Microbes are metabolically adaptable organisms that can adapt to contaminants by using them as a source of nutrients during growth. However, effective bioremediation necessitates a thorough understanding of microbial diversity and its roles in contaminated habitats. The current study employed a shotgun metagenomic approach to better understand the biodegradation-related genes in a contaminated urban riverine system. Various catabolic genes were mapped in the riverine system to metabolize xenobiotic pollutants such as Benzoate, Drug metabolism, Nitrotoluene, Aminobenzoate, and Polycyclic Aromatic Hydrocarbons. The analysis suggested the presence of aerobic and anaerobic catabolism pathways for xenobiotic pollutant degradation. Notably, catabolism patterns of several aromatic compounds culminated in the Benzoate degradation pathway, implying that it is a plausible core mechanism for autochthonous bacterial communities. Further, analysis using the RemeDB database mapped various plastic and dye-degrading enzymes. Furthermore, the diversity indices revealed only little differences (R^2 value of 18%) between the city and non-city riverine stretches, demonstrating the industrialization influence. Overall, the present work would lay a foundation for future investigations into river cleaning initiatives and the use of such microbes for bioremediation.

Yadav, R., *Rajput, V., & Dharne, M. (2023).* Environmental research, 231(Pt 1), 116128.

2.3.1. Introduction

Water conservation and safeguarding is a dire need to save all life forms. Industrial and population explosion along the river basins have severely impacted river health and is the primary cause of water quality deterioration (Suthar et al., 2009). Industrial development provides immense opportunity and transforms human life with social and economic developments; however, it has brought unpleasant environmental pollution resulting in the dumping of various toxic contaminants and xenobiotics to the water bodies, especially in the rivers traversing the urban settlements (Malla et al., 2018; Mittal et al., 2019).

Urban rivers provide numerous benefits to city people; nonetheless, urban river pollution is becoming increasingly severe (Wang et al., 2012; Suthar et al., 2009). Untreated sewage is one of the main reasons for their contamination and pollution (Marathe et al., 2017). Azodyes, Polycyclic aromatic hydrocarbons (PAH), halogenated compounds, nitroaromatic compounds, triazines and many more are persistent chemicals with adverse and toxic effects (Godheja et al., 2016). Microbial-based bioremediation, utilizing the metabolic capability of microbes, is one of the sustainable ways to transform these recalcitrant pollutants (Azubuike et al., 2016). However, when it comes to the isolation of such microbes, two significant questions arise. Firstly, their source of isolation, followed by their diversity and growing conditions. The isolation of potential microbes and their ease of cultivation form the basis of successful bioremediation. With the development of Next Generation Sequencing technologies (NGSTs), it is now possible to deduce detailed genomic, metagenomic and functional microbes from any environment. NGSTs research on the bioremediation process gives critical early insights into the biodegradative routes, detoxification mechanism, and diversity of detoxifying bacteria (Malla et al., 2018). This NGS-based information, thus, can be implicated in strategizing the bioremediation process of any environment.

We employed MinION-based shotgun sequencing to unveil the complexity and diversity of the xenobiotic degradation genes and mechanisms operating in the urban riverine system. The main aim is to i) investigate the degradation mechanism of various xenobiotic pollutants, ii). Understand the variety of pollutant-degrading enzymes in the riverine system. This thorough study will enable us to accurately isolate and extract the well-adapted autochthonous microbial community for bioremediation.

2.3.2. Experimental

2.3.2.1. Metagenomic data processing and analysis

Albacore (v2.3.4) was used for base-calling the FAST5 reads, and demultiplexing was carried out using qcat (v1.0.1), followed by adapter trimming using Porechop (v0.2.4). The MG-RAST (Keegan et al., 2016) with E-value cut-off =1×e−5 and sequence identity of 60%, using the KEGG database, was used to predict xenobiotic degradation genes. The taxonomy-based filtering of the xenobiotic gene was performed by creating a filter for Bacteria only (RefSeq taxonomic annotations) followed by a functional filter of Xenobiotics degradation and metabolism in MG-RAST. DIAMOND (Buchfink et al., 2015) was used for the alignment with a parameter of "diamond blastx -e 0.00001 --id 60 --more-sensitive" for RemeDB analysis (Sankara et al., 2019). The R packages phyloseq (McMurdie and Homes, 2013) and microbiome (Lahti et al., 2017) were used for alpha diversity and beta diversity analysis.

2.3.2.2. Statistical analysis

The samples were randomly sampled (set. seed of 100) to the lowest denomination for alpha and beta diversity estimation. The Principal Coordinate Analysis (PCoA) was constructed using the Bray-Curtis dissimilarity metric to estimate beta diversity, and significance was assessed using the Permanova (Adonis test, 0.05 p-value threshold) and betadisper test (p-value cutoff of 0.05) was used to determine the compositional heterogeneity.

The Shapiro-Wilk normality test (p-value cutoff of 0.05) and Q-Q plot were used to examine the data distribution. STAMP (Statistical Analysis of Metagenomic Profiles) (Parks et al., 2014) was used to compare significant features between city and non-city samples at Xenobiotic level 3. Welch's t-test (p-value threshold of 0.05) and Benjamini-Hochberg FDR were used for statistical significance assessment and multiple test corrections.

2.3.3. Results and Discussion

Microbes are metabolically highly diverse, possessing an extensive array of enzymes for efficiently transforming and cycling complex recalcitrant compounds (Wainwright et al., 1999). Several physicochemical parameters, studies (Borthakur et al., 2016; Yewale et al., 2019) and reports (Water Quality data, CPCB) have implicated the grim condition of rivers. The KEGG database was employed for the predictive functional analysis, which allocated the metagenomic reads to broadly six functional modules at level 1 of classification, viz., Metabolism, Genetic Information Processing, Environmental Information Processing, Cellular Processes, Human Diseases, and Organismal Systems. Metabolism is a critical ubiquitous process for the survival of any living being, and thus, most of the reads (~60%) were assigned to the Metabolism module. Maximum reads were mapped to essential functions such as Amino acid metabolism (35.46%), followed by Carbohydrate metabolism (22.69%), Energy metabolism (10.95%), and eight other categories including Xenobiotics biodegradation and metabolism (1.46%) (Fig. 2.14.). Furthermore, the analysis using the RemeDB database resulted in the prediction of more than 350 pollutant degrading enzymes involved in hydrocarbon, plastic and dye degradation.

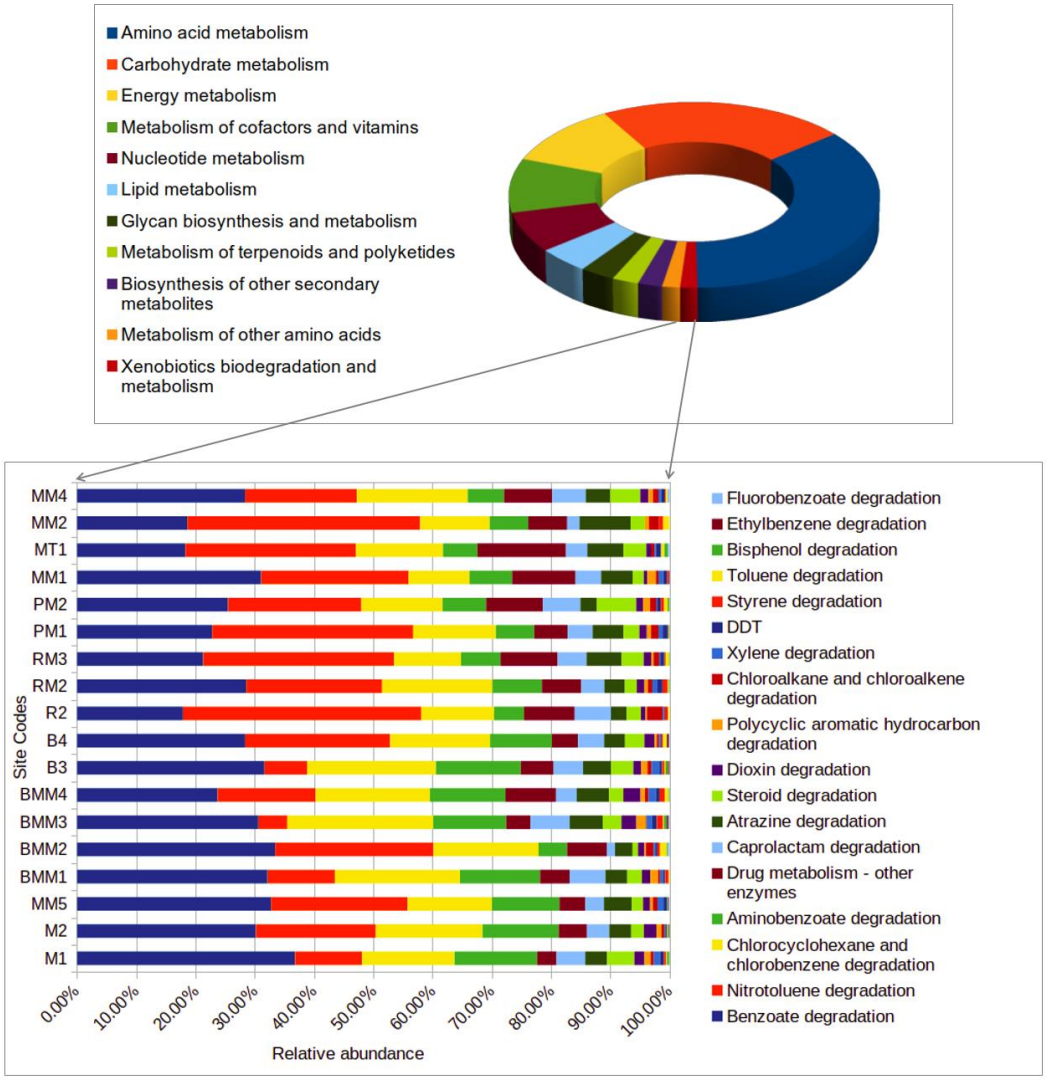

Fig. 2.14. Metabolism hierarchy and the distribution of degradation genes for various xenobiotic pollutants. The stacked bar plot describes the relative abundance of the catabolic genes observed for each category of the pollutants across the length of the riverine system. (City sites: R2, RM2, RM3, PM2, PM1, MM1, MM2, MT1, MM4 and non-city sites M1, M2, MM5, BMM1, BMM2, BMM3, BMM4, B3, B4).

2.3.3.1. Predictive composite pathway for pollutant degradation in the riverine system

Aromatic compounds are one of the most widely disseminated classes of organic compounds and are among nature's most prevalent and persistent pollutants (Valderrama et al., 2012). The analysis predicted diverse genes involved in degrading a wide variety of xenobiotic compounds at level 3 classification of KEGG orthology. Among these, the majority of the reads

were mapped to the genes involved in benzoate degradation (28%) (Significantly higher in non-city samples (Fig. 2.13.), WT test, BH FDR corrections, p-value<0.05), (Fig. 2.15.) which is a typical human xenobiotic metabolite.

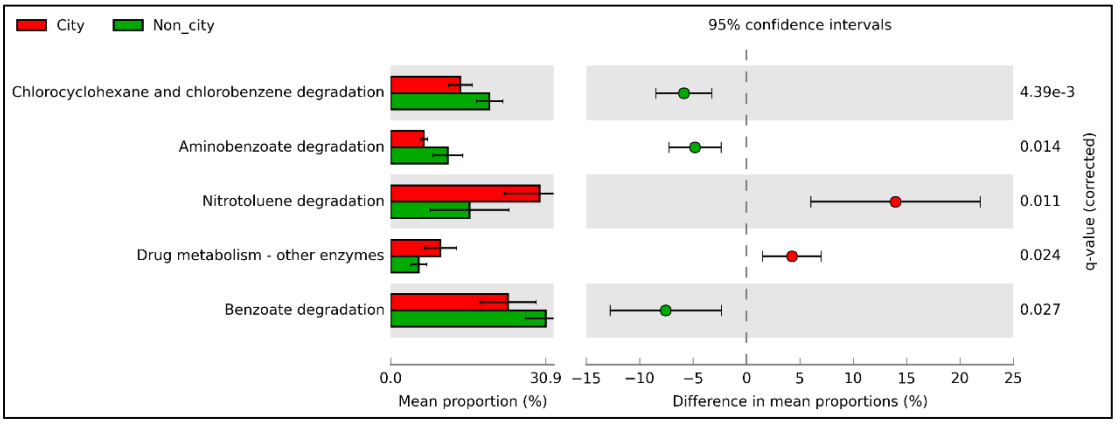

Fig. 2.15. Differentially abundant degradation genes. The extended error bar plot indicates the significant (Welsch's t-test, p<0.05, Benjamini-Hochberg FDR corrections) group of degradation genes between the city and non-city river stretch.

Furthermore, higher abundance of the genes involved in degradation and metabolism of major pollutants such as Nitrotoluene (NT) (20.46%), Chlorocyclohexane and Chlorobenzene (CCH: CB) (17.12%), Aminobenzoate (AB) (10.18%), Drug metabolism (DM) (6.43%), Caprolactam (4.51%), Atrazine (4.43%), Steroid (3.10%), and Dioxin (1.48%) were observed. Considerable reads (>0.5%) were also mapped to the genes involved in the degradation of Chloroalkane and chloroalkene, Polycyclic aromatic hydrocarbons (PAH), and Xylene. Notably, the genes involved in the degradation of AB, benzoate, and CCH: CB were significantly higher in non-city samples, whereas PDE for NT and DM were higher in city sites. (Welch's t-test, Benjamini-Hochberg FDR corrections, p<0.05) (Fig. 2.15.). Based on the detection of the different xenobiotic degrading enzymes, we propose a probable composite degradation pathway (Fig. 2.16.) operational in the riverine system under study.

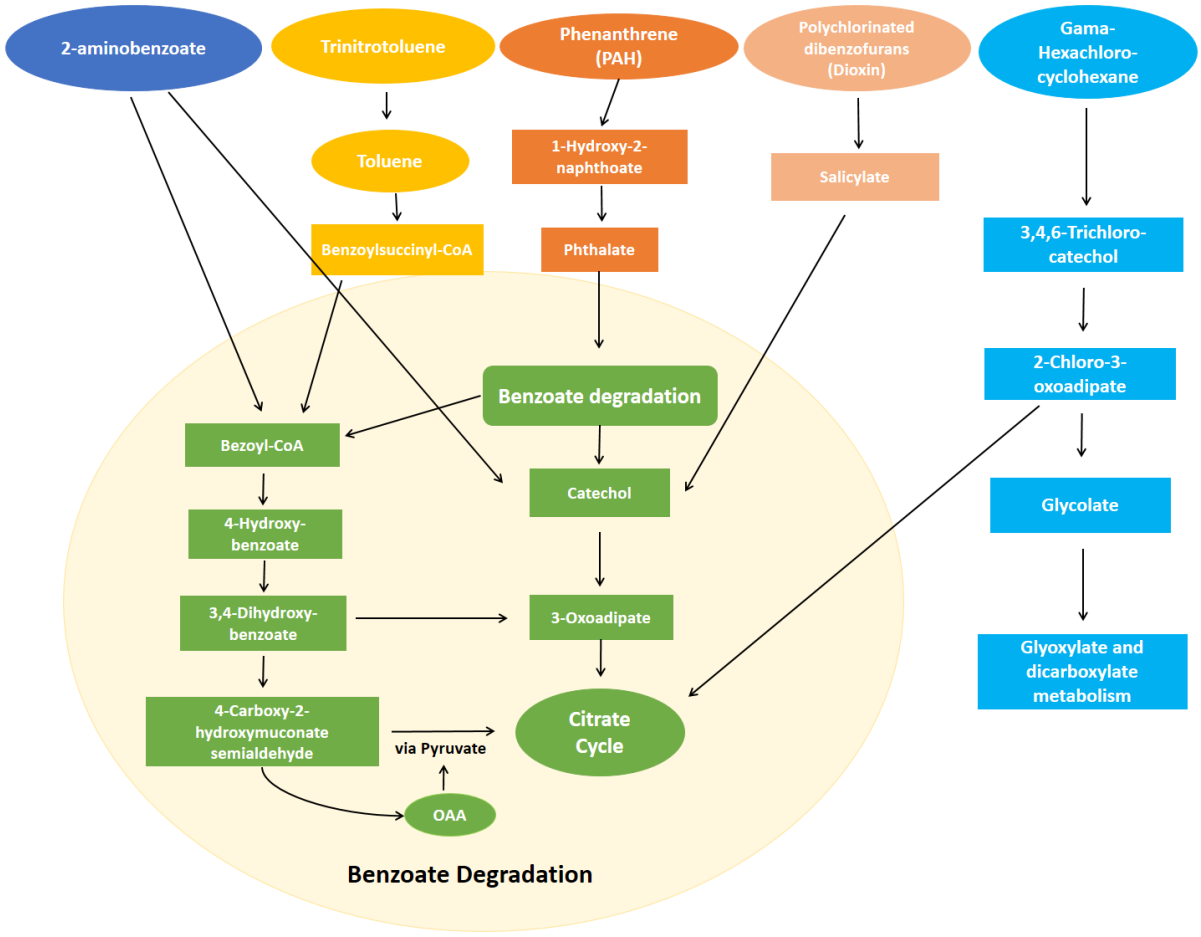

Fig. 2.16. Predicted composite pathway. The schematic depicts the predicted composite catabolic pathway operational in the riverine system.

Benzoate degradation

Benzoate is the most common model for studying the metabolism of aromatic compounds (Valderrama et al., 2012). Various key genes involved in Benzoate degradation were predicted in the analysis. The closer inspection suggested a predominance of two pathways (Fig. 2.16.) for Benzoate degradation in the riverine system. The essential genes *benB-xylY* and *benD-xylL* encoding Benzoate 1,2-dioxygenase beta subunit and dihydroxycyclohexadiene carboxylate dehydrogenase, respectively, convert Benzoate to catechol (Table 2.5.).

Table 2.5. List of the predicted catabolic genes for the predicted composite pathway.

Xenobiotic Pollutant Degradation	Probable Pathways operating in the riverine system	Gene/Enzymes detected in the riverine system
1. Benzoate Degradation	a. Benzoate degradation via 3-Oxoadipate pathway with Catechol as an intermediate b. Benzoate degradation via Benzoyl-CoA c. Other genes	*benB-xylY, benD-xylL, catA, catB, catC, pcaD, pcaI, pcaF.* *badA, hbaBCD, pobA, pcaBC, ligABIJ, oah, badK.* *badDEFGHI, aliA, aliB, benC-xylZ, cmtD, dhbB, dch,hbaA, pcaH,* EC1.14.13.12, EC:1.3.1.62, EC:4.1.1.77, EC:5.3.2.-.
2. Nitrotoluene degradation	a. Trinitrotoluene to Toluene degradation via formation of 2,4,6-Triaminotoluene b. Other genes	E1.12.99.6L and E1.12.99.6S, *dsrA, dsrB,* and *nemA.* *nfnB, nfsB.*
3. Hexachlorocyclohexane (HCH) degradation	a. HCH degradation to either Glycolate and dicarboxylate metabolism or Citrate cycle via Chloroacetate b. Other genes	*catA,* EC3.1.1.45, EC1.3.1.32, EC:3.8.1.2, EC:3.8.1.3. *tfdB, catB, dmpB, bphC, bedC1,* EC1.14.13.7, EC:1.14.13.50, EC:1.14.12.-, EC:1.13.11.37
4. 2-Aminobenzoate (ABz) degradation	a. ABz degradation via Benzoyl-CoA b. ABz degradation via Catechol c. Other genes	*abmG, abmA* *antA* *vanAB, antC, desB, mdlC,* EC:3.1.3.41, EC:4.1.1.61, EC:5.1.2.2.
5. Phenanthrene	a. Phenanthrene degradation via Phthalate	*phdF, nidD, phdJ,* EC:1.14.12.7, EC:4.1.1.55

Additionally, the presence of other essential genes such as *catB, catC, pcaD, pcaI,* and *pcaF* genes confirmed the benzoate degradation via an aerobic β-ketoadipate pathway (Table 2.4.)

with catechol as an intermediate (Kanehisa and Goto et al., 2000; Kanehisa et al., 2019; Yoon et al., 2007; Valderrama et al., 2012).

Benzoate ⇢ Catechol ⇢ 3-Oxoadipate-enol-lactone ⇢ TCA

The other pathway that seemed to be operating in the riverine system was Benzoate-CoA ligase (*badA*), an anaerobic pathway (Harwood et al., 1998; Valderrama et al., 2012). The Benzoate is converted to Benzoyl-CoA by Benzoate-CoA ligase, followed by its conversion to 4-Hydroxy-benzoate via an essential enzyme 4-hydroxy benzoyl-CoA reductase. Further, the presence of *pobA* and *pcaBC* suggested catabolism of 4-Hydroxy-benzoate via formation of β-ketoadipate and detection of *lig* genes, *oah* and *badK* indicated its metabolism to Citrate cycle (tricarboxylic acid (TCA) cycle) via formation of 4-Caboxy-2-hydroxymuconate semialdehyde Table 1 (Kanehisa and Goto et al., 2000; Kanehisa et al., 2019).

Benzoate → Benzoyl-CoA ⇢4-hydroxybenzoate ⇢3-Oxoadipate ⇢TCA

↓

4-Carboxy-2-hydroxymuconate semialdehyde

⇣

TCA

Altogether, the riverine system harbours both aerobic as well as anaerobic machinery for the metabolism of Benzoate.

Nitrotoluene transformation

The stability in Nitrotoluene structure is provided by the aromatic ring structure and nitro groups, making Nitrotoluene degradation very challenging (Serrano-Gonzalez et al., 2018). The analysis predicted Nitrotoluene degradation genes such as *nemA*, *dsrA* and *dsrB* (Table 2.5.) and the hydrogenase enzymes. Based on these, we can conclude that the Trinitrotoluene (TNT) was probably transformed via 2,4,6-TNT entering into Toluene degradation. Little abundance (0.3% of total xenobiotic degradation genes) of the Toluene

degradation gene was observed in the riverine system. Further, the presence of *bbs* genes in the riverine system suggested anaerobic degradation of toluene via benzyl-succinate to the subsequent modification to Benzoyl Co-A, which ultimately enters the Benzoate degradation pathway (Fig. 2.16.) (Kanehisa and Goto et al., 2000; Kanehisa et al., 2019).

a. TNT → 4-Hydroxylamino-2,6-dinitrotoluene ⇢ 2,4,6-Triaminotoluene ⇢ Toluene

b. Toluene → Benzyl-succinate ⇢ Benzoylsuccinyl-CoA → Benzoyl-Co⇢ Benzoate degradation

Hexachlorocyclohexane (HCH) degradation

Lindane (γ-HCH) is a widely used insecticide of organochlorine nature, and its isomers are persistent toxic organic pollutants (Singh & Kuhad, 2002). Despite detecting a vast number of genes for Lindane degradation, an essential gene (*LinA, LinB* and *LinC*) was absent. However, the detection of various enzymes catechol 1,2-dioxygenase, carboxymethylenebutenolidase, maleylacetate reductase, 2-haloacid dehalogenase, and haloacetate dehalogenase (Table 2.5.) probably indicated γ-HCH degradation via formation of 2-Chloro-3-oxoadipate.

γ-HCH ⇢ 2-Chloro-3-oxoadipate → Chloroacetate → Glycolate → GDM

↓

TCA

2-Aminobenzoate and Polycyclic Aromatic Hydrocarbon (PAH) degradation

The *abmG* and *antA* genes (Table 2.5.) implicated the presence of aerobic and anaerobic 2-Aminobenzoate degradation in the riverine system (Lochmeyer et al., 1992). The *abmG* gene produces a ligase that converts 2-aminobenzoate to 2-aminobenzoyl-CoA. The 2-Amino-benzoyl-CoA is transformed into Benzoyl-CoA, ultimately entering the Benzoate degradation pathway (Kanehisa and Goto et al., 2000; Kanehisa et al., 2019). Anthranilate 1,2-dioxygenase encoded by the *antA* gene converts 2-Aminobenzoate to catechol. PAH are ubiquitous pollutants generated primarily due to incomplete combustion of organic materials (Abdel-

Shafy & Mansour, 2016). We did not observe an enrichment of genes responsible for the degradation of high molecular weight PAH compounds such as Benzo[a]pyrene. However, genes were detected involved in the degradation of Phenanthrene (Low Molecular weight PAH compound). Prevalence of *phdF*, *nidD*, *phdJ*, *phthalate 4,5-dioxygenase*, and *4,5-dihydroxyphthalate decarboxylase* (Table 2.5.) indicated phenanthrene degradation via the Benzoate degradation pathway with phthalate as an intermediate (Kanehisa and Goto et al., 2000; Kanehisa et al., 2019).

Phenanthrene ⇢ 1-Hydroxy-2-naphthoate ⇢ Phthalate ⇢ Benzoate degradation

Several genes involved in the metabolism of other xenobiotic compounds, such as Atrazine, Drug metabolism, Bisphenol, Xylene and Steroid, were not detected. Thus, the most suitable pathway for their degradation in the riverine system could not be predicted. Furthermore, as most of the catabolic pathway converged to the Benzoate degradation pathway, it appeared to be the central pathway for the aromatic compound degradation in the riverine system.

2.3.3.2. Pollutant Degrading Enzymes (PDE) in the riverine system

The RemeDB database analysis (Fig. 2.17.) provided extensive information on the PDE. Phenylacetaldehyde dehydrogenase and Catalase peroxidase were the most abundant PDE predicted in the riverine system. Phenylacetaldehyde dehydrogenase, encoded by the *styD* gene, is a component of *styABCD* operon.

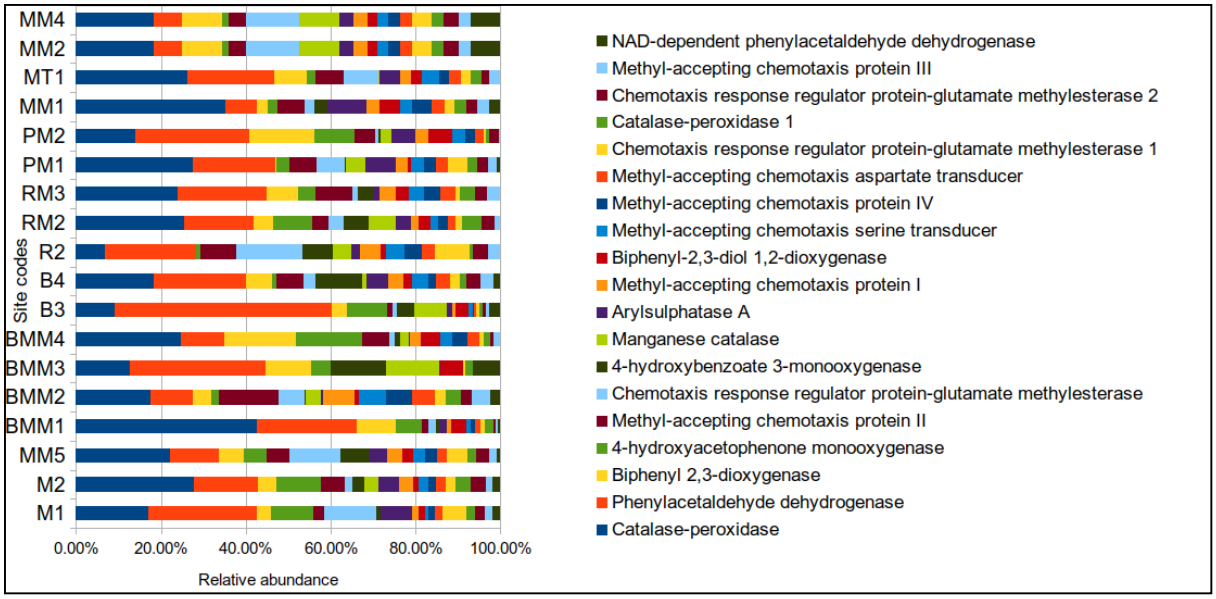

Fig. 2.17. Pollutant degradating Enzymes (PDE). The stacked bar plot depicts the relative abundance (1%) of the PDE involved in the degradation of various pollutants. RemeDB database was used for the analysis. (City sites: R2, RM2, RM3, PM2, PM1, MM1, MM2, MT1, MM4 and non-city sites M1, M2, MM5, BMM1, BMM2, BMM3, BMM4, B3, B4).

This plastic-degrading enzyme is involved in the catabolism of styrene, which is a building block of Polystyrene (Mooney et al., 2006). Peroxidases are oxidoreductases involved in degrading recalcitrant dyes and other xenobiotic pollutants such as Phenol (Bansal & Kanwar, 2013). In addition to these, PDE, Biphenyl 2,3-dioxygenase, 4-hydroxybenzoate monooxygenase, Aryl sulphatase A, and Manganese catalase were considerably detected that are involved in hydrocarbons and dye degradation (Subramanian et al., 2020). Enzymes such as Chemotaxis response regulator protein-glutamate methylesterase, methyl-accepting chemotaxis proteins, Methyl accepting chemotaxis serine, and aspartate transducer were prevalent (>1%) in the samples. Chemotaxis allows microbes to sense environmental cues and react positively or negatively towards particular chemicals (Ud-Din & Roujeinikova, 2017). It is believed to enhance xenobiotic pollutants' bioavailability and biodegradation ability (Ahmad et al., 2020). Overall, the analysis indicated the presence of a wide range of PDE for plastic, dye and hydrocarbon degradation in the riverine system.

2.3.3.3. Abundance for xenobiotic category filtered by taxonomic entities

The xenobiotic degradation genes were taxonomically mapped to 19 bacterial phyla. A majority of the degrading genes (72.46) (Fig. 2.18.A.) were assigned to Proteobacteria, followed by >1% genes assigned to *Actinobacteria, Chloroflexi, Bacteriodetes, Acidobacteria, Firmicutes,* and *Cyanobacteria*.

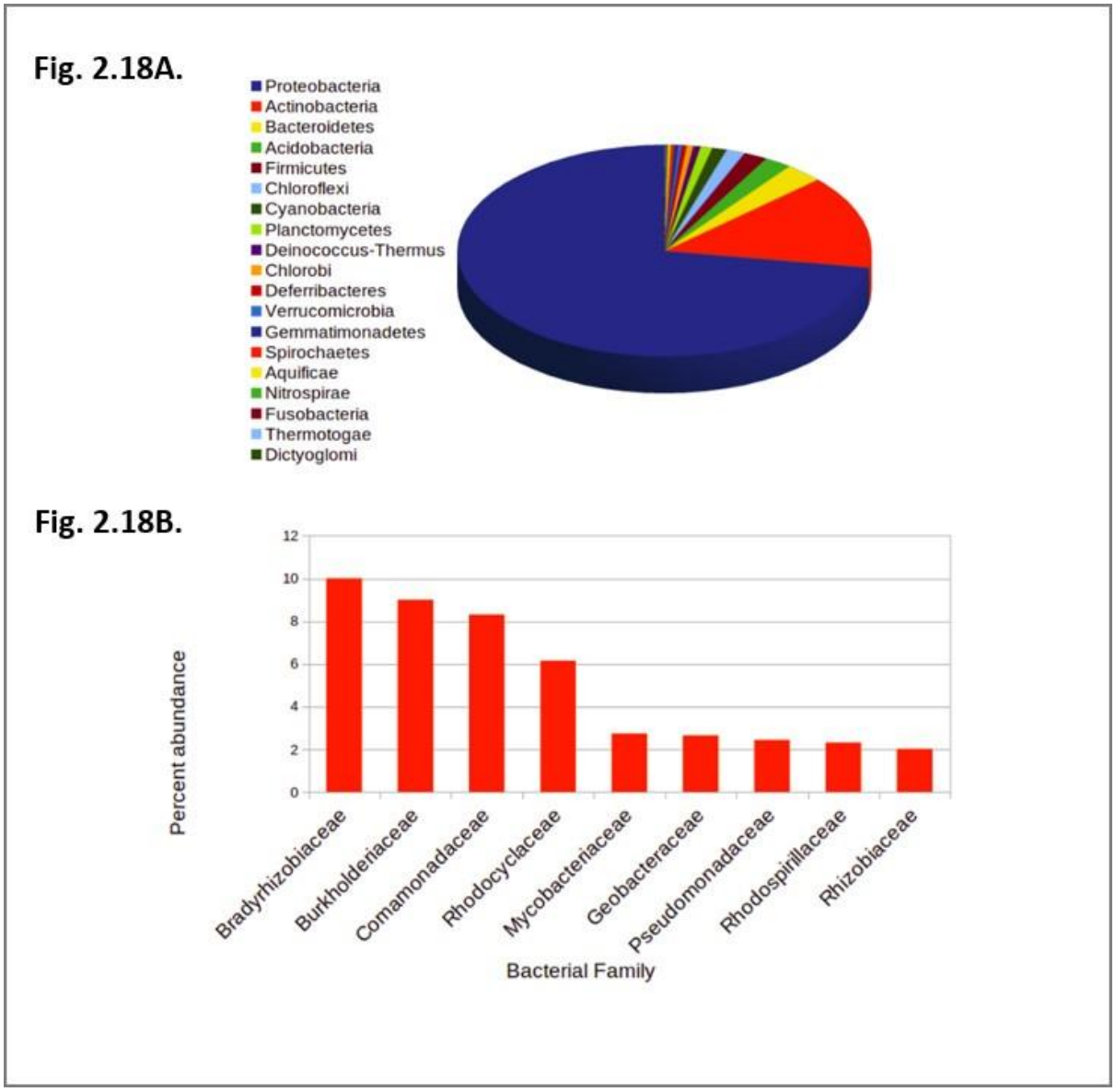

Fig. 2.18. Abundance for xenobiotic category filtered by taxonomic entities. The stacked bar blot depicts the relative abundance of xenobiotic gene abundance filtered by bacteria taxa **A.** at Phylum Level and **B.** at Family level of classification (Top 10).

At the lower taxonomy level, a predominance of genes was assigned to the bacterial family (Fig. 2.18.B.) *Bradyrhizobiaceae, Burkholderiaceae, and Comamonadaceae. Rhodopseudomonas*, a phototrophic non-sulfur purple bacteria belonging to *Bradyrhizobaceae*, is a significant participant in the degradation of aromatic compounds (Rahalkar et al., 1991; Egland et al., 2001). *Burkholderiaceae*, a *Betaproteobacteria,* is widely reported for degrading various aromatic compounds (Pérez-Pantoja et al., 2011). The members of this group, such as *Burkholderia*, *Cupriavidus* and *Ralstonia*, are versatile degraders of aromatic compounds, Nitroaromatic compounds, Trichloroethylene and various others (Xu et al., 2018; Ryan et al., 2007; Lykidis et al., 2010). Members of *Comamonadaceae* have been reported to have biodegradation potential towards a vast array of aromatic and priority pollutants (Lee et al., 2019). Apart from these taxa, higher abundance of genes was filtered by the members of *Mycobacteriaceae, Rhodocyclaceae*, *Pseudomonadaceae*, and *Geobacteraceae,* which are well-known for their efficiency in pollutant degradation (Singleton, 1994; Kleemann & Meckenstock, 2011; Galazka et al., 2018), thus indicating their prevalence and involvement in xenobiotic degradation in the riverine system.

2.3.3.4. Xenobiotic degrading gene diversity across the riverine stretch

The functional level of the xenobiotic category was used to analyze the variety of xenobiotic degrading genes in the riverine system. The alpha diversity using richness estimators Chao1 and Observed indices, and diversity estimators Shannon function and Simpson index (Fig. 2.19., Fig. 2.20.A) revealed significantly higher diversity of the non-city stretch compared to the city samples ($p<0.05$). The PDE diversity and heterogeneity between the city and non-city stretch were further analyzed by estimating beta diversity. The PCoA plot (Fig. 2.20B.) using NMDS matrices suggested significant differences between the city and non-city stretch (riverine stretch) in terms of xenobiotic functional metabolism with lower heterogeneous dispersion among the groups (Permanova, Adonis test $p<0.05$, R^2 value= 0.18;

Beta-disper p>0.05). The river type did not impact the diversity of xenobiotic degrading genes (Permanova, Adonis test p>0.05). Despite the significant differences, the lower R^2 value (only 17 % of differences were explained) suggested a shallow impact of the riverine stretch. Moreover, the significant difference observed could be attributed to the confluence site BMM2 (confluence of Mula-Mutha with Bhima River), reflecting significant differences from the other sites. Due to the convergence of different rivers, confluence sites are more prone to changes in their genetic structure (Samson et al., 2019).

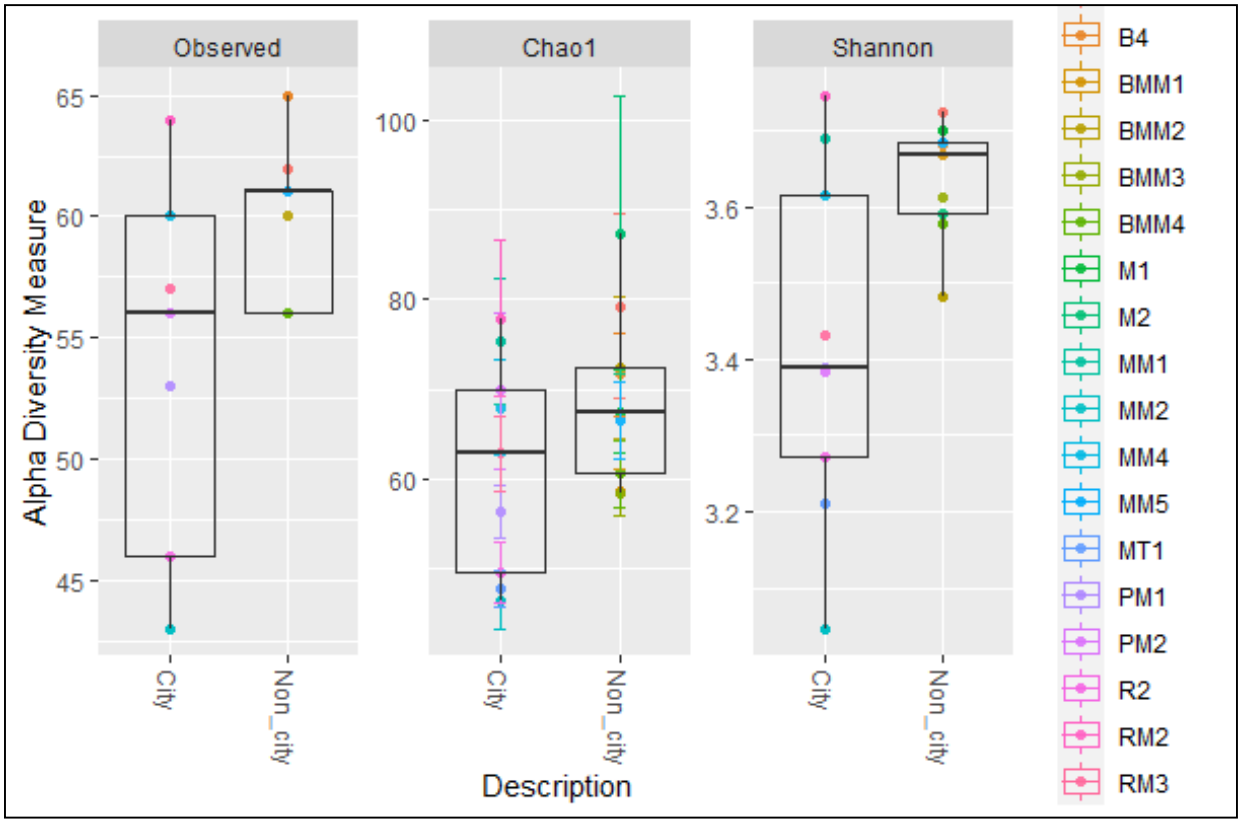

Fig. 2.19. Alpha diversity estimation. Box-plot explaining alpha diversity using richness and diversity estimators.

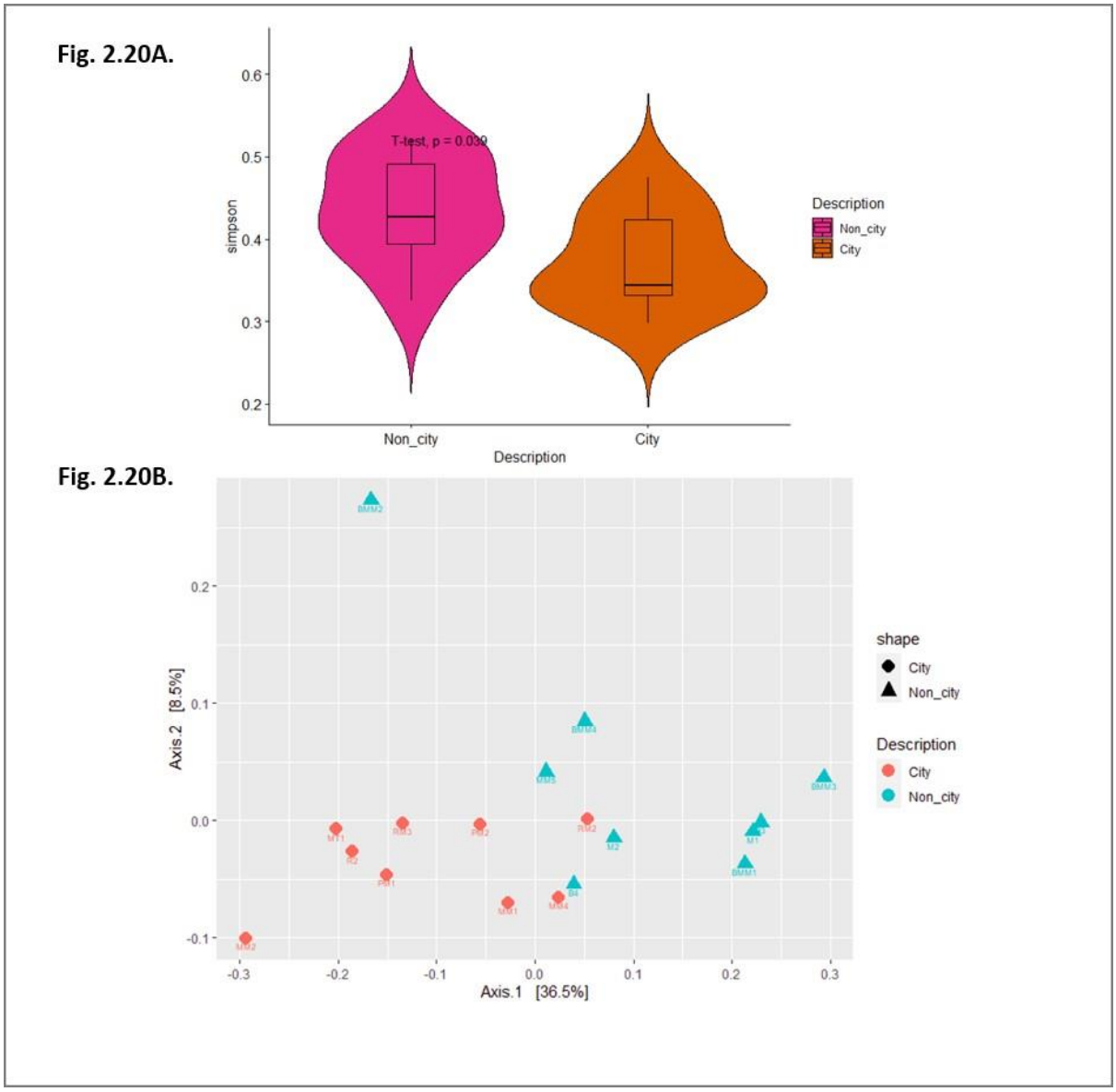

Fig. 2.20. Diversity of xenobiotic metabolism genes. A. Violin Plot for the assessment of the alpha diversity using Simpson indices (T-test, $p<0.05$), **B.** Beta diversity estimation using PCoA plot with Bray-Curtis dissimilarity measure (Permanova, Adonis test, $p<0.05$, R^2 value 0.18, Beta-disper test- $p>0.05$).

2.3.4. Conclusion

The shotgun metagenomics investigation of the riverine system revealed potential genes involved in the bioremediation of xenobiotics and their plausible detoxification mechanisms. Such genes and enzymatic pathways could aid microbes to thrive in such a

polluted and harsh environment. The functional investigation suggested the utilization of both aerobic and anaerobic catabolism for the potential degradation of the pollutants. Benzoate degradation appears to be a central pathway for the catabolism of most aromatic compounds. Moreover, the diversity analysis suggested marginal differences in the xenobiotic transformation potential between the city and non-city riverine stretches, indicating the impact of urbanization and the associated pollution in the non-riverine stretch. Overall, the present investigative study would serve as a fundamental basis for utilizing metagenome-aided functional information to design improved strategies to remediate such contaminated water bodies and explore such microbes for bioremediation applications.

2.3.5. References

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Chapter 3. To understand the impact of mass bathing event (Wari) on the river microbial ecology

Chapter 3. To understand the impact of mass bathing event (Wari) on the river microbial ecology

Abstract

Mass bathing and religious gatherings can pose considerable public health issues, dramatically changing river microbial ecology. During the Pandharpur Wari, an annual pilgrimage in Maharashtra, India, millions of faithful devotees carry the footprints of the saint poets and pay prayer to Lord Vitthal on the 11th day of the moon's waxing phase (Ashadi Ekadashi). Religious pilgrims go approximately 250 kilometres to take holy baths in two holy rivers, the Indrayani at Alandi and the Bhima River at Pandharpur. In this study, we investigated the impact of spiritual mass bathing on environmental changes (in terms of river microbial community structure and functions). We understood the dynamics of the pathogenic potentials and antibiotic resistance during such anthropogenic activities. Alterations in alpha and beta diversity were recorded in both river's bathing and post-bathing water samples, indicating significant spatiotemporal microbial diversity and functional changes. The findings revealed significant differences in the abundance of virulence genes between bathing and post-bathing samples, as well as an increase in priority skin and enteric pathogens like *Acinetobacter baumannii, Streptococcus pyogenes, Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Pseudomonas aeruginosa* during the bathing event. Furthermore, we observed a considerable increase in antibiotic resistance in Bhima and Indrayani river bathing samples. This is the first thorough metagenomic assessment of the impact of religious mass-bathing on the riverine ecology.

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3.1. Introduction

The river water quality is significantly deteriorated due to the increased magnitude of anthropogenic activities (Labbate et al., 2016; Wang et al., 2018). Consequently, most rivers worldwide face severe threats of elevated antimicrobial resistance (AMR) and deadly waterborne diseases (Yewale et al., 2019; Marathe et al., 2017; Wang et al., 2020; Hamner et al., 2019). Anthropogenic activities such as the ritual mass bathing during Kumabh-Mela, where millions of devotees plunge into rivers with a belief to wash their sins (Jani et al., 2018a; Jani et al., 2018b), increase stress on the river ecosystem several-fold (Tyagi et al., 2013). Similar to Kumbh Mela, the Pandharpur Wari, commonly known as "Wari", is also one of the most significant religious events in India, undertaken by millions of Warkaris (pilgrims) for more than 800 years (Mathesul et al., 2016).

Pandharpur is a small town in the Solapur district of Maharashtra, India, regarded as "Heaven on Earth" by the devotees (Baad, 2016). As per the tradition in Hindu religion, the Ashadi Ekadashi (the 11th day in the moon's waxing phase) is considered auspicious to undertake Wari. Overall, there are four Waris that are conducted in Hindu months: Ashadh (June, which attracts the most number of devotees), Kartik (November), Magh (February), and Chaitra (April). The devotees form a small group ('dindis') that carries the footwear (padukas) of a saint-poets in a palkhi (palanquin) (Baad, 2016; Koiso, 2017). They walk about 250 kilometres in western Maharashtra, from Dehu and Alandi to Pandharpur, to pay their obeisance to Lord Vitthal on the sacred event of Ashadi Ekadashi (Koiso et al., 2016). River Indrayani and River Bhima (also known as Chandrabhaga in Pandharpur because of their half-moon form) are respected holy rivers. The Bhima River travels 860 kilometres through southeast India before joining the Krishna River. At the same time, the Indrayani River rises in the Sahyadri mountains and flows east to reach the Bhima. During the Wari festival, millions

of devotees immersed in the hymn pay their respects to Lord Vitthal and take a holy bath in the Indrayani and Bhima rivers at Alandi and Pandharpur, respectively.

Understanding the source of organic pollution in river water and developing knowledge about the implications of mass bathing is important (Purohit et al., 2020). These mass gathering events observe communal activities, posing a potential waterborne illness risk (Vortmann et al., 2015). Also, several studies explained the probable consequences of mass anthropogenic activities on public health and the river ecosystem (Tyagi et al., 2013; Jani et al., 2018a; Blyth et al., 2010; Shafi et al., 2008; Memish et al., 2012). A targeted amplicon sequencing study by Jani et al. in 2018a gave an account of the alterations in microbial diversity in the Godavari River during the Kumbh Mela. Furthermore, the influence of mass gatherings on public health is undoubtedly inevitable and requires utmost monitoring and precautions (Shafi et al., 2008). Collectively, these events might lead to alterations in the microbial ecology of the rivers, therefore affecting the overall indigenous functioning and quality of the aquatic ecosystem. Comprehensive shotgun metagenomics has the potential to provide a detailed understanding of the broader changes that occur during such mass gathering events. As per our best knowledge, in-depth studies addressing the impact of mass-bathing events on the river ecosystem still need to be made available. The paucity of information regarding the overall ecological changes during such enormous anthropogenic stress calls for a comprehensive investigation.

Here, we aimed to investigate the changes in the overall microbial ecology, their functionality and potential health impacts associated with religious mass bathing events. Moreover, particular emphasis was given to assessing antibiotic resistance and pathogenicity changes during mass bathing in Wari using the latest shotgun-based MinION nanopore sequencing technology.

3.2. Experimental

3.2.1. Sample collection details

The sampling was conducted during the bathing events at two locations, i.e., Alandi of Indrayani River and Pandharpur of Bhima River. Also, we collected the post-bathing samples from the same sites to evaluate the influence of mass bathing. Water samples were collected in gamma-irradiated sterile containers from two locations i). at Alandi, on the bank of River Indrayani (n=8, n is the number of samples) and from ii). at Pandharpur, on the bank of River Bhima (the sampling site is also known as Chandrabhaga) (n=6). The sample codes and their description are as follows: River Indrayani at Alandi (Bathing sample codes: UB01, BSL01, BSR01, and DB01; Post-bathing sample codes: UB02, BSL02, BSR02, and DB02). River Bhima at Pandharpur (Bathing sample codes: UB03, BS03, DB03; Post-bathing sample codes: UB04, BS04, DB04) (Fig. 3.1.).

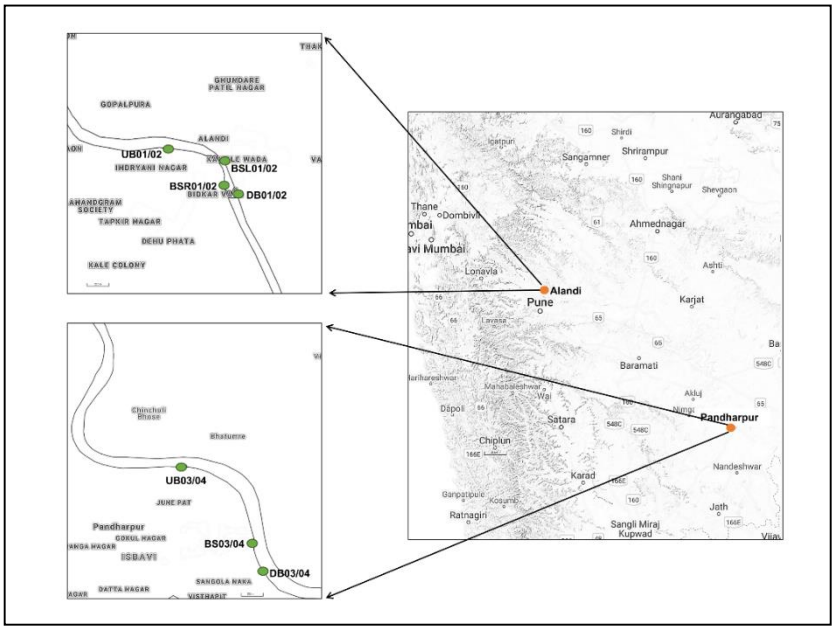

Fig. 3.1. Map illustrating the sampling locations. The water samples were collected from the Indrayani River at Alandi (Bathing samples: UB01, BSL01, BSR01, and DB01; post bathing samples: UB02, BSL02, BSR02, and DB02) and from Bhima River at Pandharpur (Bathing samples: UB03, BS03, and DB03; Post bathing samples: UB04, BS04, and DB04).

3.2.2. DNA extraction, Library preparation, and metagenomic sequencing

The water was filtered in successive filtration processes in sterile conditions using a sterile water filter assembly. The coarse filtration of 500 ml of water was carried out using sterile 11µm pore-sized Whatman Grade 1 Qualitative Filter paper. The filtrate collected was further subjected to filtration using a sterile 0.4 µm PES membrane filter (Millipore Express). The resultant filtrate was re-filtered using a 0.22 µm PES membrane filter (Millipore Express). The filter paper was stored at -80° C in a sterile Petri plate until DNA extraction. The DNA extraction was carried out using a DNeasy PowerWater Kit, Qiagen, as per the protocol mentioned by the manufacturer. The DNA quality and concentrations were checked by Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific) followed by its qubit analysis on Qubit fluorometer (Thermo Fisher Scientific) using Qubit High sensitivity (HS) assay kit (Thermo Fisher Scientific Q32851, respectively). The DNA was further stored at -80° C until further processing.

DNA having a purity of 1.7 to 1.9 (260/280) was used for library preparation. The library preparation was initiated with 1.1 µg/48 µl DNA using 1D Ligation sequencing kit SQK-LSK109. Briefly, the following protocol was followed:

a. End-repair was performed using 3.5µl of each NEBNext FFPE repair buffer, Ultra II End-prep buffer, 2µl of FFPE repair mix and 3µl of Ultra II End-prep enzyme mix. The reaction was thermally cycled at 20°C for 10 minutes and 65°C for 10 minutes.

b. 2.5µl of NBD104 and 114, 25µl of Blunt/TA Ligase Master Mix was mixed with 600ng (Qubit estimated) of repaired DNA for barcoding (incubated at 28°C for 10 minutes).

c. Lastly, all the barcoded samples were pooled (each 100ng) and were mixed with 8µl of Adapter mix II (AMII) and 60µl Blunt/TA ligase (incubated at 28°C for 10 minutes). The library was eluted by elution buffer (EB) at 37°C for 10 minutes.

Each step mentioned above was followed by DNA purification using 60µl of AMPure beads and 70% ethanol on a Magnetic Separation Rack (New England BioLabs, #S1509S).

3.2.3. MinION data processing and analysis and statistical analysis

The base-calling was conducted using Albacore (v2.3.4), and demultiplexing was performed using qcat (v1.0.1). The adapter trimming from the end and the middle of the reads were carried out using Porechop (v0.2.4). Kaiju (Menzel, 2016) was used for the taxonomic classification. NanoARG (Arango-Argoty et al., 2019) was used for the prediction of Antibiotic resistance genes (ARGs) and Mobile genetic elements (MGE). NanoARG uses DIAMOND for the alignment of reads and bedtools to cluster the local best hits into regions (Arango-Argoty et al., 2020). We used Virulence factors database (VFDB) (Chen et al., 2005) for mapping of virulence genes and pathogens. Briefly, we used DIAMOND (Buchfink et al., 2015) for the alignment with a parameter of (evalue of 0.00001, --more-sensitive, --identity of 60%). MG-RAST (Glass et al., 2010) with E-value cut-off =1×e−5 and sequence identity of 60% using the KEGG database was used for functional annotation. Furthermore, R packages such as phyloseq (McMurdie & Homes, 2013), microbiome (Lahti & Shetty, 2017), and MicrobiomeAnalyst (Dhariwal et al., 2017) were used for generating alpha diversity, beta-diversity plots and other statistical analysis. All the data were rarefied before comparison using QIIME. Percent difference was calculated by dividing the differences between the two values with the mean of two values followed by multiplication with hundred. The data-sets supporting the conclusions of this article are available in the MG-RAST in the project entitled as “Wari_2019”. Following are the MG-RAST Ids for the metagenome samples (Sample ID-MGRAST ID): DB01-mgm4869419.3, UB02-mgm4869420.3, BSL02- mgm4869421.3, DB03- mgm4869422.3, BS04- mgm4869423.3, DB04- mgm4869424.3, BSR02- mgm4869425.3, BSR01-mgm4869426.3, BSL01- mgm4869427.3, UB01- mgm4869428.3, BS03- mgm4869429.3, UB03- mgm4869430.3, UB04- mgm4869431.3, DB02- mgm4869432.3

QIIME was used for data rarefaction (Caporaso et al., 2010). Shapiro-Wilk normality test (p-value threshold of 0.05), followed by the confirmation using a Q-Q plot, was used to assess the data distribution. The Bray-Curtis dissimilarity measure was used to examine beta diversity, and statistical significance was checked using the Adonis and Permanova test (p-value threshold of 0.05). STAMP (Statistical Analysis of Metagenomic Profiles) (Parks et al., 2014) was used for analyzing the differentially abundant significant features between bathing and post-bathing samples. Here, we used Welch's t-test (p-value threshold of 0.05) for normally distributed data and White's non-parameter test (WP test) (p-value threshold of 0.05) for the data which was not normally distributed. Furthermore, the statistical significance of the alpha diversity data between bathing and post-bathing samples was checked using the Kruskal-Wallis test (p-value threshold of 0.05) and t-test (p-value threshold of 0.05).

3.3. Results and Discussion

Around 18 GB of base-called data with an average quality score and length of 8.75 and 4 Kb, respectively, was generated after MinION-based shotgun sequencing. Overall, 70% of total reads were classified using the nr_euk database by employing Kaiju, among which ~93 % were assigned to bacteria, and 0.8% were assigned to archaebacteria. The overall read statistics are listed in Table 3.1. **Table 3.1.** describes the read statistics for each sample

3.3.1. Influence of mass bathing on the microbial community

In the bathing samples of Bhima River, the *Actinobacteria* (Welsch's two-sided t-test, p-value <0.05) (mean%±Standard deviation%) (39.93±5.29) were highly prevalent, followed by *Proteobacteria* (21.60±1.85), *Planctomycetes* (10.66±1.87), *Bacteroidetes* (5.2±0.62) (Fig. 3.2A). The bathing samples of Indrayani possessed significantly higher prevalence of *Proteobacteria* (72.21±16.07) (WP test, p-value <0.05), however lower (48.11%) at the upstream site (UB01), followed by *Bacteriodetes* (6.43±5.86), *Firmicutes* (4.45±0.50) (WP test

p-value<0.05), and *Actinobacteria* (4.81±4.39) (Fig. 3.3.A). in the post-bathing samples, we found a significant increase (WP test, p-value<0.05) in the population of *Actinobacteria* (17.76±1.33), *Verrucomicrobia* (9.99±1.55) and *Planctomycetes* (2.21±0.19). A higher enrichment of *Rhodocyclaceae* (33.61±6.96) at the BSL01, BSR01 and DB01 were observed at the family level of classification. In contrast, there was a decrease in its prevalence (0.47±0.06) in post-bathing samples of the Indrayani River (Fig. 3.3B). The bacterial family such as *Commamonadaceae* was relatively higher in the post-bathing samples of both the rivers. The *Mycobacteriaceae* (21.14±3.85) was the most dominant group in the bathing sample. In contrast, its prevalence slightly decreased (10.54±1.24) in the post-bathing samples of Bhima River (Fig. 3.2B).

Sample ID	River	Events	Classified Reads	Unclassified Reads	Reads assigned to Bacteria	Reads assigned to Archaebacteria
UB01	Indrayani	Bathing	175346	203318	150836	2290
BSL01	Indrayani	Bathing	157191	45890	153431	680
BSR01	Indrayani	Bathing	229414	86361	222734	963
DB01	Indrayani	Bathing	160026	24063	157721	470
UB02	Indrayani	Post-bathing	85764	30705	79413	1041
BSL02	Indrayani	Post-bathing	143692	77660	133748	963
BSR02	Indrayani	Post-bathing	132126	57065	122726	1841
DB02	Indrayani	Post-bathing	155748	55831	145179	1358
UB03	Bhima	Bathing	148798	53349	137139	1095
BS03	Bhima	Bathing	168443	47403	154159	1203
DB03	Bhima	Bathing	174576	65399	158451	1395
UB04	Bhima	Post-bathing	113741	58727	106375	816
BS04	Bhima	Post-bathing	85823	35972	76414	814
DB04	Bhima	Post-bathing	21924	5173	19750	194

Fig. 3.2A.

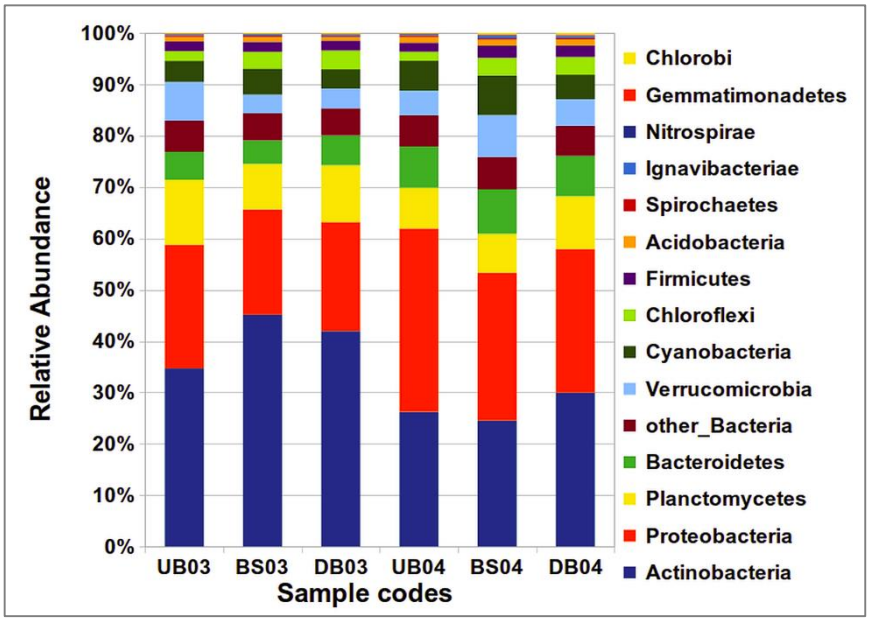

Fig. 3.2B.

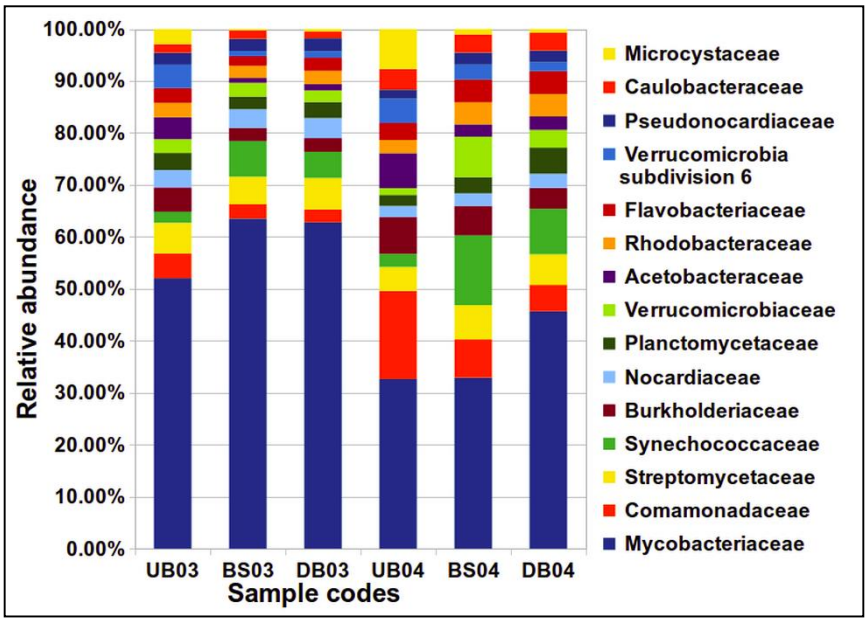

Fig 3.2. Bacterial community pattern of Bhima River during Wari. A. Bacterial Phyla in Bhima River. **B.** Bacterial family in Bhima River. (UB01, DB01, BSL01, BSR01, UB03, BS03, DB03 are Bathing samples of respective rivers whereas UB02, DB02, BSL02, BSR02, UB04, BS04, and DB04 are Post-bathing samples of respective rivers).

Fig. 3.3A.

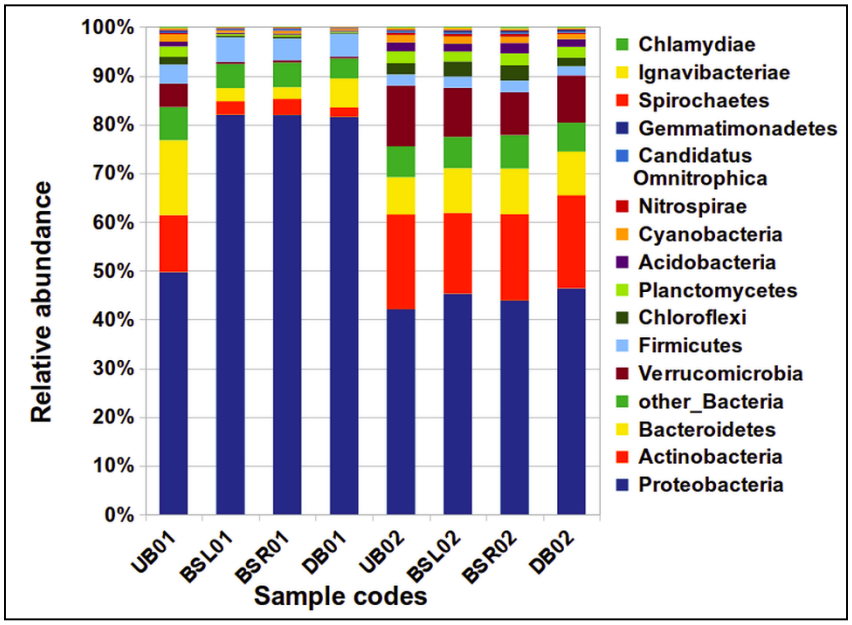

Fig. 3.3B.

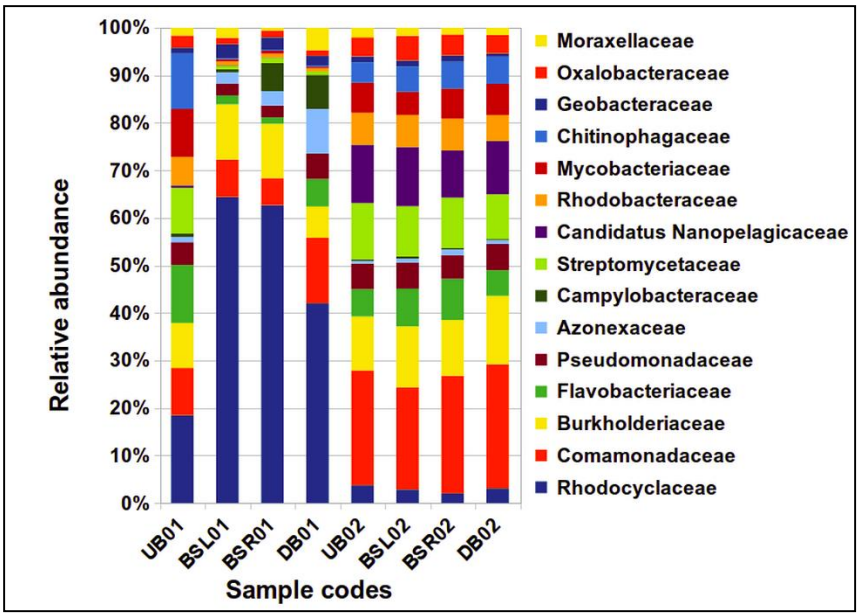

Fig. 3.3. Bacterial community pattern of Indrayani River during Wari. A. Bacterial phyla in Indrayani River. **B.** Bacterial family in Indrayani River. (UB01, DB01, BSL01, BSR01, UB03, BS03, DB03 are Bathing samples of respective rivers whereas UB02, DB02, BSL02, BSR02, UB04, BS04, and DB04 are Post-bathing samples of respective rivers).

The members of *Euryarchaeota* were the most abundant (55.98±3.93, 65.60±4.77 and 65.93±2.14, 64.59±4.90) archaeal phylum during bathing as well as the post-bathing event in Bhima and Indrayani River, respectively (Fig. 3.4A and B). The *Candidatus Bathyarcheaota* and *Thaumarcheota* were other considerable archaeal phyla prevalent during bathing and post-bathing in both rivers.

Fig. 3.4A.

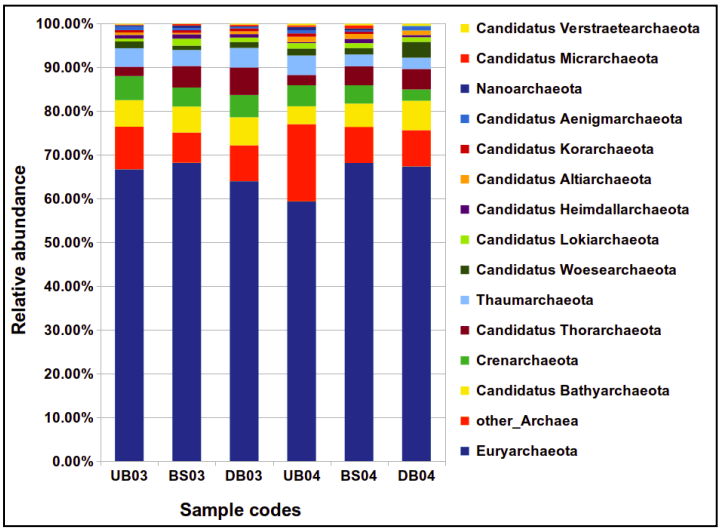

Fig. 3.4B.

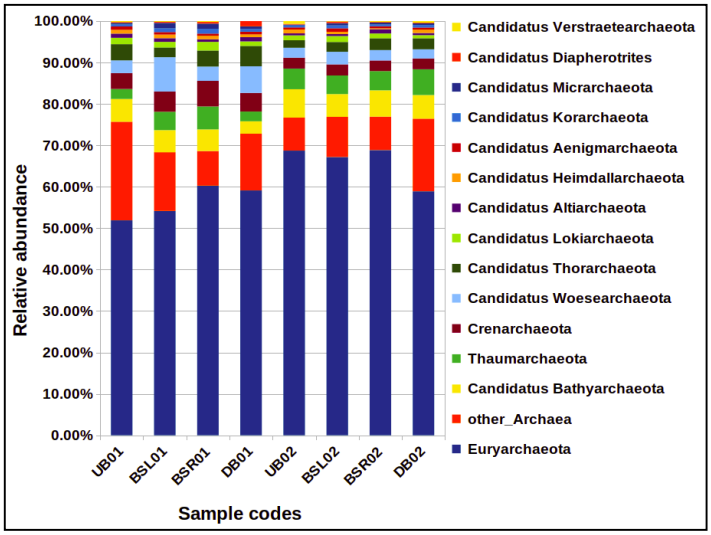

Fig. 3.4. Archaebacterial community pattern a phylum level. A. in Bhima River and 1B. in Indrayani River. (UB01, DB01, BSL01, BSR01, UB03, BS03, DB03 are Bathing samples of

respective rivers whereas UB02, DB02, BSL02, BSR02, UB04, BS04, and DB04 are Post-bathing samples of respective rivers).

Anthropogenic activities and the pollutants profoundly impact the overall microbial ecology of the riverine system (Jani et al., 2018b; Li et al., 2018). Significant variability in the abundance of predominant bacterial populations, such as *Actinobacteria, Proteobacteria, Firmicutes,* and others, was observed between both rivers' bathing and post-bathing samples. Moreover, a higher prevalence of skin and gut-associated bacterial phyla, such as *Actinobacteria, Proteobacteria, Firmicutes*, and *Bacteriodetes*, was recorded in the bathing samples, implying the influence of bathing as well as open defaecation observed during Wari (Jani et al., 2018b; Grice & Segre, 2011; Thursby & Juge, 2017). The sites under study are religiously significant and primarily witness gatherings throughout time, making them more vulnerable to anthropogenic actions. However, the number of pilgrims surged dramatically during the Wari event, making the ecological perturbations more obvious. Thus, the rise in *Rhodocyclaceae* members and the decline in the common freshwater *Commamonadaceae* could be ascribed to anthropogenic disturbances that result in increased carbon loading (Moon et al., 2018; Balmonte et al., 2016). Additionally, the increase in *Mycobateriaceae*, a group containing many known human pathogens (Malhotra et al., 2017), further implicates the ill effects of such mass bathing events.

3.3.2. Influence on microbial diversity

Around 18.45% and ~6.78% (Shannon indices), differences in the bacterial diversity between the bathing and post-bathing samples of the Indrayani and Bhima Rivers, respectively, were recorded. The bacterial population showed significantly lower (t-test, p-value<0.05) (Fig. 3.5A), while the archaebacteria displayed higher diversity during the time of bathing in the Bhima River (Figs. 3.6A and 3.6B). Similarly, observations were recorded (Fig. 3.5C) during the bathing event in Indrayani River (Supplementary Fig. 3.6B). Notably, spatially, sites

(UB01, UB03) had relatively higher bacterial diversity in both rivers. Furthermore, we observed significant differences in the microbial communities (Figs. 3.5B and 3.5D; Supplementary Figs. 3.6B and 3.6D) (significant for bacteria and archaebacteria from Indrayani River, Permanova (Adonis test) p-value<0.05, betadisper p-value>0.05).

Fig 3.5A.

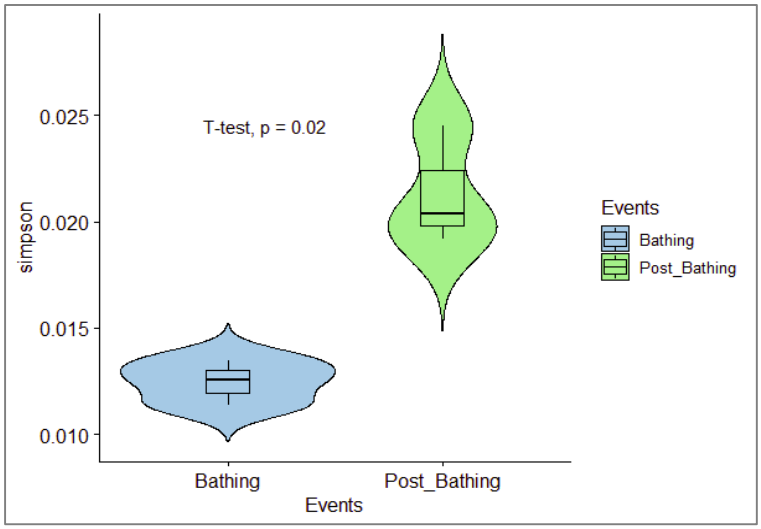

Fig. 3.5B.

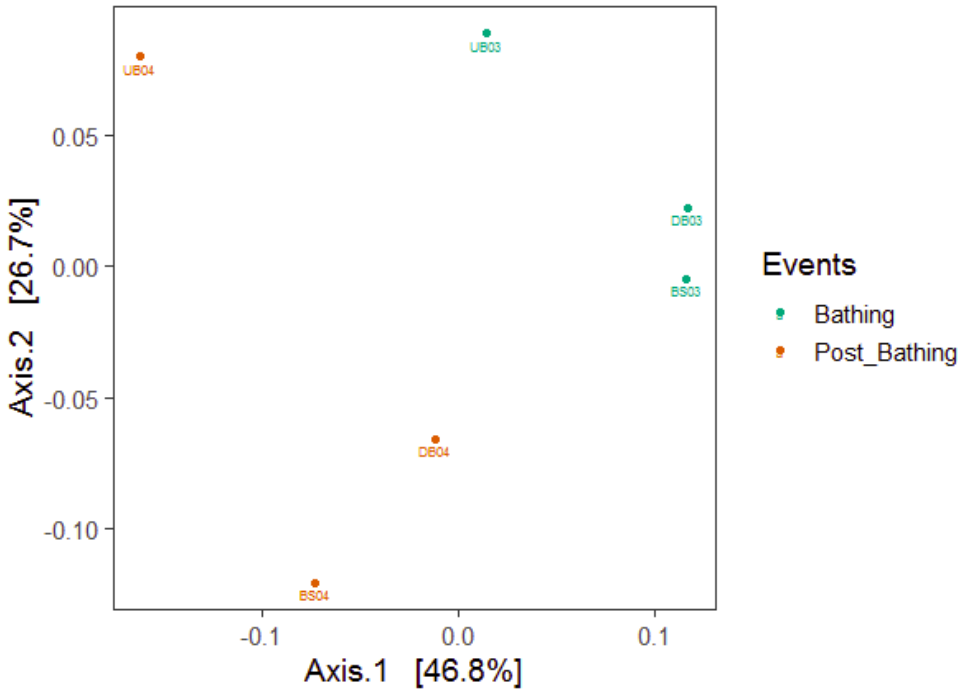

Fig 3.5C.

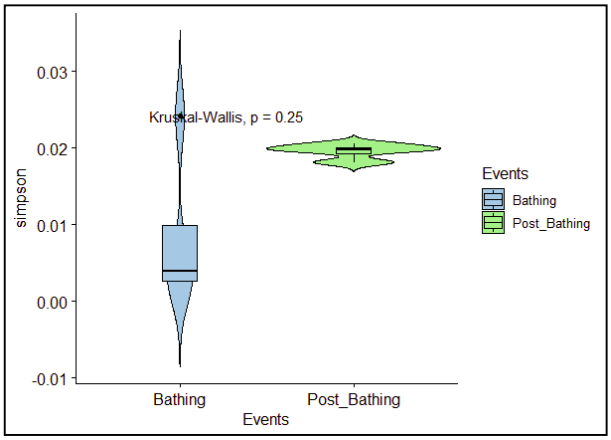

Fig 3.5D.

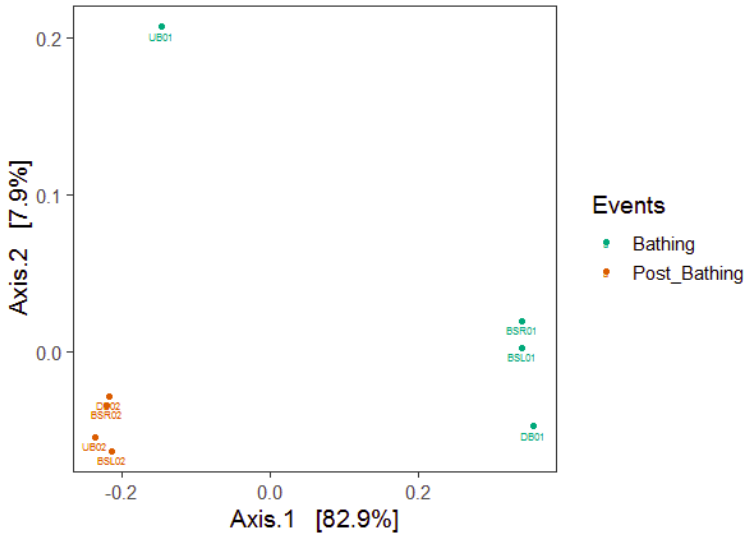

Fig. 3.5. Spatiotemporal variations of Bacterial Community. A and C..Violin plot depicting alpha diversity between bathing and post-bathing samples of Bhima River (t-test p-value <0.05) and Indrayani River (Kruskal-Wallis test, p-value>0.05) respectively. B and D. Beta diversity estimation (PCoA plot using Bray-Curtis dissimilarity matrix) of River Bhima (Adonis test and Permanova p>0.05) and Indrayani (Adonis test and Permanova p<0.05) respectively during Wari.

Fig. 3.6A.

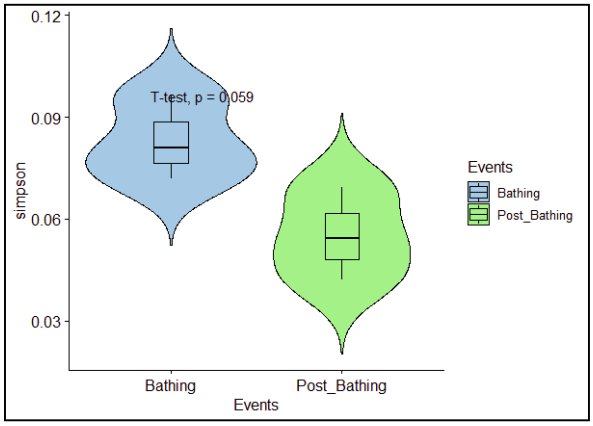

Fig. 3.6B.

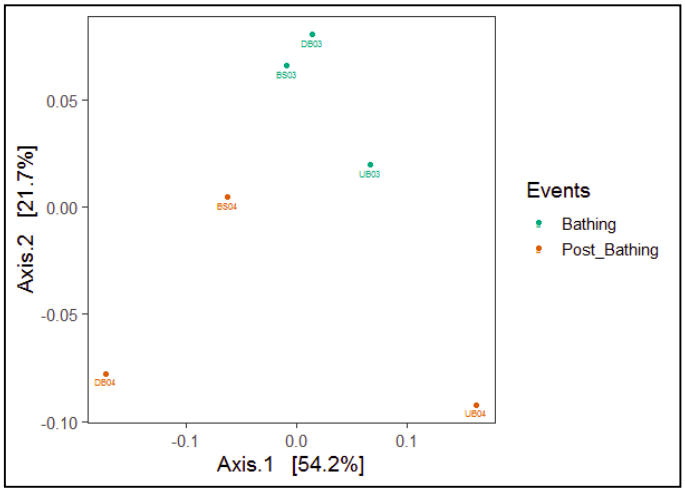

Fig. 3.6C.

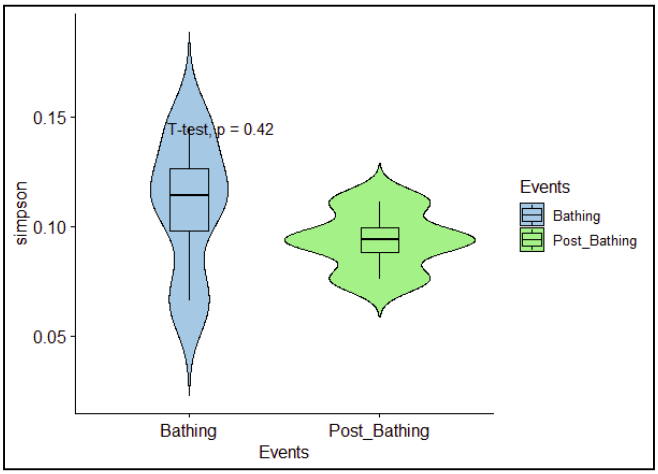

Fig. 3.6D.

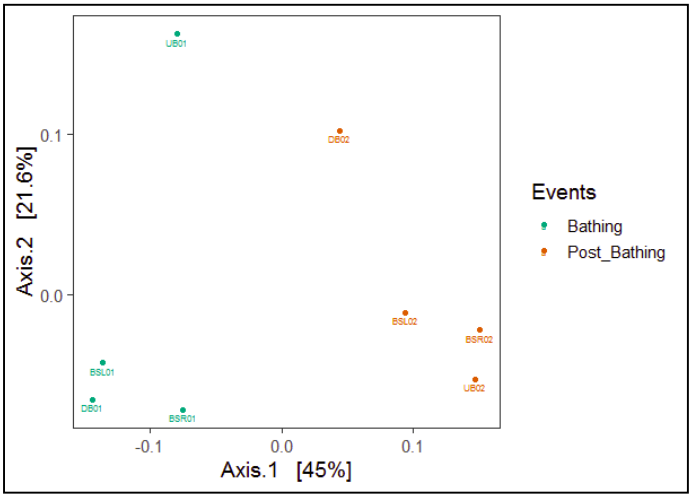

Fig. 3.6. Spatiotemporal variations of Archaebacterial Community. A and C. Violin plot depicting alpha diversity between bathing and post-bathing samples of Bhima River (t-test p-value <0.05) and Indrayani River (Kruskal-Wallis test, p-value>0.05) respectively. **B and D.** Beta diversity estimation (PCoA plot using Bray-Curtis dissimilarity matrix) of River Bhima (Adonis test and Permanova p>0.05) and Indrayani (Adonis test and Permanova p<0.05) respectively during Wari.

Further, in-silico functional gene analysis using the MG-RAST tool and the ordination analysis (Fig. 3.7A and 3.7B) revealed functional dissimilarity for the bathing and post-bathing samples of Indrayani (Permanova (Adonis test) p-value<0.05, R2=0.45 and betadisper p-value>0.05) and Bhima (Permanova, p>0.05).

When it comes to aquatic ecosystems, a dynamic and vulnerable ecosystem, anthropogenic stress has a wide range of consequences on the microbial structural pattern and their functionality (Nogales et al., 2011; Won et al., 2017). Jani et al., 2018a have reported decreased bacterial richness and diversity during the Kumbh Mela. Similarly, the present analysis detected notable alterations in the alpha diversity during the bathing and post-bathing events, bathing samples of both rivers displayed significantly lower bacterial diversity. Moreover, we observed higher differences in the rivers' overall microbial richness and diversity between the bathing and post-bathing samples. Additionally, higher diversity in the upstream

samples (UB03, UB01) could be attributed to lesser usage of these sites during mass bathing. Further, the beta diversity analysis unveiled the critical impact of mass bathing.

Fig. 3.7A.

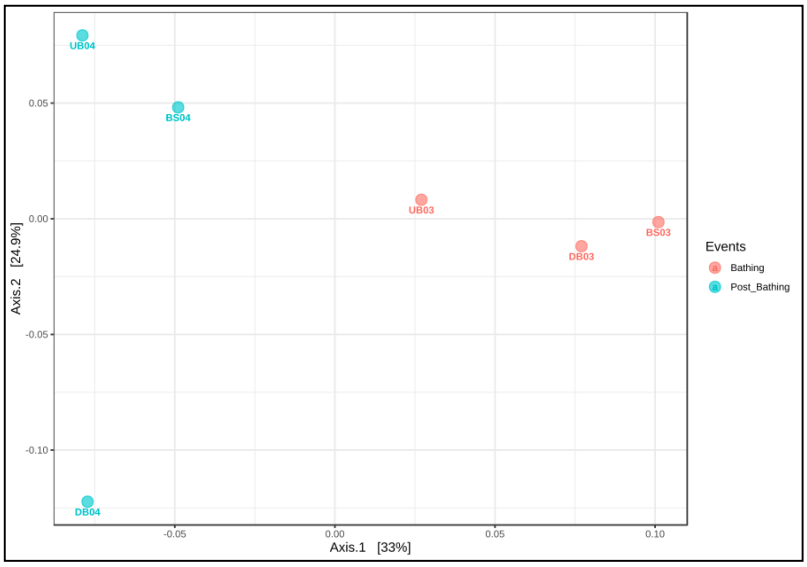

Fig. 3.7B.

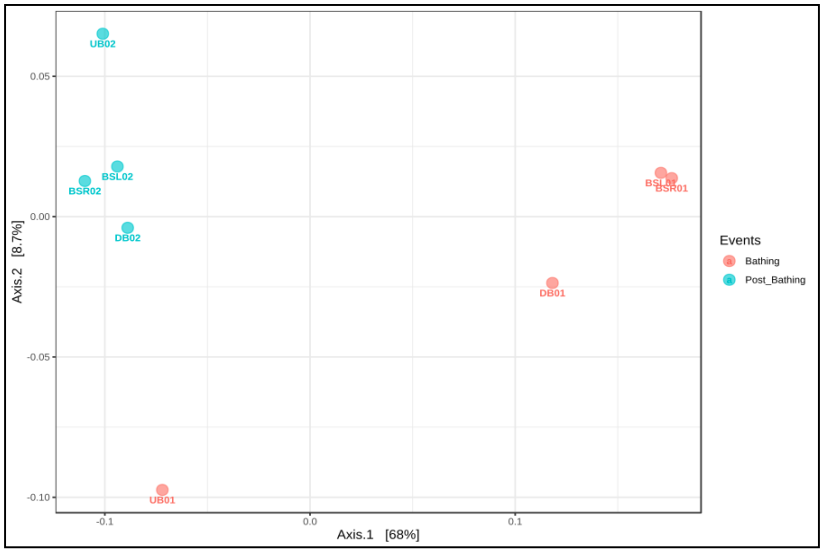

Fig. 3.7. PCoA plot explaining the variations in the functionality A. and B. Beta diversity of functional genes in Bhima and Indrayani River (Adonis test, $p<0.05$) respectively.

The higher R^2 value (0.58 and 0.48 for bacteria and archaebacteria, respectively) strongly suggested the influence of mass bathing. The alterations in the microbial community also lead to changes in the functional potentials of the riverine system. We observed substantial differences in the functionality of the river ecosystem during the mass bathing. The observed spatiotemporal alterations in the microbial community and functionality can be associated with the input of allochthonous microbes in these rivers during mass bathing.

3.3.3. Virulence and antibiotic resistance

The Virulence Factor Database (VFDB) was used to get insights into the alterations of virulence genes during the Wari event. The Permanova analysis suggested the crucial impact of mass bathing as significant changes ($p<0.05$, $R2=0.19$) were recorded in the virulence genes (VG) of the Indrayani River. Further, our analysis showed enrichment of VG mapping (ranging from 11% to 80% higher prevalence in bathing samples) (Fig. 3.8.) to pathogens such as *Acinetobacter baumannii*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* and the members of the *Enterobacteriaceae* group during the bathing event.

Fig. 3.8.

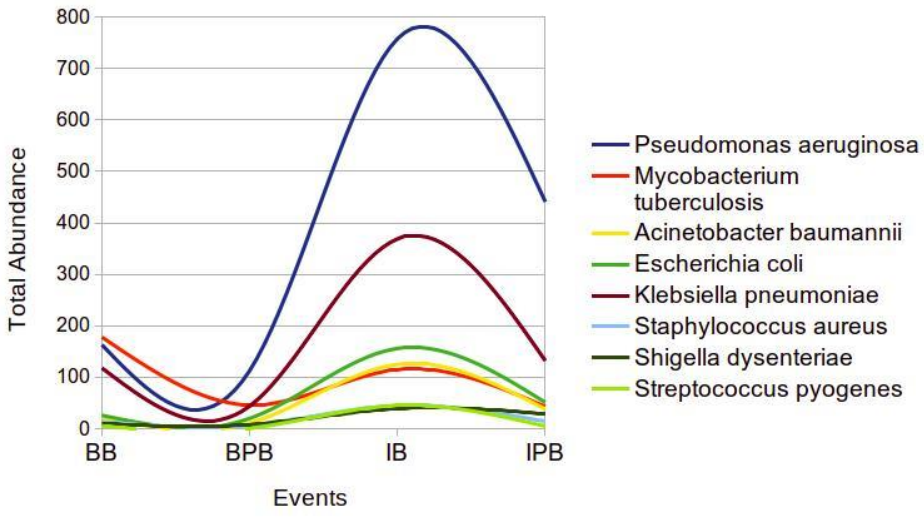

Fig. 3.8. Influence of mass bathing on the priority pathogens. BB- Bhima bathing samples; BPB- Bhima post-bathing samples; IB- Indrayani bathing samples; and IPB- Indrayani post-bathing samples.

The type of microbial community determines the water quality, as pathogen-containing water will have grave consequences on human health (Azzam et al., 2017). Previously, influenza outbreaks were reported during the mass gatherings of World Youth Day 2018 (Blyth et al., 2010). During such mass bathing events, millions of people interact, gargle, and directly contact the water. Thus, such events allow the infectious and multi-drug resistant pathogens to propagate. The significant dissimilarity in the virulence gene contents among the bathing and post-bathing samples indicates the addition of pathogens during the mass bathing. Since mass bathing involves the influx of a large number of pilgrims, we gave particular emphasis to the skin and intestinal disease-related pathogens. These virulence genes predicted were mapped to various global priority and ESKAPE pathogens such as *Acinetobacter baumannii, Staphylococcus aureus, Streptococcus pyogenes, Mycobacterium tuberculosis* and others, possessing the potential to cause multiple intestinal and skin-related infections (Asokan et al., 2019; Findley & Grice, 2014; Mulani et al., 2019). The finding confirms the increase in the potential of water-borne infections and displays the rivers' dismal conditions during mass bathing.

A resistance against 29 categories of antibiotics was predicted in the analysis (Fig. 3.9). The analysis revealed 142 and 333 antibiotic-resistant genes (ARGs) sub-types in Bhima and Indrayani Rivers, respectively, with the predominance of ARGs that provide resistance to the multiple drugs. The *rpoB2* (14.81±5.92 and 10.19±2.31), *ugd* (24.14±0.86 and 0.86±0.45), *bacA* (20.63±11.23 and 4.38±1.66), *penA* (21.42±12.75 and 3.59±1.43), *mdtC* (23.13±14.5 and 1.87±0.34), and *ompR* (18.45±9.93 and 6.55±1.0) were among the top abundant antibiotic

resistance genes predicted during bathing and post-bathing events in Indrayani River respectively.

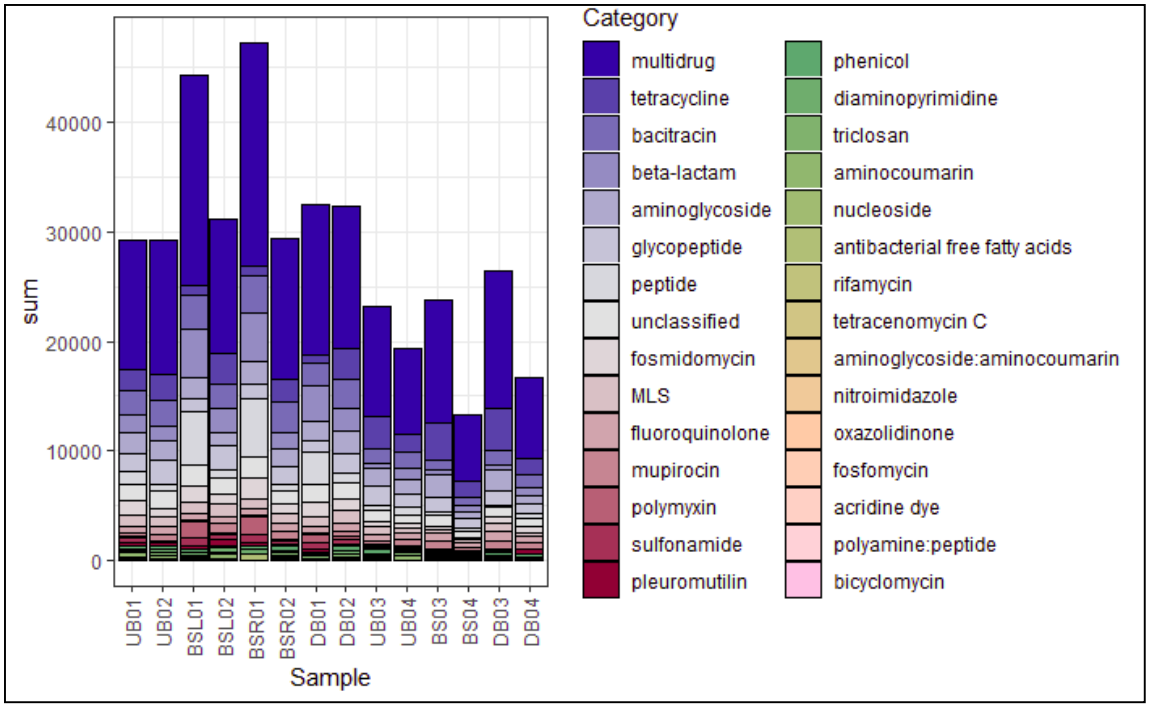

Fig. 3.9. Impact of mass bathing on Antibiotic resistance. The abundance of ARGs during bathing and post bathing in both the rivers.

Likewise, *rpoB2* (23.08±4.65 and 10.25±5.02), *efpA* (27.36±8.53 and 5.97±3.66), *tetA* (24.03±4.95 and 9.3±5.73), *tap* (27.10±9.70 and 6.22±3.53), and *ompR* (23.31±3.98 and 10.02±6.35) were among the dominant ARGs in the Bhima River during the bathing and post-bathing events in Bhima River, respectively. Moreover, the significant (Fig. 3.10A and 3.10B) variations of ARGs during the bathing and post-bathing in the Bhima and Indrayani Rivers (Permanova, Adonis $p<0.05$) indicate the allochthonous inputs of ARGs during such events. We also observed higher differences in MGEs (Fig. 3.11.), such as transposases, recombinase and integrases between the bathing and post-bathing samples of Bhima and Indrayani River, respectively. AMR is currently a dangerous threat to global health and is progressing at an unmatched pace (https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance).

Fig. 3.10A.

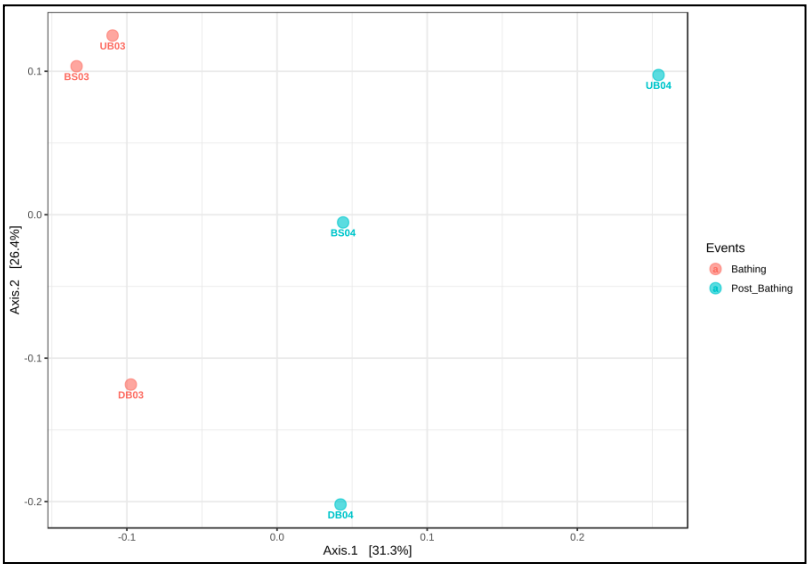

Fig. 3.10B.

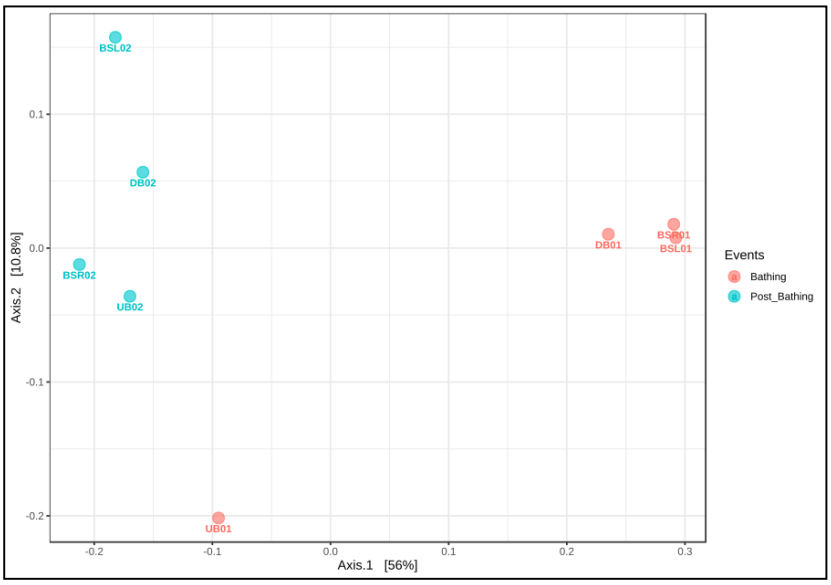

Fig. 3.10. Variations of ARGs during the bathing and post-bathing in the Bhima (A) and Indrayani Rivers (B).

Our analysis detected ARGs against all the clinically significant classes of antibiotics. The inflated levels of ARGs in the bathing samples further substantiated the impact of mass bathing in these rivers. The river ecosystem will be a suitable niche for interacting with these

MDR pathogens. As a result, it will facilitate the emergence and propagation of newer antibiotic resistance. When considering the AMR problem, it is also essential to consider the mobilome of the pathogens that amplify this antibiotic resistance via horizontal transfer of such ARGs (Stokes & Gillings, 2011). This notable increase of MGEs such as transposases, recombinase and integrases in bathing samples further exacerbates the antibiotic resistance problems during such bathing events. The mass bathing and the associated anthropogenic activities resulted in significant alterations in the overall microbial ecology and functionality.

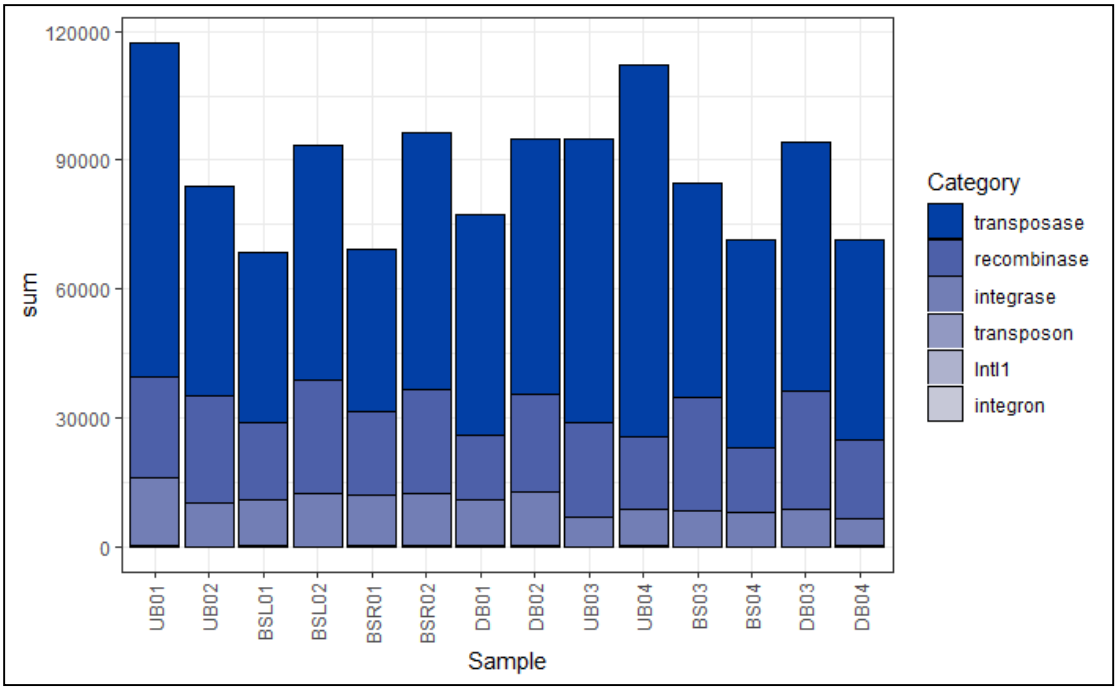

Fig. 3.11. Influence of mass bathing on Mobilome. (UB01, DB01, BSL01, BSR01, UB03, BS03, DB03 are Bathing samples of respective rivers whereas UB02, DB02, BSL02, BSR02, UB04, BS04, and DB04 are Post-bathing samples of respective rivers).

3.4. Conclusion

Wari is a unique religious event symbolizing unity that brings millions of devotees from different castes together. This comprehensive study aimed to unravel the microbial ecological changes in the river ecosystem and monitor the functional changes, mainly aiming at AMR. This information would help the government plan the upcoming Wari event more meticulously

to minimize river pollution due to open defecation and reduce the chance of infections. To our knowledge, this comprehensive shotgun-based study is the first report addressing the implication of mass gathering and bathing during the event of Wari. Overall, we observed changes in the levels of virulence genes and ARGs during the bathing event of Wari, which resulted in higher differences in their prevalence between the bathing and post-bathing samples. A higher prevalence of priority pathogens and their virulence genes during bathing further provides a potential opportunity for the pathogens to spread rapidly. Moreover, the spatiotemporal variations in the overall microbial ecology collectively illustrated the impact of mass bathing. The comparison with the post-bathing samples helped us to conclude about the ecological alterations occurring in the river during bathing. As we observed, post-bathing samples relatively recovered (lesser prevalence of ARGs, virulence and increase in the bacterial diversity compared to bathing samples) from the sudden impact of the mass bathing; however, the uncertainty remains. Therefore, our future studies would undertake the long-term monitoring of these rivers after the Wari event to delineate the nature of its impact on the riverine ecosystem. Altogether, our results emphasize a need for proper surveillance, planning and infrastructure developments to tackle the challenges of water pollution and risks associated with public health.

3.5. References

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Chapter 4. To map the microbiome and functional aspects of Water Hyacinth

Chapter 4. To map the microbiome and functional aspects of water hyacinth

Abstract

Water hyacinth (WH) is a perennial, widespread, free-floating intrusive aquatic plant with a prolific reproductive and distribution rate, covering entire water bodies and affecting overall biogeochemistry. Despite their unalterable significance, the WH microbiome remains unexplored in detail. Here, we conducted a shotgun sequencing analysis of WH root-associated microbiome from urban water bodies to reveal the diversity drivers and functional association. The beta-diversity analysis using PCoA plot with Bray-Curtis dissimilarity matrix indicated that the rhizobiome of the water hyacinths is significantly shaped by the type of the aquatic bodies (River vs lake) (ANOSIM-R of 0.94 to 0.98 and PERMANOVA ADONIS R^2 of 0.36 to 0.54). The higher ANOSIM R and ADONIS R^2 values suggested temporal variations (River WH_2020 vs WH_2022) river WH, which could probably be attributed to the transient taxa as there was a higher sharing of core bacteria (48%). Also, the WH rhizobiome significantly varied (R= 0.46 to 1.0 and R^2 of 0.18 to 0.52) from its water bodies. Furthermore, utilizing the REMEDB database, functional mining predicted 140 pollutant-degrading enzymes (PDEs) involved in the breakdown of diverse xenobiotic pollutants such as hydrocarbons, plastics, and aromatic dyes. The increased number of metal and biocide resistance genes emphasized the durability of resistant bacteria that aided WH in environmental cleanup applications.

4.1. Introduction

The commonly known Water hyacinth (WH, *Eichhornia crassipes, Pontenericeae*) is a rapidly growing intrusive gliding macrophyte that originated in South America's Amazon basin and has now been successfully introduced in almost all the continents (Ávila et al., 2019; Xu et al., 2022; Villamagna and Murphy et al., 2010). They form profuse quantities of thick, interlocking mats on the surface of various aquatic bodies such as rivers, wetlands, and lakes amid their higher reproductive rate, different modes of reproduction, and root structure (Ávila et al., 2019; Xu et al., 2022). Due to their extreme mat growth, they completely cover the aquatic bodies, hindering sunlight and oxygen penetration in the lower layers significantly affecting aquatic flora and fauna (Galgali et al., 2023). Strategies such as mechanical dredging and removal, chemical herbicides, and biological control agents, such as fungi, have been employed to prevent the proliferation of WH (Xu et al., 2022). These efforts are environmentally unsustainable and attract substantial economic costs.

Owing to their root adsorbing abilities and higher carbon and hydrogen contents, water hyacinths have been widely implicated as a phytoremediation agent to remove heavy metals, dyes and other recalcitrant pollutants (Li et al., 2021; Delgado et al., 1993; Madikizela et al., 2021). Aquatic plants are a promising ecological niche for microbial diversification and propagation, particularly the roots functioning as hot spots for microbial diversity. This rhizobiome plays an integral role in direct nutrient cycling, eradicating contaminants, denitrification and phosphate uptake, impacting dissolved oxygen and generally have a mutualistic relationship with the host plants (Ávila et al., 2019; Achá et al., 2005; Pramanic et al., 2023). Comprehending the WH-associated microbial population that significantly influences the overall nutrient cycling and biochemical process is critical. Previous studies have provided an understanding of these plant-associated microbes' role in reducing diverse pollutants and metals (Anudechakul et al., 2015; Achá et al., 2005). Further, a study by Luo et

al., 2015, also conveyed a synergistic connection of Fusarium with water hyacinth when exposed to heavy metals. Ávila et al., 2019, using 16S rRNA amplicon sequencing, thoroughly learned the prevailing structure and roles of the WH rhizobiome in Amazon and Pantanal wetlands, highlighting the spatiotemporal changes. Another 16S rRNA-based study by Sharma et al., 2021, gave an interpretation of the microbiome associated with WH of the Hindon River in India. These studies gave a synopsis of the WH microbiome and indicated putative functions based on the 16S rRNA study; however, the influence of the water and different aquatic bodies on the WH rhizobiome has not been investigated so far. Although the function of WH is well-known in environmental bioremediation, knowledge of WH-associated microbial genes and enzymes for xenobiotic metabolism is not yet reported.

In the current study, we sought to examine the effect of surrounding water (i.e. environment) on the WH microbiome, how the WH microbiome varies from two different types of aquatic bodies (Lake vs River water hyacinths) experiencing different ecological processes, the temporal deviations (changes in core and transient microbiome) in WH microbiome, and energy metabolism. Since WH is vigorously explored for environmental bioremediation, we also investigated the possibilities of the WH-associated microbiome to ascertain their function in aiding WH in remediation. The present shotgun metagenomic-based study was carried out to define the relationship of the WH microbiome with its surrounding environment, habitat, and role in the bioremediation of harmful pollutants. Eventually, this study will help us apprehend WH propagation in various aquatic bodies, specifically in urban rivers and lakes, which shall have marked socio-economic impacts.

4.2. Experimental

4.2.1. Sampling details

Major rivers in Pune, which include River Mula, Ramnadi, Mutha, Mula-Mutha and Pawana, traverse the urban settlements and are in deteriorated conditions. These rivers are lavishly covered with water hyacinths, providing breeding environments for mosquitoes, resulting in a spike in dengue cases (Removal of water hyacinth, Citizens and water hyacinth). The regional governing body in Pune recently declared around 500 million INR for tackling these invasive weeds (Removal of water hyacinth).

In the current study, WH and corresponding samples were collected from four sites (three river sites (River Mula and Ramnadi) and one Lake (Pashan) in Pune City, India. The locations were named A1 (Mula River, 18.565264, 73.788792), A2 (Ramnadi River, 18.548429, 73.79844), A3 (Mula River, 18.567607, 73.796593) and (Pashan Lake, 18.535551, 73.787922). The sampling was carried out in March 2020, and the follow-up sampling was taken out in 2022 in the same month from the same sites. The temperature in March in Pune city ranges from 30 to 35°C, and water hyacinth starts blossoming at these sites from January till the rainy season (June to August). During the first sampling, 11 WH samples were collected in sterile Hidispo bags (PW038, Himedia), triplicate from each site (A1, A2, A3, and WH), along with the four water samples (1 from each site) and were immediately sealed and transported in dry ice. Around 1 litre of water was collected in sterile polypropylene bottles. In the second sampling, we collected 8 WH samples (from three sites, A1, A2, and A3) and three corresponding water samples. The sample details are mentioned in (Table 4.1.). Due to WH's inaccessibility, we did not conduct sampling from Pashan Lake in 2022. The following are the abbreviations used from samples in this study: WH_2020 and WH_2022 (Water hyacinths sampled in Year 2020 and 2022, respectively); Water_2020 and Water_2022 (Water samples from river sites A1, A2, and A3 sampled in Year 2020 and 2022, respectively);

WH_Lake/LWH_20 (Water hyacinths sampled from Pashan Lake in 2020; Water_Lake (Water sample from Lake sampled in year 2020). A1, A2, and A3 are the riverine sites for 2020 and 2022. WH is an abbreviation for Water Hyacinth.

Table 4.1. The following table provides details of water hyacinth and water samples. WH= Water Hyacinth. Here, Mula and Ramnadi are rivers and Pashan is a lake.

Sr. No.	Sample codes	Group code	Sample type	Aquatic body	Broad codes	River/Lake	Year of sampling
1	A1_1_20	A1_20	WH	River	WH_2020	Mula	2020
2	A1_2_20	A1_20	WH	River	WH_2020	Mula	2020
3	A1_3_20	A1_20	WH	River	WH_2020	Mula	2020
4	A2_1_20	A2_20	WH	River	WH_2020	Ramandi	2020
5	A2_2_20	A2_20	WH	River	WH_2020	Ramnadi	2020
6	A2_3_20	A2_20	WH	River	WH_2020	Ramnadi	2020
7	A3_2_20	A3_20	WH	River	WH_2020	Mula	2020
8	A3_3_20	A3_20	WH	River	WH_2020	Mula	2020
9	A1W_20	A1W_20	Water	River	Water_2020	Mula	2020
10	A2W_20	A2W_20	Water	River	Water_2020	Ramnadi	2020
11	A3W_20	A3W_20	Water	River	Water_2020	Mula	2020
12	A1_1_22	A1_22	WH	River	WH_2022	Mula	2022
13	A1_2_22	A1_22	WH	River	WH_2022	Mula	2022
14	A1_3_22	A1_22	WH	River	WH_2022	Mula	2022
15	A2_1_22	A2_22	WH	River	WH_2022	Ramandi	2022
16	A2_2_22	A2_22	WH	River	WH_2022	Ramnadi	2022
17	A2_3_22	A2_22	WH	River	WH_2022	Ramnadi	2022
18	A3_2_22	A3_22	WH	River	WH_2022	Mula	2022
19	A3_3_22	A3_22	WH	River	WH_2022	Mula	2022
20	A1W_22	A1W_22	Water	River	Water_2022	Mula	2022
21	A2W_22	A2W_22	Water	River	Water_2022	Ramnadi	2022
22	A3W_22	A3W_22	Water	River	Water_2022	Mula	2022
23	LWH1_20	LWH_20	WH	Lake	WH_Lake	Pashan	2020
24	LWH2_20	LWH_20	WH	Lake	WH_Lake	Pashan	2020
25	LWH3_20	LWH_20	WH	Lake	WH_Lake	Pashan	2020
26	LW_20	LW_20	Water	Lake	Water_Lake	Pashan	2020

4.2.2. Sample processing for DNA extraction

The roots were cleaned with sterile distilled water (DW) and cut into a uniform size of 2 cm, followed by gentle washing in sterile DW twice to remove loosely bound microbial cells. These roots were further crushed in the sterile mortar and pestle. Around 250 mg of the crumbled roots were processed for DNA extraction using DNeasy PowerSoil Pro Kit (Qiagen, 47014) per the manufacturer's instructions. The water samples were centrifuged at 7000 rpm/5

minutes to remove coarse suspended particles. Further, the samples were successively filtrated, first using a 0.4 uM pore size filter, followed by 0.22 uM filter paper (PES membrane filter, Himedia). The 0.22 uM filter was used for DNA extraction using the RNeasy Power water kit (Qiagen, 14700) as per the manufacturer's instructions. The resultant DNA was analysed for purity (260/280) using NanoDrop (ThermoFischer NanoDrop Lite spectrophotometer), and concentration was estimated using Qubit dsDNA HS Assay Kit (Invitrogen, Q32851) using Qubit Fluorometer (Qubit 4 Fluorometer, Invitrogen).

4.2.3. Library preparation for DNA sequencing

1D Ligation sequencing kit SQK-LSK-109 was used for library preparation. Barcoding was carried out using EXP-NBD104 and NBD114. The library was prepared per the manufacturer's instructions with the following modifications. The initial DNA concentration was 1.1ug/48ul for End-repair. The end-repair was performed at 20°C for 20 minutes, 65°C for 10 minutes and held at 4°C. The adapter and barcode ligation step was extended for 10-minute incubation than was mentioned in the procedure. Qubit was used to quantify the resultant library, and 450 ng was loaded onto FLO-MIN-106D, R9 Version.

4.2.4. Data analysis and statistics

Basecalling and demultiplexing were performed using Guppy software (V 5.0.11) (Wick et al., 2019). Read trimming using a Q-score of 8 and a read length of 500bp was carried out using NanoFilt (v 2.7.1). The taxonomic classification was performed using the nr_euk database (released in Aril, 2020) using the Kaiju tool (Menzel et al., 2016) with default Greedy mode using an in-house customized script. Further, functions were annotated using the KEGG database in MG-RAST (Keegan et al., 2016). The RemeDB (Sankara Subramanian et al., 2020) was used for analyzing pollutant degrading enzymes (PDEs) using the DIAMOND alignment tool (v 0.9.26) (Buchfink et al., 2015) using parameters for long reads (e-value of 10-5, identity of 60, --long reads (-F 15, --range-culling, k 1), subject cover 40, --more-sensitive). Similarly,

the BacMet database (v 2.0) (Pal et al., 2014) was used for biocide and metal resistance genes (MRGs) using the same diamond parameters. For taxonomic classification of genes from BacMet and RemeDB, reads were extracted, and kaiju was used. The downstream data analysis was performed in the R program and R studio (Version). Microbiome and Phyloseq R packages were used (McMurdie & Holmes, 2013; Lahti & Shetty, 2017; Microbiome analysis). STAMP (v) (Parks et al., 2014) was used for differential abundance analysis, and plotting was done in GraphPad PRISM (8.0.2). The beta diversity analysis was performed using the Bray-Curtis distance method, and alpha diversity was analyzed using Shannon and chao1 indices. The data were normalized using Deseq2 normalization (Love et al., 2014). Further, ANOSIM and PERMANOVA using ADONIS-2 were used to interpret the statistics of beta diversity. The data was rarefied using the "rarefy_even_depth" function of phyloseq in R before alpha diversity calculation. The rarefaction of microbiome data still remains to be a topic of debate (McMurdie et al., 2014; Cameron et al., 2021). In the present study, normalized data was used for comparative analysis. For alpha diversity, we used the t-test and Wilcoxon test, depending upon the normality distribution of the data, which was performed in R using the Shapiro-Wilk normality test and Q-Q normalization plot. The differential abundance analysis in STAMP was performed for two groups using two-sided Welch's t-test, White's non-parametric test and ANOVA for multiple groups using the Benjamini–Hochberg FDR correction test. The differential abundant analysis was performed on rarefied data, which was rarefied using the "single_rarefaction.py" command in QIIME. The bacterial core was analyzed using a detection of 0.001 and a prevalence of 50% and was plotted using Venny (v 2.1.0). The R-scripts are provided in Supplementary information, and data is available with NCBI accession number PRJNA942025.

4.3. Results and Discussion

We used the nr_euk database to predict the microbial taxonomy pattern prevailing in the water hyacinth and corresponding environmental samples. Around 87% of the total reads were classified as bacterial, and ~1 % were mapped to archaebacteria. The details of the read distribution are stated in Table 4.2.

Table 4.2. The following table provide the details of total classified and unclassified reads after taxonomic classification by Kaiju using nr_euk database.

Sr. No.	Sample codes	Classified	Unclassified
1	A1W_20	114333	13844
2	A1W_22	49378	71167
3	A1_1_20	43854	27369
4	A1_1_22	73377	57338
5	A1_2_20	51899	35879
6	A1_2_22	42339	33142
7	A1_3_20	17695	8585
8	A1_3_22	48764	76276
9	A2W_20	45339	4443
10	A2W_22	232396	123263
11	A2_1_20	63395	52819
12	A2_1_22	147821	64386
13	A2_2_20	54521	37168
14	A2_2_22	64763	40147
15	A2_3_20	73577	52396
16	A2_3_22	41730	83081
17	A3W_20	68296	11797
18	A3W_22	83954	113734
19	A3_2_20	43969	15951
20	A3_2_22	164639	250970
21	A3_3_20	38670	26902
22	A3_3_22	68929	85046
23	LWH1_20	23889	195162
24	LWH2_20	44763	352037
25	LWH3_20	99416	395122
26	LW_20	145425	122904

The phylum level analysis revealed *Proteobacteria* (Mean relative abundance (MRA) % in WH_2020 and WH_2022) (54.78±3.2% and 61.58±35%) as the predominant phylum, followed by *Bacteriodetes* (9.6±1.195% and 6.85±1.14%), *Actinobacteria* (6.87±1.13% and 7.1±1.19%), *Firmicutes* (4.04±0.56% and 3.8±1.05%), *Chloroflexi* (3.22±1.44% and

3.21±1.46%), *Verrucomicrobia* (3.71±0.5% and 1.98±0.5%), *Cyanobacteria* (2.61±1.8% and 0.86±0.26%), *Planctomycetes* (2.0±0.36% and 2.50±1.17%), *Acidobacteria* (1.75±0.42% and 2.16±1.31%), and *Spirochaetes* (1.36±0.4% and 0.46±0.1%) in WH (Figure 4.1A.).

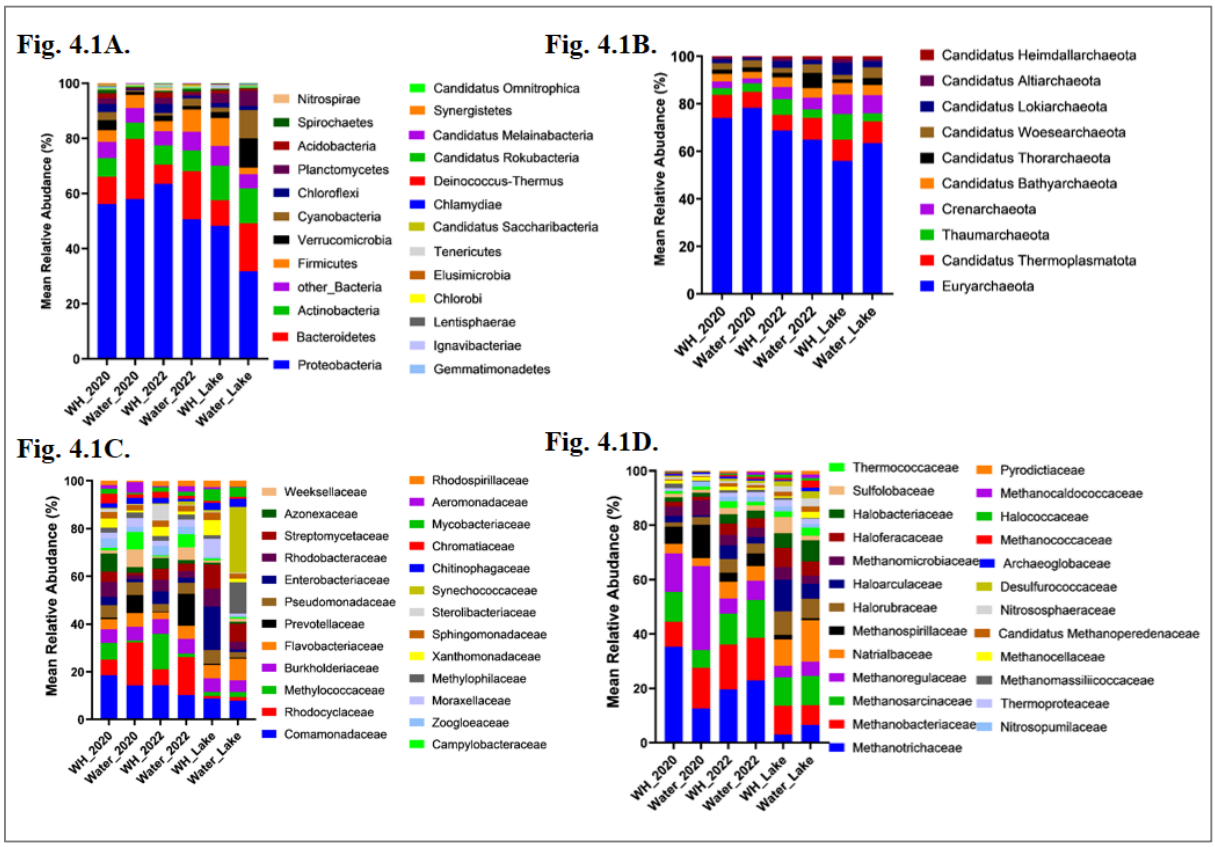

Fig. 4.1. Microbial community pattern. The figure illustrates the percent mean relative abundance. of **A**. Bacterial phyla (top25), **B.** Archaebacterial phylum, **C.** Bacterial families (top 25), and **D.** Archaebacterial families (top 25) in WH_2020 (Water hyacinths from rivers in 2020 sampling), WH_2022 (Water Hyacinths from rivers in 2022), Water_2020 (Water samples from river in 2020), Water_2022 (Water samples from river in 2022), WH_Lake (LWH_20) (Water Hyacinths from Pashan Lake in 2020), Water_Lake (LW_20) (Water from Pashan Lake in 2020).

The members of the phylum *Euryarchaeota* were the most prevalent (MRA 50% to 66%) in the WH of lake, river, and water samples. The other prominent phyla (MRA>1%) observed were *Candidatus Thermoplasmota, Thaumarchaeota, Crenarchaeota, Candidatus Bathyarchaeota, Candidatus Thorarchaeota, Candidatus Woesearchaeota, and Candidatus*

Lokiarchaeota. Methanomicrobia, Halobacteria (Figure 4.1B.), *Methanomicrobia, Thermoplasmata, Thermoprotei,* and *Thermococci* were most abundant at the class level (Fig. 4.2.).

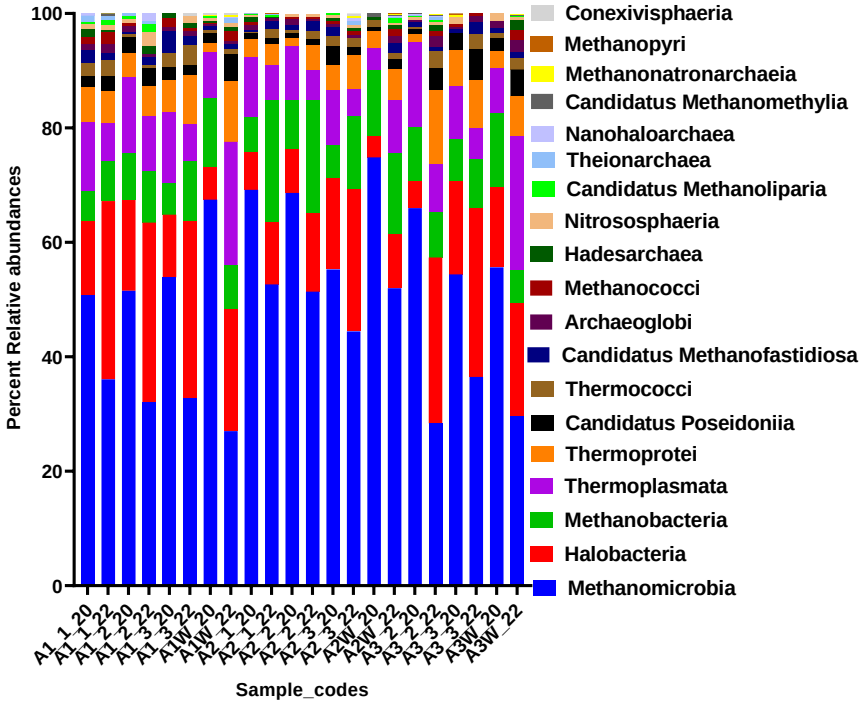

Fig. 4.2. The stacked bar illustrates top archaebacteria at class level

Substantial differences in the WH microbial diversity by the type of aquatic bodies (River and Lake), with its surrounding environment (water), and on a temporal scale were observed. Moreover, our analysis of WH rhizobiome also indicated their remediating ability and tolerance to biocides and metals. We observed multifarious bacterial communities associated with the river water hyacinth, particularly *Proteobacteria* being the most dominant, followed by *Bacteriodetes, Actinobacteria, Firmicutes, Chloroflexi,* and *Verrucomicrobia* (Figure 4.1.). A previous study on *E. crassipes* from Hindon River, India, by Sharma et al. 2021, observed similar numerically abundant bacterial taxa. In comparison to the WH from wetlands (Ávila et al., 2019), we observed a rather higher proportion of *Bacteroidetes* members (10%) in the river WH. Additionally, a higher prevalence of *Euryarchaeota* was observed in river WH, whereas,

in wetlands, the *Woesearchaeota* phylum was more dominant (Figure 4.1.). Overall, these differing observations can be attributed to the higher occurrence of these taxa in the rivers of Pune city (Yadav et al., 2021b), the effect of geography, and the difference in the type of aquatic ecosystems (Ávila et al., 2019).

4.3.1. Habitats drive the variance in the rhizobiome of Water Hyacinth

Lake WH showed a significantly higher preponderance of *Actinobacteria* and *Spirochaetes* but a rather lower *Proteobacterial* population than river WH (Welch's t-test, Benjamini–Hochberg FDR, $p<0.05$, Fig. 4.3.).

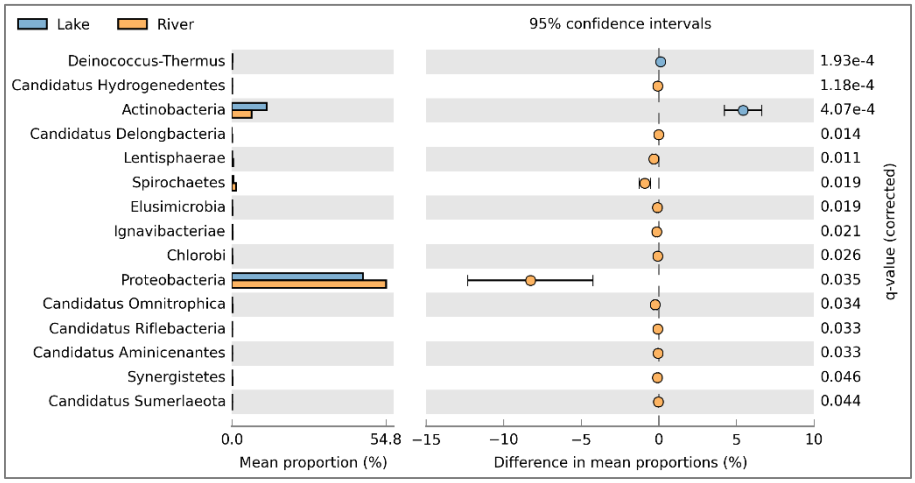

Fig. 4.3. Extended error bar plot showing differentially abundant bacterial phylum (Welch's t-test, Benjamini–Hochberg FDR, $p<0.05$) between Lake and River.

The lake-river comparison of WH rhizobiome showed a remarkably higher prevalence of *Cytophagia* and *Actinobacteria* in the lake and differential enrichment of *Betaproteobacteria* and *Deltaproteobacteria* in river WH. Further, Lake WH showed a very high prevalence of *Streptomycetaceae*, *Nocardiaceae*, *Rhizobiaceae*, *Rhodobacteraceae*, *Mycobacteriaceae*, and *Phyllobacteriaceae*. In disparity, *Azonexaceae*, *Methylococcaceae*, *Chromatiaceae*, *Rhodocyclaceae*, *Comamonadaceae*, *Zoogleaceae*, *Desulfobacteriaceae*, *Desulfobulbaceae*, and *Desulfovibrionaceae* were more prominent in river WH (Welch's t-test, Benjamini–Hochberg FDR, $p<0.05$).

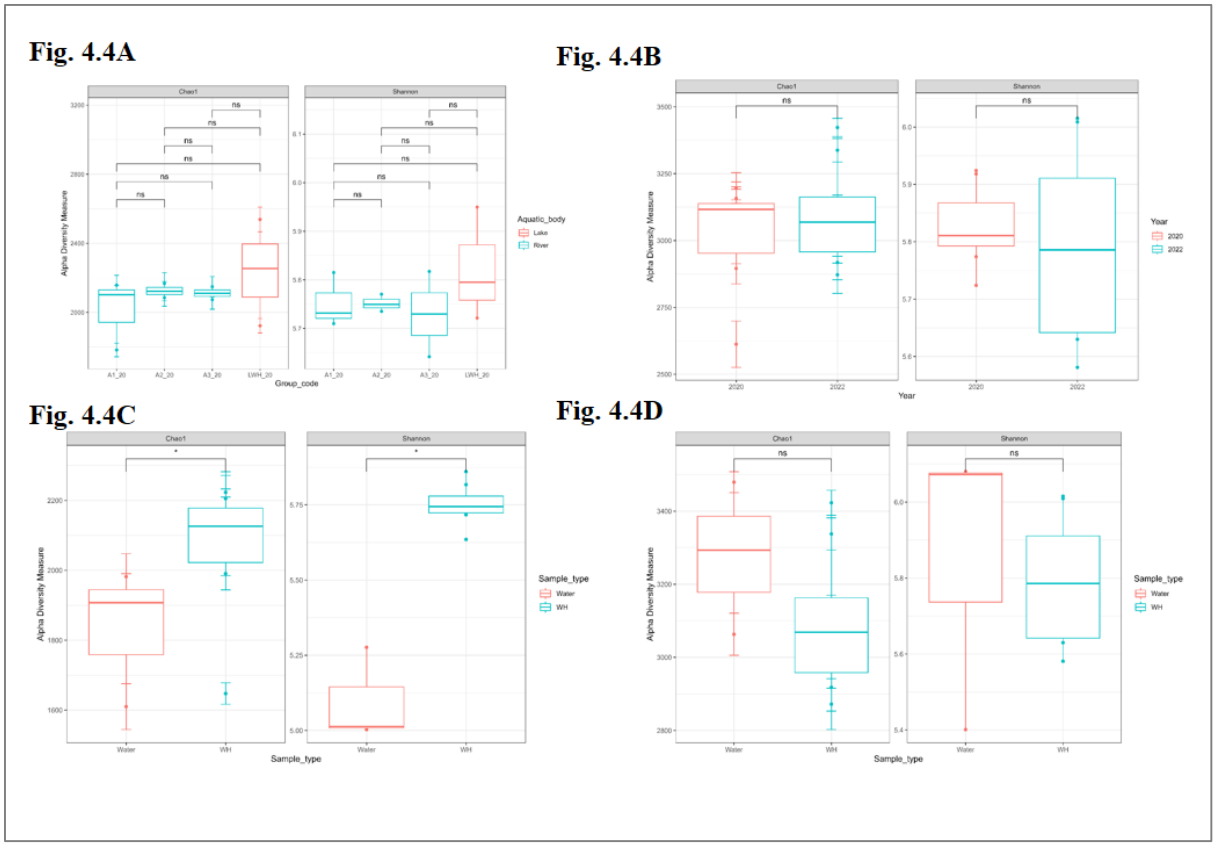

Fig. 4.4. Bacterial Alpha Diversity. Box plots illustrates the alpha diversity indices chao1 and Shannon of **A**. Alpha diversity between Lake WH and River WH (t-test, $p>0.05$), **B.** Alpha diversity of River WH_2020 and WH_2022 (t-test, $p>0.05$), **C.** Water hyacinth of 2020 and Water samples 2020 (Wilcoxon test, $p<0.05$) and **D.** Alpha diversity of WH_2022 and Water_2022 (t-test, $p>0.05$). A1_20, A2_20, A3_20 are water hyacinths from three riverine sites and LWH_20 are water hyacinth from Pashan Lake site.

The diversity contrasts were observed in the WH rhizobiome from the lake and river. Bacterial alpha diversity was more increased in Lake WH (WH_20) compared to the water hyacinths (A1_20, A2_20, A3_20) of rivers (Figure 4.4). Significantly more heightened microbial diversity differences were discerned after lake-river WH comparison (Fig. 4.5B and 4.5A). Although there was higher similarity in the core bacteria of Lake WH and River WH (37.8%), we also observed a highly unique (42.7%) core bacterial population in Lake water hyacinths (Fig. 4.6A.).

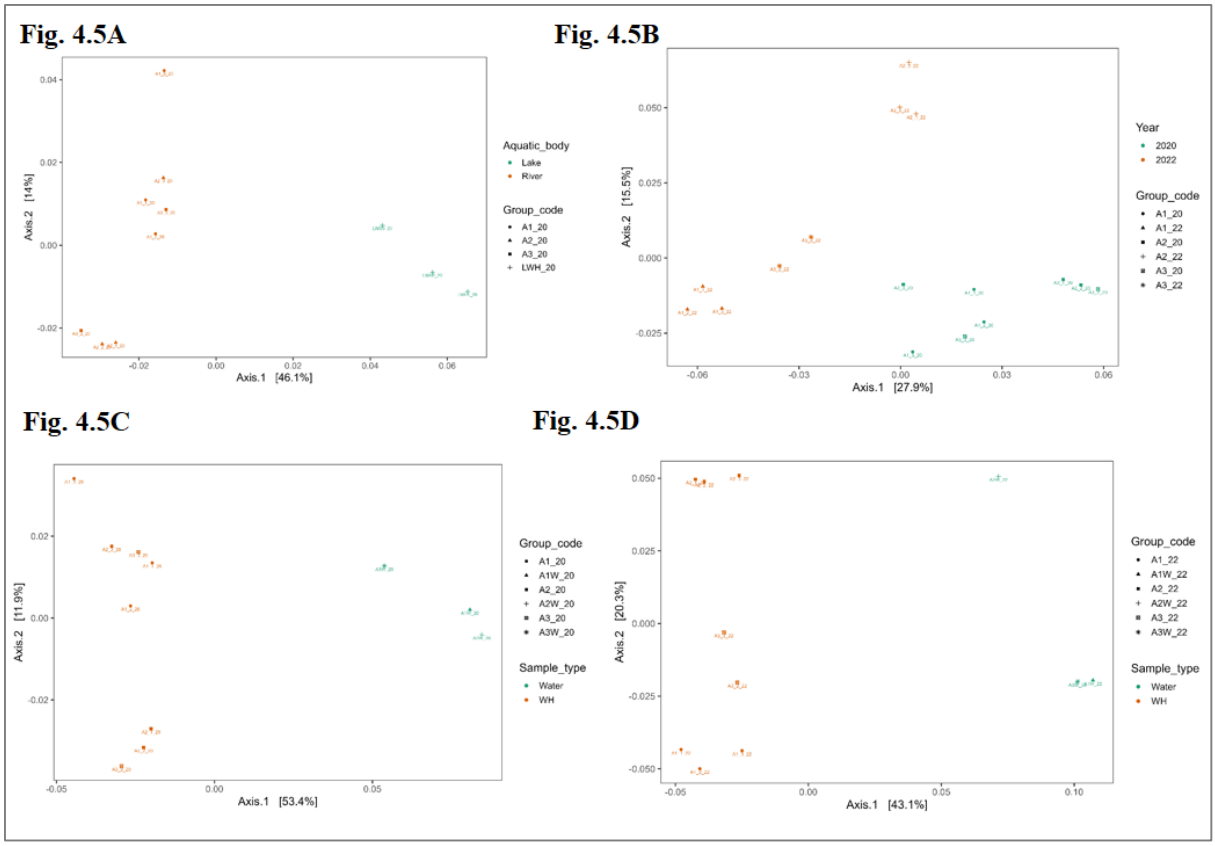

Fig. 4.5. Bacterial Beta Diversity. PCoA plots using Bray-Curtis distance. **A.** Between Lake WH (LWH_20) and River WH ($p<0.05$, ANOSIM R=0.98, PERMANOVA ADONIS R2 =0.58, Beta-disper >0.05). **B.** Between River WH_2020 vs WH_2022 ($p<0.05$, R=1, R2 =0.81, Beta-disper>0.05), **C.** River Water_2020 and WH_2020 ($p<0.05$, R=1, R2 =0.52, Beta-disper>0.05), **D.** River Water_2022 and WH_2022 ($p<0.05$, R=1, R2 =0.41, Beta-disper>0.05).

Opposing observations were noted in the microbiome of WH from Lake, where a remarkably higher enrichment of *Actinobacteria* and *Spirochaetes* and a lower abundance of *Proteobacteria* was observed than in the river water hyacinths (Welch's t-test, Benjamini–Hochberg FDR, $p<0.05$). Similar observations were recorded at the lower level of taxonomy, where bacterial families such as *Streptomycetaceae, Rhizobiaceae, Rhodobacteraceae, Nocardiaceae, Mycobacteriaceae,* and *Phyllobacteriaceae* were significantly (Welch's t-test, Benjamini–Hochberg FDR, $p<0.05$) enriched in Lake WH compared to river WH showing the differential abundance of *Azonexaceae, Methylococcaceae, Chromatiaceae, Rhodocyclaceae, Comamonadaceae, Zoogleaceae, Desulfobacteriaceae, Desulfobulbaceae,* and

Desulfovibrionaceae. Although insignificant, we observed more archaebacterial lineages such as *Natrialbaceae, Methanosarcinaceae,* and *Halorubraceae* in the Lake WH than in the river WH (Fig. 4.1D.).

Fig. 4.6. Venn diagram for shared and exclusive bacterial families. The core was calculated using detection 0.001 and prevalence of 50%. A. Core comparison between WH of lake and river, B. Core comparison between WH of 2020 and 2022, C. Core comparison of WH_2020 vs Water 2020, D. Core comparison of WH_2022 and water 2022

Moreover, the Lake WH microbiome displayed higher microbial alpha diversity than the River WH. Additionally, we observed significantly distinct clustering of the microbiome of Lake WH and River WH upon beta diversity estimation. The higher ANOSIM R-value and PERMANOVA R^2 value using the ADONIS-2 test (R-value and R^2 value for bacteria and Archaea (0.98, 0.57%) and (0.94, 0.36%), respectively) suggested water body type as a decisive explanatory variable for microbial shaping of water hyacinth (Fig. 4.5A.) Rivers and lakes are distinct habitats with different ecological processes, anthropogenic impacts, and multiple biotic interactions that determine the microbiological community (Tang et al., 2020). The core

microbiome analysis further substantiated the influence of habitats. Although the river and Lake WH shared 37 % of their core bacterial population, around 43% of the core bacteria were exclusive to Lake WH (Fig. 4.6.). Among these core bacteria, some of the dominant bacterial families unique to Lake WH were *Zhaonellaceae*, *Weeksellaceae*, *Synechococcaceae*, *Rhodospirillaceae*, *Pseudonocardiaceae*, *Cytophagaceae*, and others. Overall, these observations suggested the prominent influence of the aquatic bodies in determining the water hyacinth's microbial community; however, additional physicochemical studies are required to substantiate this.

4.3.2. Temporal variations visible in transient microbiome

Verrucomicrobia, *Bacteriodetes*, and *Spirochaetes* were significantly more elevated in WH 2020 compared to 2022 (Welch's t-test, Benjamini–Hochberg FDR, $p<0.05$, Fig. 4.7.).

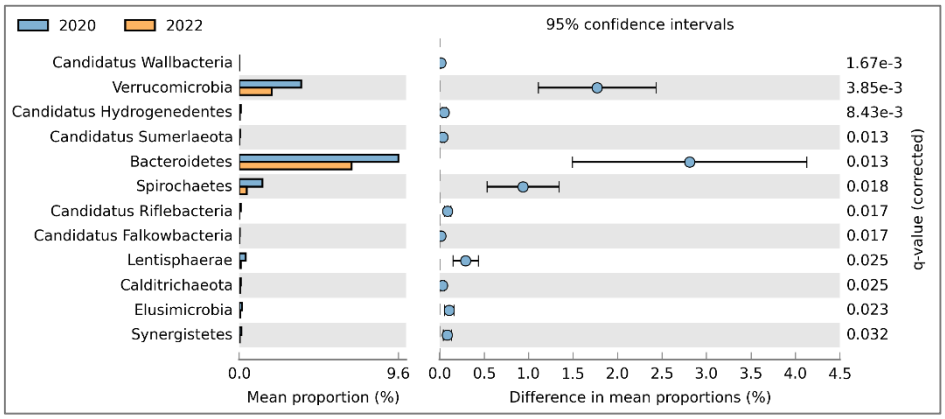

Fig. 4.7. Extended error bar plot showing differentially abundant bacterial phyla (Welch's t-test, Benjamini–Hochberg FDR, $p<0.05$) between WH_2020 and WH_2022.

Betaproteobacteria, *Gammaproteobacteria*, and *Alphaproteobacteria* comprise the most prevalent bacterial classes of the WH microbiome. *Alphaproteobacteria* were quite higher 2022, whereas *Bacteroidia*, *Spirochaetia*, *Verrucomicrobia*, and *Opitutae* mainly prevailed in WH 2020. We observed a higher and significant abundance of *Methylococcaceae*, *Rhodobacteriaceae*, *Azonexaceae*, and *Xanthomonadaceae* in WH_2022 (Welch's t-test, Benjamini–Hochberg FDR, $p<0.05$). The microbial communities showed similar richness and

diversity and no significant spatiotemporal differences (Fig. 4.4.). Significantly higher microbial diversity differences were discerned after temporal WH comparison (Fig. 4.5B and 4.5A). The core microbiome analysis was carried out at the family level of classification. Comparing WH_2020 with WH_2022 revealed that most core bacteria were similar (47.7%), with the exclusive core as 24% and 27%, respectively (Fig. 4.6B). Upon comparison of the River WH microbiome in the year 2020 (WH_2020) and 2022 (WH_2022), we observed differential enrichment of microbial taxa. The bacterial phylum *Verrucomicrobia, Bacteriodetes,* and *Spirochaetes* were significantly higher in WH_2020 than in WH_2022. Similarly, the bacterial families such as *Prevotellaceae, Bacteroidaceae, Spirochaetaceae, Desulfovibrionaceae,* and *Desulfobacteraceae* were significantly higher in WH_2020 and *Gallionellaceae* and *Hyphomicrobiaceae* in WH_2022. We did not observe differential preference in the archaebacterial population; however, we observed a higher prevalence of *Methanotrichaceae* in WH_2020 samples (Mean abundance of 14% vs 8% in WH_2022). Unlike the Lake WH microbiome, the core microbiome in the river on a temporal scale was much conserved (48% of the core was common), with only 24% to 27% being unique, which can be attributed to the environmental habitat (Fig. 4.6.). We did not observe a significant temporal influence on the overall alpha diversity but observed significant beta diversity differences in the microbiome of WH_2020 and 2022. The ANOSIM and PERMANOVA revealed significantly higher differences with R-values of 1.0 for bacteria and 0.41 for archaebacteria, suggesting distinct microbial cluster formation and R^2 values of 0.81% for bacteria and 0.17% for archaea, indicating that time could be another factor for diversity changes along with other physical and chemical factors on a longitudinal scale, which needs to be studied. (Fig. 4.5B). These results revealed changes in the overall microbiome of water hyacinth in rivers across time. Despite the observed variations, most of the core microbiomes in the river water hyacinth of 2020 and 2022 were conserved (Fig. 4.6B.).

4.3.3. WH microbiome is distinct from its surrounding water

The differential enrichment of microbes in water hyacinth was quite prominent compared to their corresponding water samples. *Bacteroidetes* and *Firmicutes* were significantly higher in the water samples of the year 2020 and 2022, respectively, whereas the WH carried significantly higher members of *Planctomycetes, Spirochaetes, Verrucomicrobia, Acidobacteria,* and *Chloroflexi* (Fig. 4.8. and 4.9.).

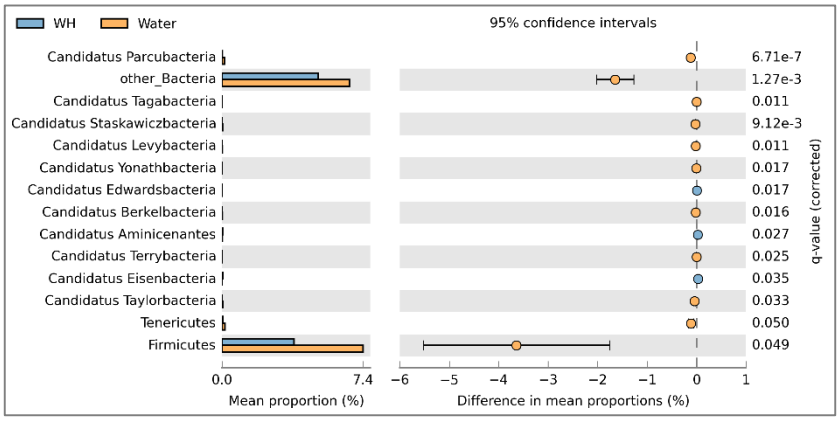

Fig. 4.8. Extended error bar plot showing differentially abundant bacterial phyla (Welch's t-test, Benjamini–Hochberg FDR, $p<0.05$) between Water_2022 and WH_2022.

Bacteroidetes and *Firmicutes* are highly prevailing in rivers from urban cities (Lin et al., 2019; Yadav et al., 2021b). Along with freshwater bacterial groups such as *Flavobacteraceae*, we observed a significantly more elevated abundance of gut-related and pathogen-containing groups such as *Campylobacteraceae*, *Burkholderiaceae*, and *Prevotellaceae* in water samples than in water hyacinth (Bernardet and Nakagawa et al., 2006; Magana-ArachchI). Further, the organisms documented for bioremediation (Loredanan et al., 2017; Zhang et al., 2021; Yadav et al., 2020a), such as *Azonexaceae, Xanthomonadaceae, Rhodobacteriaceae, Rhodobacteraceae*, *Azonexaceae*, and few others were numerically more abundant in the WH than water.

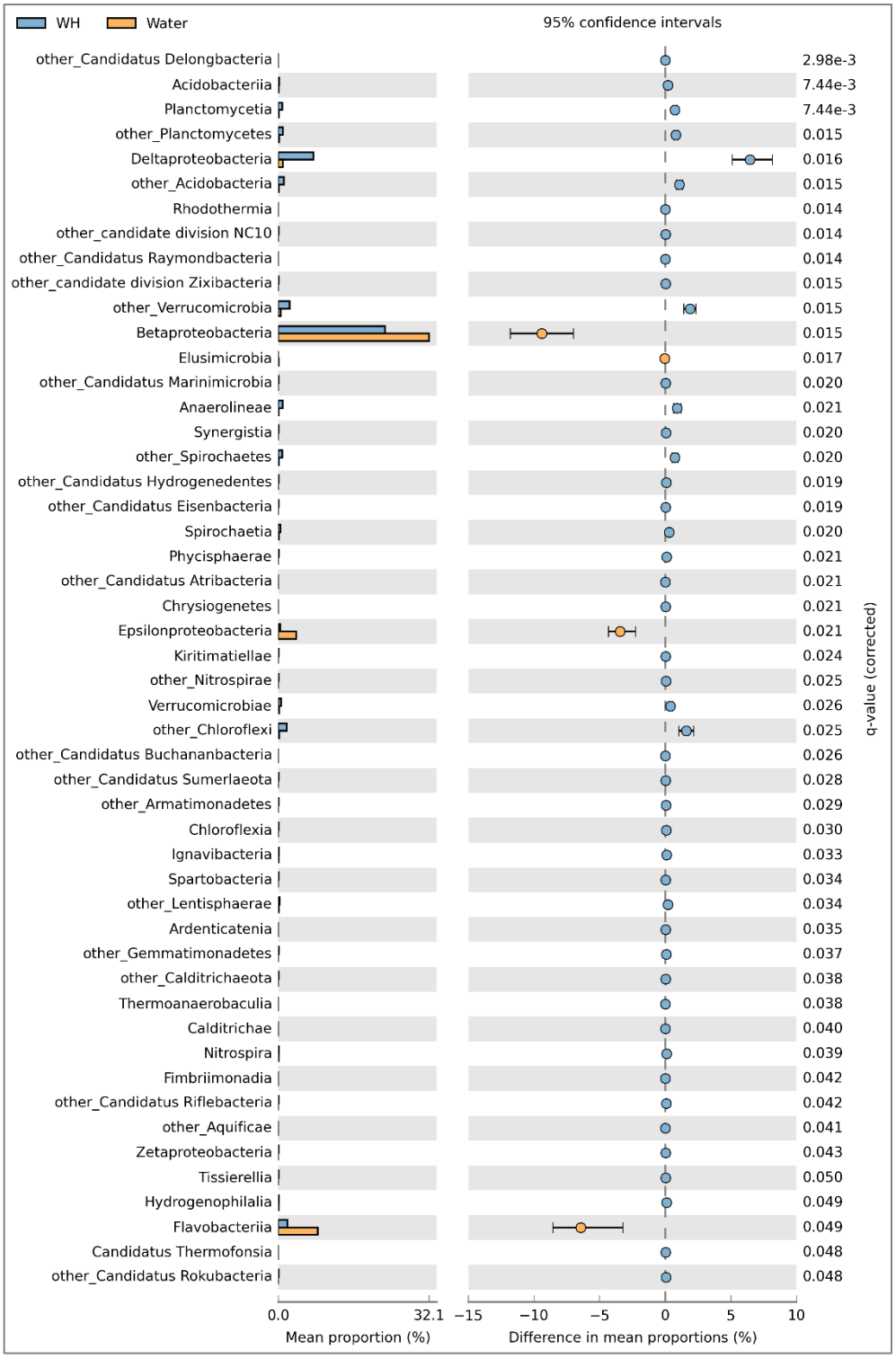

Fig. 4.9. Extended error bar plot showing differentially abundant bacterial class (Welch's t-test, Benjamini–Hochberg FDR, $p<0.05$) between Water_2020 and WH_2020.

Also, significantly higher occurrences of methanogens and methanotrophs such as *Methanotrichaceae*, *Methylococcaceae*, and *Methanocellaceae* were observed in WH than

in water. Interestingly, most of the core microbiome was shared between water hyacinth and water; however, WH still had 45% and 42% unique core bacterial families in 2020 and 2022, respectively (Fig. 4.6.). These observations indicate that WH has a stable core microbiome, which is probably maintained over time irrespective of its environment and is also supported by the earlier observations with temporal and aquatic body comparison. Furthermore, from the diversity perspective, we observed significantly higher alpha diversity in the WH microbiome than in water samples. Upon beta diversity estimation using the Bray-Curtis distance measure, we observed robust clustering and variance explanation value (ANOSIM R of 0.46 to 1.0 and R^2 of 0.18 to 0.52), indicating significantly higher diversity differences between the water and WH microbiome (Fig. 4.5.). In the future, it will be interesting to understand detailed molecular insights, including the chemical signalling and interactions between the roots and surrounding microbes in water to maintain such selectivity.

4.3.4. Energy metabolism and microbial-associated bioremediation

Metabolic processes were ascribed to 58% of bacterial and archaeal metagenomic data. In addition, at the KEGG level 2 functional module, 6% and 1.1% of reads were assigned to Energy Metabolism and 1.1% to Xenobiotic Metabolism, respectively. In energy metabolism, oxidative phosphorylation functions are encoded for by 50% of the predicted genes (52% in WH_2020, 52% in WH_2022, and 49.8% in Lake WH (WH_2020)), followed by nitrogen (21.2%), methane (12.5%), sulfur (7.48%), carbon fixation (5.40%), and photosynthesis (1.08%) (Figure 4.10A.). In xenobiotic metabolic functions, maximum reads were assigned to Nitrotoluene (28.4%), Benzoate (26.36%), Chlorocyclohexane and chlorobenzene (18.79%), Aminobenzoate (8.15%), Drug (4.02%), Atrazine (3.55%), Caprolactam (2.95%), Dioxin (2.13%), Xylene (1.69%), and Steroid (1.6%) (Figure 4.10B.). Pollutant Degrading Enzymes (PDE for hydrocarbon, plastic, and dye degradation) in the WH microbiome were predicted using RemeDB. The Catalase peroxidase (10%) was the most prevalent PDE predicted in WH

rhizobiome, followed by Protocatechuate 4,5-dioxygenase (5.5%), Chemotaxis response regulator protein-glutamate methylesterase (5.38%), 2-nitropropane dioxygenase (5.17%%), and Glyoxalase/bleomycin resistance (3.16%) (Figure 4.10C.).

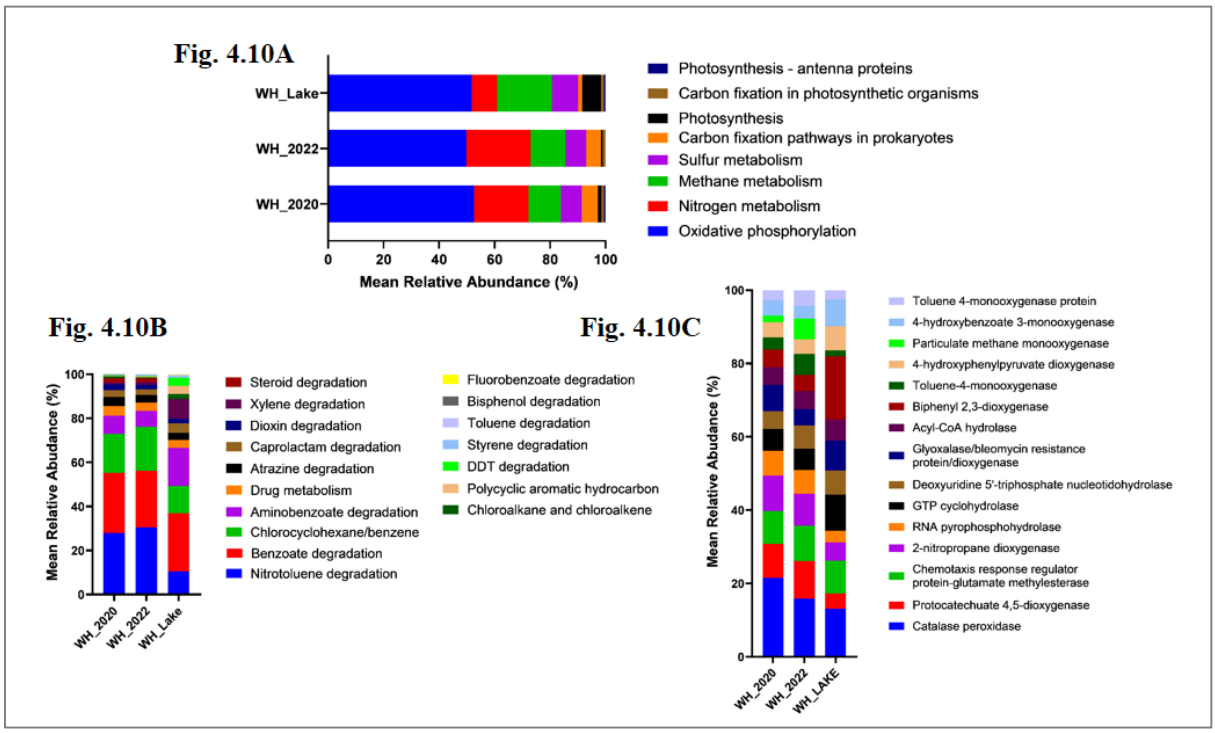

Fig. 4.10. Energy and Bioremediation metabolism of WH microbiome. The stacked bar plot illustrates 4A. Prevalence of Energy metabolism pathways, 4B. Xenobiotic metabolism, 4C. Top 15 Pollutant degrading enzymes in WH microbiome using RemeDB. The gene level information is provided in supplementary tables. WH_Lake (LWH_20) are water hyacinths from Lake, WH_2020 and WH_2022 are water hyacinths from rivers in year 2020 and 2022, respectively.

The taxonomic classification of the PDEs showed the predominance of *Proteobacterial* classes, with *Betaproteobacteria* (64.4%), *Gammaproteobacteria* (11.48%), and *Alphaproteobacteria* (7.12%). The other prominent bacterial types (>0.2%) contributing to PDEs in WH rhizobiome were *Actinobacteria, Deltaproteobacteria, Falvobacteria, Cytophagia, Hydrogenophilalia,* and *Bacteroidia*. Further, we observed a higher abundance (>2%) of *Hydrogenophaga, Ideonella, Rubrivivax,* and *Dechloromonas* at the genus level. The

other numerically prominent members (>1%) were *Acidovorax*, *Methylomonas*, *Pseudomonas*, *Aquabcterium*, *Thauera*, and *Sphaerotilus* (Fig. 4.11 and Fig. 4.12.).

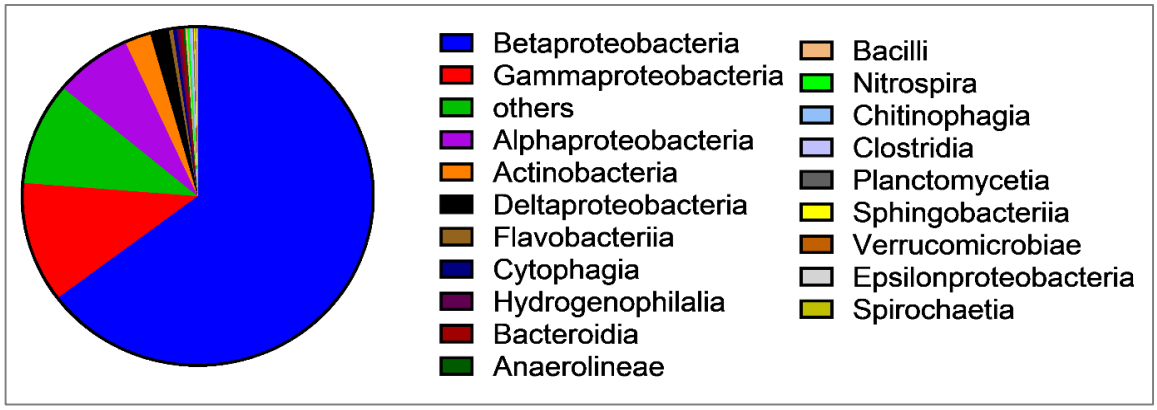

Fig. 4.11. Bacterial taxa at class level encoding for PDEs

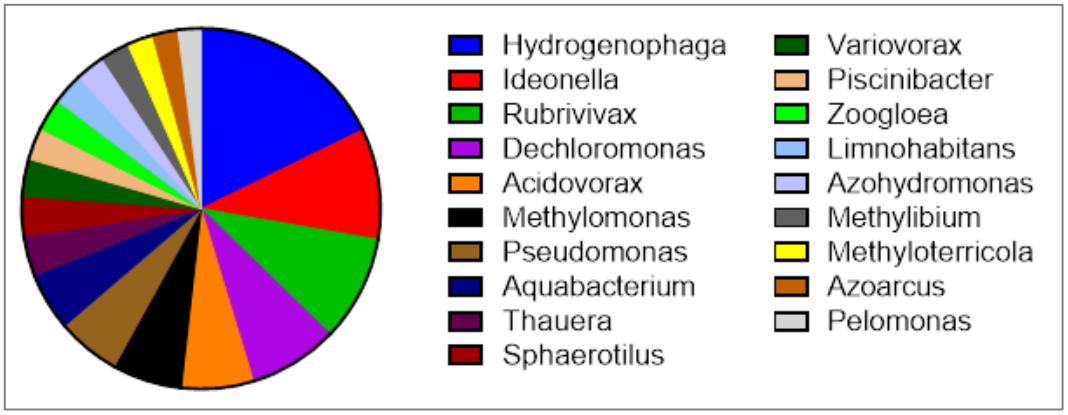

Fig. 4.12. Bacterial taxa at genus level encoding for PDEs

As primary energy-deriving metabolic functions in water hyacinth, root-associated methanogenesis and oxidation were reported previously by Ávila et al., 2021. Our study predicted oxidative phosphorylation (aerobic process) and nitrogen metabolism as the probable prevailing energy metabolism functions, followed by methane, sulphur, and carbon metabolism. A considerable amount of nitrogen intake usually inflows the urban aquatic bodies amid higher anthropogenic activities (Yu et al., 2020). The nitrification genes (amoA, B, C), denitrification genes (nir, nar, nor, nos, nap), and core-forming bacterial groups such as *Acetobacteraceae*, *Bradyrhizobiaceae*, and *Rhizobiaceae* predominance indicated a higher

prevalence of nitrogen metabolism in WH of urban aquatic bodies. The functional mining indicated a possibly higher prevalence of methane metabolism in Lake WH (19%) compared to River WH (11%). In contrast, nitrogen metabolism was predicted to be more prevalent in river WH (23%) than in Lake WH (9%). Studies (Xiao et al., 2017; Hilt et al., 2022; Kosten et al., 2016) have reported that methane concentrations in aquatic bodies are highest in the vegetated zones by macrophytes. The core microbiome of the WH consisted of the methane-metabolizing archaebacterial groups (Liu, 2010; Costa et al., 2019) such as *Methanobacteriaceae, Methanobacteriaceae, Methanocellaceae, Methanomicrobiaceae, Methanoregulaceae, Methanosarcinaceae, Methanospirillaceae, Methanotrichaceae* suggesting that the root-associated archaebacteria might drive methane metabolism in WH. Further, the abundance of bacterial groups (core microbiome) such as *Desulfobacterales, Desulfovibrionales,* and *Desulfuromonadales* can be linked to sulfur metabolism in water hyacinths. Such sulphate-reducing bacteria are reported to be associated with water hyacinth (Achá et al., 2005). Several studies have reported the use of WH to reduce water pollutants such as organic dyes, metals, and insecticides, mainly attributing their removal to the functional groups (—PO4, C=O, and C—H) on the root surfaces (Madikizela et al., 2021; Delgado et al., 1993). A study by Shehzadi et al., 2016, also reported the enhancement in the remediation of pollutants with the associated endophytic bacteria of *E.crassipes,* facilitating bioremediation. Certain microbes extract energy from metabolizing xenobiotic compounds, especially in this study's polluted river ecosystem. 1.1% of the total reads were assigned to xenobiotic degradation and metabolism genes, whereas top reads were mapped to genes and enzymes for Nitrotoluene and Benzoate degradation. Our previous study (Yadav et al., 2021a) from the same river sites revealed similar enrichment of genes in sediments involved in the degradation of these compounds. The WH in the present study are from polluted rivers and are often exposed to pollutants, which might play a role in determining the microbial structure of

WH. Further, to understand other remediating functionalities, we used the RemeDB database to predict pollutant-degrading enzymes (PDEs) in the microbial population associated with water hyacinth. Our analysis predicted over 140 PDEs in the WH microbiome reported to be involved in dye, hydrocarbon, and plastic degradation. Various dye-degrading enzymes such as Catalase peroxidase and other peroxidases, Catechol 1,2-dioxygenase; hydrocarbon utilizing enzymes such as monooxygenases and dioxygenases; and plastic degrading enzymes such as catalase, phenylacetate dehydrogenase, and various hydrolases were detected (Yadav et al., 2021a; Mooney et al., 2006; Sankara Subramanian et al., 2019). The taxonomic classification of the PDEs predicted a higher prevalence of *Hydrogenophaga*, *Ideonella, Rubrivivax, Dechloromonas, Thauera, Pseudomonas* and several others that are already reported for degradation of xenobiotic compounds such as aromatic hydrocarbons and plastic degradation (Fig. 4.12) (Palm et al., 2019; Dubbels et al., 2009; and Fan et al., 2019).

4.3.5. Biocides and Metal resistance genes (MRGs)

The BacMet database study showed 341 genes in the WH microbiome that encode antibacterial biocide and metal resistance. Altogether, the analysis possibly suggested higher resistance to Biocides and other compounds (Percent abundance, WH_2020 (23.97%), WH_2022 (24.27), WH_LAKE (21.13%)), followed by Metals and other compounds (13.21%, 15.6%, 21.77%), Copper (14%, 9%, 17%), Multi-metal resistance (11%, 12%, 13%), Arsenic (11%, 13%, 12%), Mercury (8%, 7%, 3%), Chromium (4%, 5%, 4%), and other heavy metals (Fig. 4.13). At the gene level, *ruv B*, a DNA helicase-mediated resistance gene, was very abundant (9%) in the WH rhizobiome, followed by *sodB* (8%), *dpsA* (7%), *merA, actP, cop, acr3, chrA, mdeA, arsC, copR, abeS, pcm, copF* (>1%) (Fig. 4.14A). The majority of the genes were categorized as *Methylomonas* (1.99%), *Dechloromonas* (1.8%), *Hydrogenophaga* (1.56%), *Nitrospira* (1.02%), *Ideonella* (0.87%), *Pseudomonas* (0.83%), *Rubrivivax* (0.82%), *Planktothricoides* (0.72%), and *Acidovorax* (0.6%) (Fig. 4.14B).

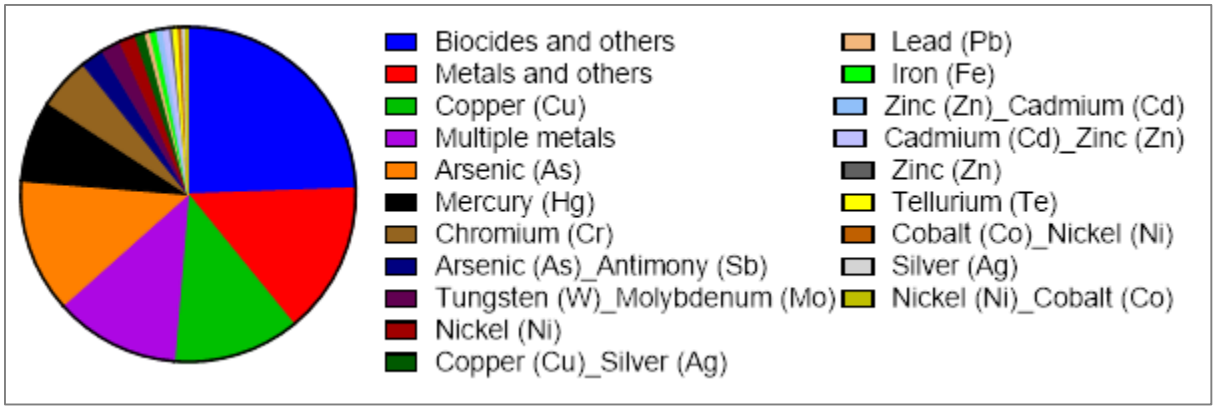

Fig. 4.13. MRGs against different biocides and metals

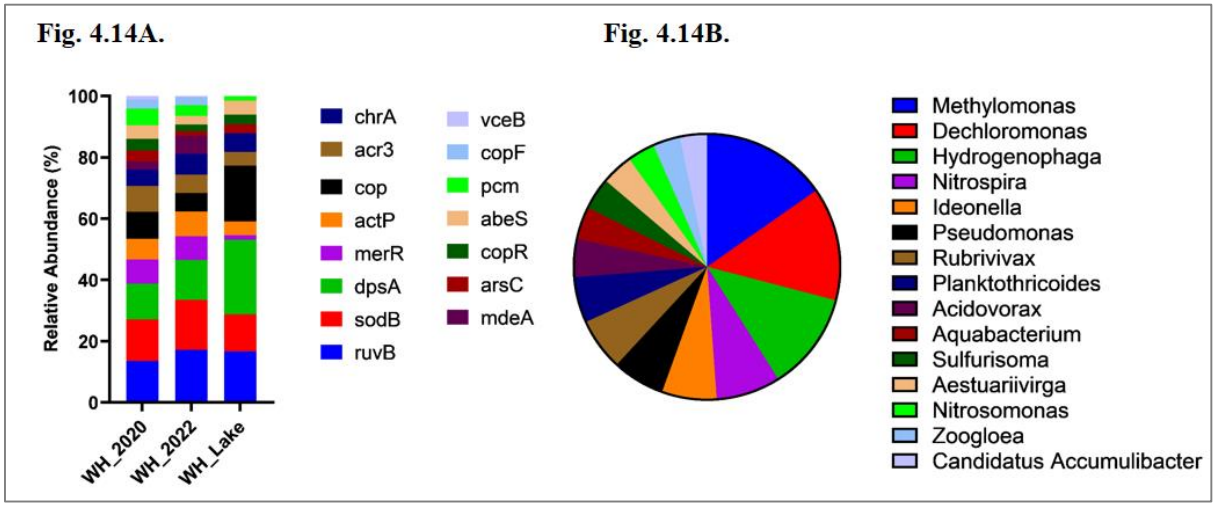

Fig. 4.14. Biocides, Metal Resistance, and bacterial classification. A. Top 15 Biocide and Metal resistance genes in WH microbiome, **B.** Prevalent bacterial genus encoding Metal and Biocide resistance. WH_Lake (LWH_20) are water hyacinths from Lake, WH_2020 and WH_2022 are water hyacinths from rivers in year 2020 and 2022, respectively.

Several studies have shown that hyacinths can remove metals and biocides efficiently (Delgado et al., 1993; Polprasert & Khatiwada, 1998; Rezania et al., 2015; Nor, 1994). Recent research has also found that plant-associated bacteria can improve metal and biocide removal (Anudechakul et al., 2015; Mahfooz et al., 2021; Kularatne et al., 2009). Plant roots are biodiversity hotspots, offering a favourable ecological niche for microbe-plant and microbe-microbe interactions (Sharma et al., 2021). Water hyacinth roots absorb heavy metals and biocides, which can be essential to the associated microbes at a lower concentration. However, at high concentrations, these could harm microbes. Different mechanisms, such as inactivation

or transformation of metals, impermeability to metals, and alteration of sites, are developed by microbes to tackle high concentrations of metals and biocides (Trevors et al., 1985). The present analysis predicted a likely higher resistance to biocides and metals by the WH microbiome. Our analysis mapped genes encoding resistance to metals such as Copper, Arsenic, Mercury, Chromium, Antimony, Lead, and Zinc. Also, higher *ruvB* and *sodB*-mediated resistance genes were mapped in the WH rhizobiome. The RuvB is an ATP-dependent helicase which can repair the DNA damage caused by toxic metals (BacMET, Miranda et al., 2005). The *sodB* encodes superoxide dismutases that are reported to protect bacterial cells against metal oxides (BacMET, Bébien et al., 2002). In addition to these two genes, around 340 metal and biocide resistance genes were mapped in WH rhizobiome, such as *dps A* for hydrogen peroxide, *mer R* for mercury, *cop* for copper resistance, act gene for multiple metals and biocides, *acr* for Arsenic and several others (BacMET). The taxonomic classification revealed bacterial genera such as *Methylomonas, Dechloromonas, Hydrogenophaga, Nitrospira, Pseudomonas, Ideonella*, and others that contributed to metal and biocide resistance. Most of these, such as *Methylomonas, Hydrogenophaga, Ideonella, Pseudomonas*, and *Thauera*, constitute the core microbiome of WH, thus indicating that metal removal by WH is possibly facilitated by its microbiome. These WH-associated metal and biocide-resisting microbiomes could also have biotechnological applications for controlling toxic metals in various environments. On the other hand, these traits could also lead to the spread and dissemination of antibiotic resistance. The evolution of antibiotic resistance is not only governed by antibiotics but also by other factors, such as metals, as they possess a common mode of action and thus could be co-selected (Li et al., 2017).

4.4. Conclusion

To summarize, we discovered various microbial communities linked with water hyacinth roots. The greater ANOSIM R-value and Adonis R2 value revealed that water body

types and time were likely explanatory variables for the observed diversity of microbes in water hyacinths. Further, temporal variations were observed in the water hyacinth microbiome that could be associated with the temporary microbial taxa, as most of the core taxa were conserved. However, forthcoming studies shall be directed towards comprehending other physical and chemical factors that vary on a longitudinal scale and influence microbial diversity. We observed significantly differentially predominant taxa in WH compared to water, such as methane and sulfur metabolizing organisms. Furthermore, in line with the earlier studies, our analysis indicated methane metabolism possibly as one of the major energy-generating mechanisms in water hyacinth. In addition to methane metabolism, a higher preponderance of sulfur-metabolizing microbial taxa constituting the core microbiome of WH was detected, implying sulfur metabolism is likely the other key energy source. Our predictive functional analysis mapped various genes to xenobiotic and pollutant degradation functions in the WH microbiome. The mapping of multiple genes known for their functionality in metal and biocide resistance in the WH metagenome suggested the probable role of WH-associated microbes in remediation. It also provides a genesis for future investigations exploring WH for bioremediation. Overall, our current metagenome-based predictive analysis provided an understanding of the microbiome associated with the water hyacinth, inferred potential factors affecting microbial diversity, and possible functional mechanisms for energy and survival.

4.5. References

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Chapter 5. To develop the microbial process for conversion of biomass-waste to 2,3-butanediol and Poly-γ-Glutamic Acid

Chapter 5. To develop the microbial process for conversion of biomass-waste to 2,3-butanediol and Poly-γ-Glutamic Acid

Chapter 5.1. 2,3-butanediol production by *Bacillus halotolerans* EX5-6

Abstract

A platform chemical, 2,3-butanediol (2,3-BDO or BDO), is a bivalent alcohol with several industrial applications. 2,3-BDO is a precursor for various industrial goods, including rubber and solvents, and it possesses biofuel properties. Several studies, over the last decade have reported microbial-based BDO biosynthesis. However, the high substrate and product recovery cost and the microbial pathogenic type (group 2) have hampered its industrial implementation. As a result, there is a demand for a more affordable feedstock that requires least or no pretreatment, unlike other lignocellulosic biomass. *Bacillus halotolerans* EX5-6, isolated from Pawana River sediment, was tested for 2,3-butanediol synthesis in this study. We got a BDO concentration of 73.19 g/L with a high yield and productivity of 0.5 g/g and 1.02 g/L/h, respectively after optimization at the 250ml flask level. In addition, rotting tomato juice was used as a substrate for BDO production to solve substrate cost issues. India is the world's second-largest producer of tomatoes; yet, due to their shorter shelf life, storage concerns, and fungal infections, most are wasted and abandoned. A process was developed wherein the rotten tomato juice was concentrated into various concentrations: 2X, 3X, and 4X. Compared to the optimized synthetic media containing costly substrates, 16.62 g/l of BDO titer was obtained from concentrated 2X rotten tomatoes with a productivity of 0.7g/l/h at the flask level, relatively higher compared to many other reports utilizing different lignocellulosic and other wastes. Additionally, the addition of optimized medium constituents in tomato concentrate had no increasing effect on the BDO production; thus, tomato waste served as a complete medium requiring no supplementation. The present study is the first report on the production of 2,3-BD by *Bacillus halotolerans*.

5.1.1. Introduction

5.1.1.1. Importance of 2,3-butanediol

The US Energy Department classifies 2,3-butanediol (2,3-BDO or BDO), a four carbon metabolic product, as a highly valued platform chemical (Yang & Zhang, 2019). It is a bivalent alcohol with a various industrial applications. It is used as a precursor material in the production of solvents, rubber, and cosmetic items, as well as in the synthesis of many medications, ointments, antifreeze agents, and liquid fuels (biofuels) (Fig. 5.1.). BDO and its derivatives are excellent contender for fuel additives due to its high heating value and low vapour pressure when compared to other liquid fuels (Fig. 5.1.). The BDO derivatives such as 1,3- butadiene and methyl ethyl ketone (MEK) have wide ranges of applications in the synthesis of rubber, plastics, resins, and several others (Fig. 5.1.). (Maina et al., 2021) Furthermore, the 2,3-BDO market is expanding quickly; by 2027, it is predicted to reach $220 million (Tsigoriyna et al., 2021; Song et al., 2019). It is a chiral compound with three isomeric forms: L-BDO (dextro- or (2S, 3S)- (+)-), D-BDO (levo- or (2R, 3R)-(-)-), and meso-BDO (2R, 3R-) (Maina et al., 2021) (Fig. 5.2.).

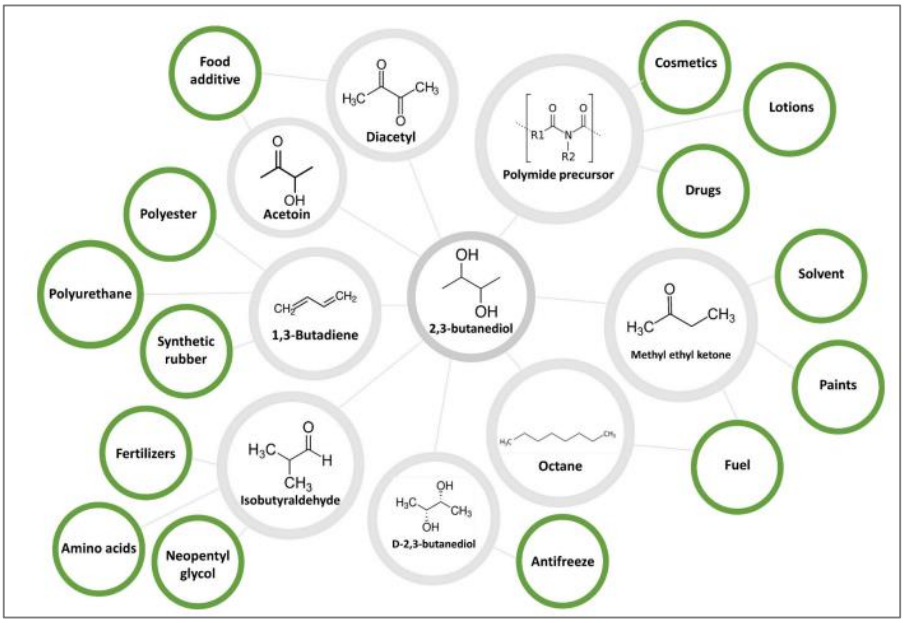

Fig. 5.1. Applications of BDO and its derivatives (Maina et al., 2021)

In all the three forms, BDO exists in crystalline, colourless, and odourless forms with high boiling point ranging from 177°C to 182°C. In biological synthesis, the formation of different stereoisomers of BDO mainly relies on the activity of butanediol hydrogenase (BDH), an enzyme involved in the ultimate step of BDO formation (Petrov and Petrova, 2021; Ji et al., 2011).

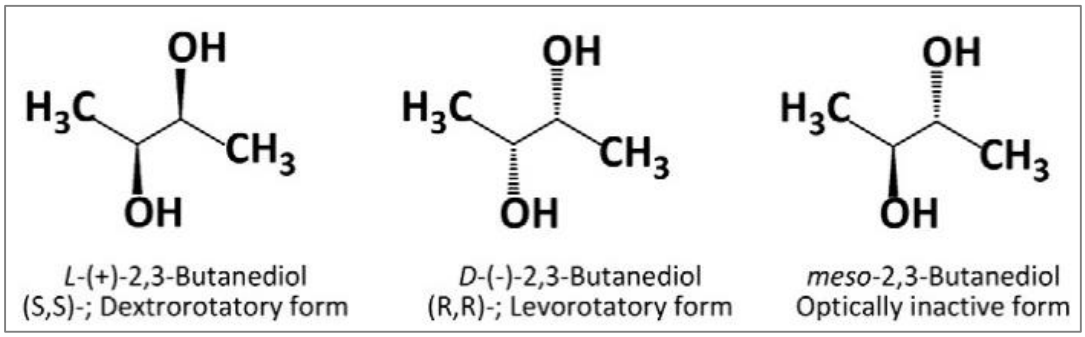

Fig. 5.2. Stereoisomers of 2,3-Butanediol (Ji et al., 2011)

5.1.1.2. Microbial production of 2,3-butanediol

2,3-BDO is chemically produced via the pyrolysis of diacetate and from butene (derived from cracked gases. The significant barriers in the chemical-based production of BDO include expensive catalysts, high production cost, the mixture of stereoisomers, and environmental concerns (Fig. 5.3.) (Song et al., 2019; Hakizimana et al., 2020). Technically, these are some of the challenges associated with BDO production. Hence, in recent years, the interest in the microbiological production of BDO has increased. Further, there is a grave environmental concern over fossil fuel exhaustion and carbon dioxide emission. Since 2,3BDO is a microbial fermentation product, bio-based BDO production constitutes an environmentally friendly approach. In addition, higher catalytic activity, specificity, lower energy consumption, and reduced number of steps improve cost-effectiveness (Maina et a., 2021; Song et al., 2019; Hakizimana et al., 2020).

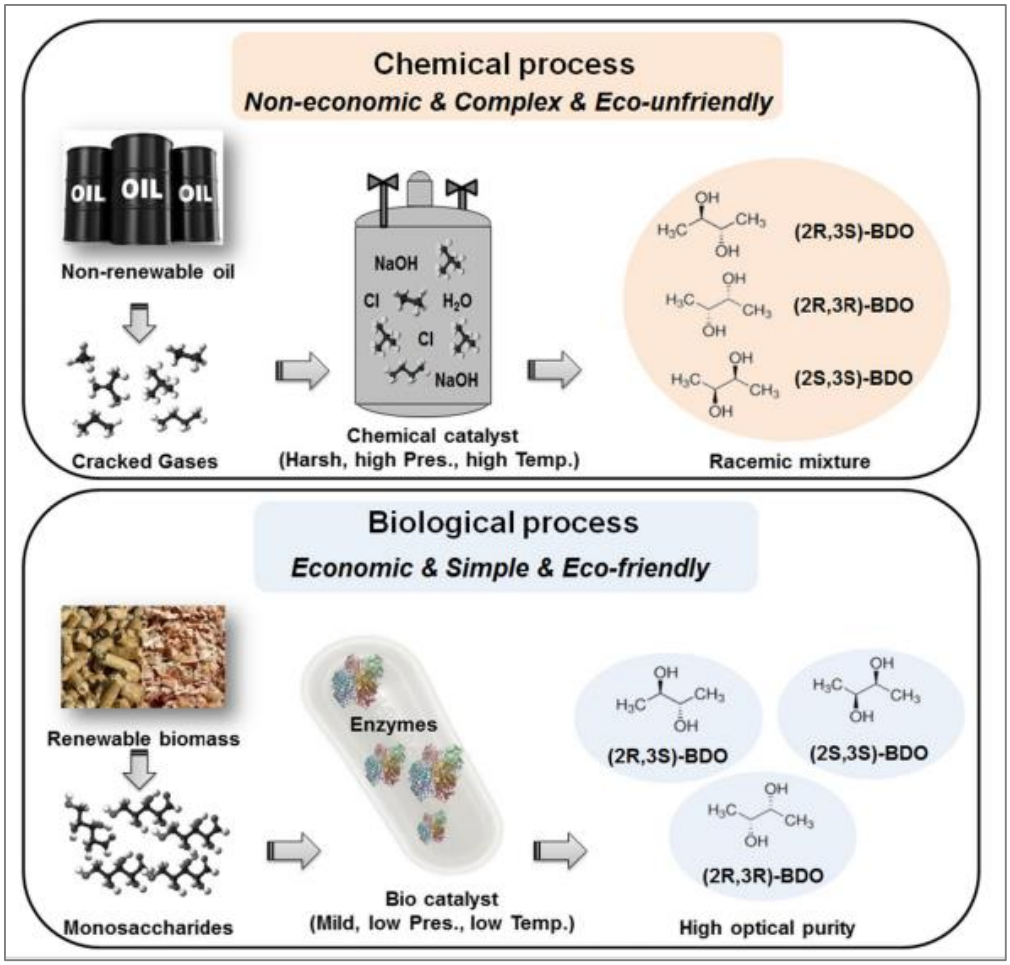

Fig. 5.3. Comparison of chemical and biological production of 2,3-BDO (Song et al., 2019)

5.1.1.2.a. Why do microbes produce 2,3-BDO

The microbial synthesis of 2,3-BDO is accompanied by mixed-acid fermentation, the most common characteristic of *Enterobacteriaceae group* along with some other bacteria. The mixed acid fermentation generally results in the production of organic acids such as lactic acid, acetic acid, and others, lowering pH in the environment. Thus, to avoid excessive acidification, microbes initiate the butanediol synthesis pathway, directing carbon flux to the alpha-acetolactate synthesis, which is ultimately converted to BDO. It is also understood that BDO, besides lowering pH, serves as a storage mechanism of carbon and energy by maintaining the NAD+/NADH ratio in the cells (Białkowska, 2016; Song et al., 2019).

5.1.1.2.b. Microorganisms for BDO syntheis

Microbial production of BDO has a history of almost ten decades. Several Gram-positive and Gram-negative microbes are reported to produce BDO belonging to genera such as *Klebsiella, Bacillus, Enterobacter, Paenibacillus,* and *Serratia*. Also, numerous non-natural producers are engineered to produce BDO, including *Lactobacillus lactis, Escherichia coli,* and *Saccharomyces cerevisiae* (Białkowska, 2016; Song et al., 2019, Hakizimana et al., 2020). Highest production is mainly reported from the bacterial family *Enterobacteriaceae* members such as *Klebsiella*, *Serratia*, and *Enterobacter* species (Table 5.1.). Owing to their wide substrate utilization and metabolic adaptability, these can produce a higher yield of BDO; however, most species belong to group 2 risk pathogenic group, limiting their industrial implementation. As a result of this, Gram-positive bacterial groups, mainly Bacillus, are now widely explored for 2,3-BDO production due to their safer characteristics. Although they require a rich nutrient medium, recent studies have suggested comparable production of BDO from Bacillus. The major advantages of *Bacillus* species over *Enterobacteriaceae* members include: Generally regarded as a Safe organism (GRAS), metabolic simplicity permitting modifications to yield optically pure isomers, ability to thrive in microaerophilic and aerophilic conditions, and can be grown at high temperatures.

Table 5.1. Biological production of 2,3-BDO by different microbes. Highest producers are listed in the following table (Information adapted from Song et al., 2019).

Sr. No.	Hosts	Genotype	Conditions	Titer (g/L)	Productivity (g/L/h)	References
1	*Klebsiella pnuemoniae*	Random Mutant	Fed-batch	150.0	4.21	Ma et al., 2009
2	*Klebsiella pnuemoniae*	*ΔadhE ΔldhA*	Fed-batch	116.0	2.21	Guo et al., 2014
3	*Klebsiella pnuemoniae*	Overexpression of	Fed-batch	101.5	2.54	Kim et al., 2012

		budA and *budB*				
4	*Klebsiella pnuemoniae*	Wild	Fed-batch	91.63	2.29	Li et al., 2014
5	*Enterobacter aerogenes*	*ΔldhA*	Fed-batch	118.0	2.19	Jung et al., 2012
6	*Enterobacter cloace*	Wild	Fed-batch	93.9	2.0	Wang et al., 2012
7	*Serratia marcescens*	Random Mutant	Fed-batch	139.0	3.49	Zhang et al., 2010
8	*Bacillus subtilis*	Multigene mutant	Fed-batch	103.7	0.224	Fu et al., 2016
9	*Bacillus licheniformis*	Wild	Fed-batch	144.7	1.14	Jurchescu et al., 2013
10	*Bacillus licheniformis*	*ΔbudC*	Fed-batch	123.7	2.95	Ge et al., 2016
11	*Bacillus amyloliquefaciens*	Overexpression of *bdh* and *gapA*	Fed-batch	132.95	2.95	Yang et al., 2013
12	*Paenibacillus polymyxa*	Wild	Fed-batch	111	2.06	Hassler et al., 2012

5.1.1.2.c. Biosynthetic pathway for BDO production

Microbes utilize variety of sugars (pentoses and hexoses), especially monosachharides, for the production of 2,3-BDO. These sugars are firstly converted to pyruvate via the Embden–Meyerhof pathway (EMP). In case of pentoses, conversion to pyruvate happens via Pentose Phosphate Pathway and EMP pathway. Most microbes carry out BDO formation via three consecutive steps which includes conversion of pyruvate to α-acetolactate to acetoin and/or diacetyl to 2,3-BDO. The pyruvate conversion to α-acetolactate is a decarboxylation reaction mediated by an enzyme α-acetolactate synthase. Further, α-acetolactate decarboxylase catalyzes the conversion of α-acetolactate to R-form of Acetoin. Also, under aerobic condition, α-acetolactate is spontaneously converted to diacetyl, which is then converted to S-form of Acetoin with the help of enzymes butanediol dehydrogenase or a diacetyl reductase. Acetoin

is a penultimate metabolite, which is converted to 2,3-BDO in a reversible reaction catalysed by 2,3-butanediol dehydrogenase. From R and S-acetoin, all the three isomers of 2,3-BDO are formed, which is mainly dependent on the activities of stereospecific enzymes: butanediol dehydrogenase and an acetoin reductase. The *Klebsiella* and *Enterobacter* strains mainly are reported to form (2S,3S)-BD and meso-form BDO. In contrast, the *bacillus* groups is reported to form (2R,3R)-BD and meso-form BDO. The mechanism of stereoisomer formation is mainly attributed to multiple enzymes for terminal step in the same organism or bifunctional terminal enzymes. Since two pyruvate molecules are required for 1 molecule of 2,3-BDO, the theoretical yield from glucose is 0.5g/g (Fig 5.4.) (Song et al., 2019).

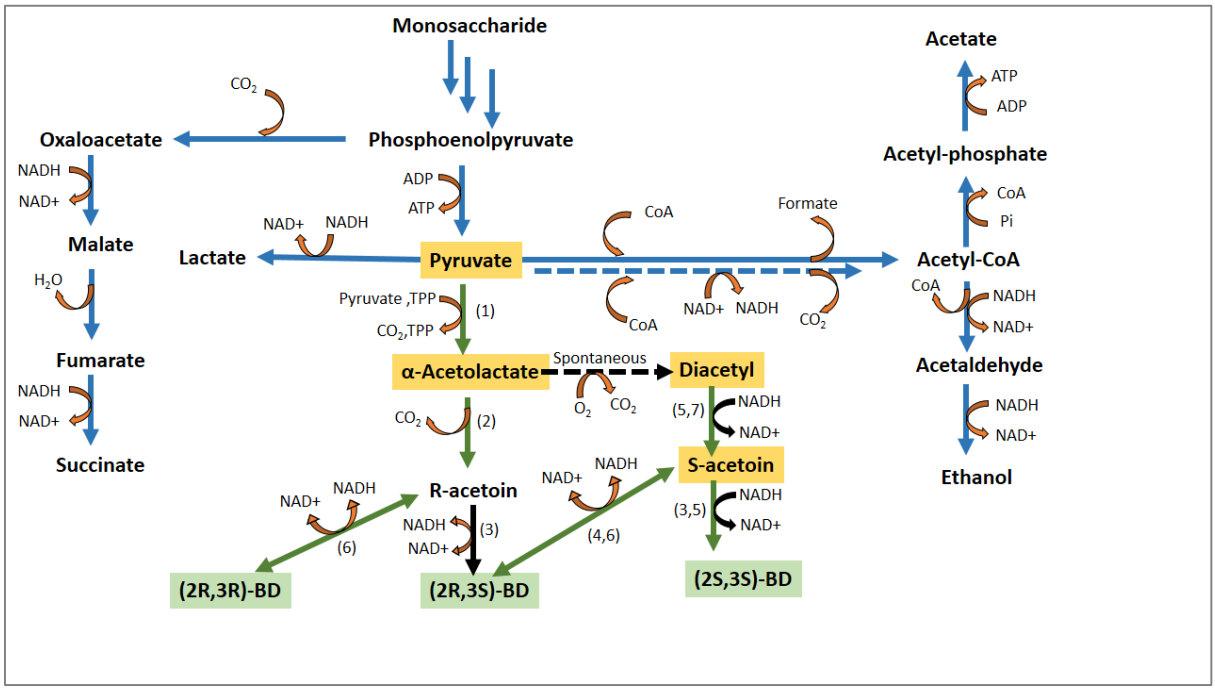

Fig. 5.4. Biological routes for 2,3-BDO production. Dashed Lines: Aerobic condition, (1). Alpha-acetolactate synthase; (2) α-acetolactate decarboxylase; (3) (2R,3S)-butanediol dehydrogenase (R-acetoin forming); (4) (2R,3S)-butanediol dehydrogenase (S-acetoin forming); (5) (2S,3S)-butanediol dehydrogenase (S-acetoin forming); (6) (2R,3R)-butanediol dehydrogenase; and (7) diacetyl reductase (S-acetoin forming). (Pathway information adapted from Song et al., 2019.

5.1.1.3. Challenges and solutions for BDO production

Microbial safety and the cost of carbon sources are two essential variables for efficient and cost-effective process development for BDO manufacture. While the former has been solved by using GRAS microorganisms, the latter remains a challenge. Several experiments have been published that use various types of waste substrates to create BDO. Using lignocellulosic hydrolysate, corn stover hydrolysate, molasses, bakery waste hydrolysate, and crude glycerol (Petrov & Petrova, 2021; Hazeena et al., 2020) is one method (Table 5.2.). Despite increased yield and productivity, the cost of production remains a barrier. The expense could be mainly related to the pretreatment method necessary to prepare the lysate and substrate availability (Fig. 5.5.). As a result, there is a need for an alternative waste source that requires minimal chemical treatment.

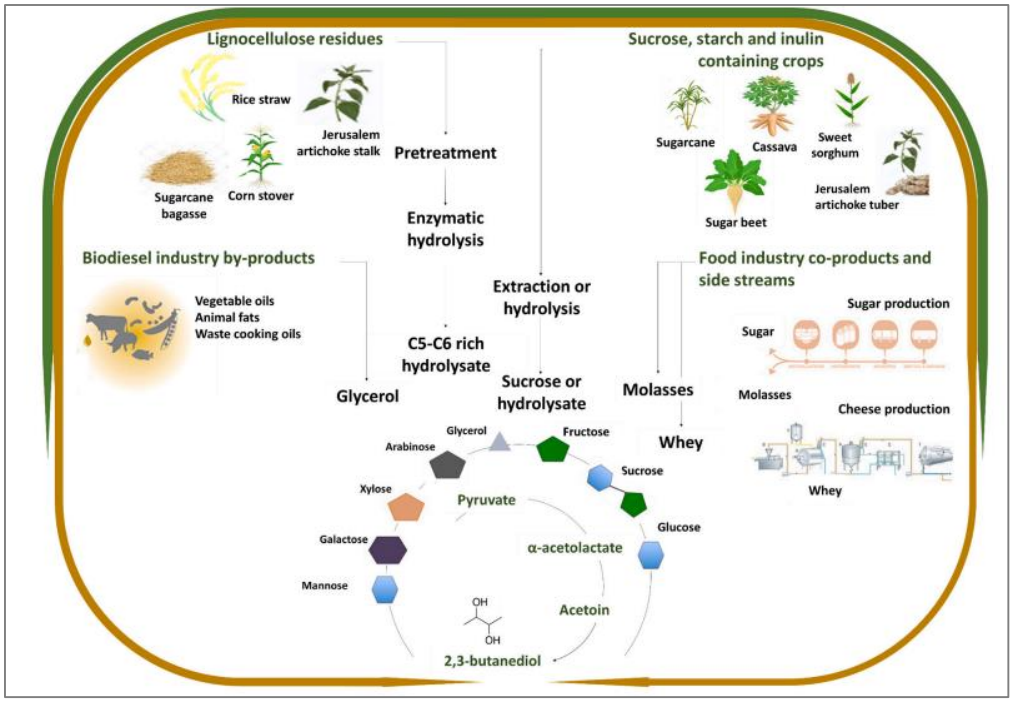

Fig. 5.5. BDO production from renewable feed stock and other biowastes (Maina et al., 2021)

Table 5.2. BDO production from biowastes

Sr. No.	Hosts	Carbon source	Additional Nitrogen source	Titer (g/L)	Productivity (g/L/h)	References
1.	*Bacillus licheniformis*	Apple pomace hydrolysate	Yeast Extract	77.6	0.42	Białkowska et al., 2015
2.	*Serratia marcescens H30*	Sweet sorghum stalk	Yeast Extract	109.4	1.4	Yuan et al., 2017
3.	*Enterobacter aerogenes*	Sugarcane bagasse hydrolysate	Yeast Extract,Casamino acids	114.3	1.49	Kim et al., 2020
4.	*Enterobacter ludwigii FMCC 204*	Fruit and vegetable hydrolysate	Yeast Extract and Malt Extract	50	0.41	Liakou et al., 2018
5.	*Enterobacter cloacae*	Sugarcane molasses	Multiple inorganic and organic nitrogen sources	140	2.59	Jung et al., 2015
6.	*Klebsiella oxytoca M3*	Crude glycerol	Yeast and casaminoacids	131.5	0.84	Cho et al., 2015
7.	*Bacillus amyloliquefaciens*	Crude glycerol: molasses	Corn steep liquor and soybean meal	102.3	1.16	Yang et al., 2015

Tomatoes are one of the most important vegetables and are a staple in many people's diets around the world. They are well-known for their role in decreasing blood cholesterol levels. China is the leading tomato grower, followed by India, which accounts for 11% of global production. According to the World Processing Tomato Council (WPTC), around 8 million tons of tomatoes processed yearly are wasted globally (Trombino et al., 2021). Tomatoes have a shorter shelf life and are consequently more susceptible to rotting owing to fungal infections and poor storage conditions. Most tomatoes are consumed fresh, with only

about 2% used to make sauce and paste. However, more than 31% of tomatoes are wasted during the peak season. Chemically, tomato consists of various nutrients and bioactive compounds, including fructose and glucose as the primary carbon sources (Ali et al., 2020). Compared to abundant lignocellulosic biomass, tomato contains non-cellulosic sugars that microbes can utilize without the need for any pretreatment process. With this hypothesis, we aimed to utilize rotten tomatoes and develop a cheap substrate to produce 2,3-butanediol with a goal to achieve sustainability.

5.1.2. Experimentals

5.1.2.1. Bacterial screening for 2,3-BDO production

Four bacterial isolates (WHEC4, WHEC3, UK1, and EX5-6) were screened for 2,3-BDO production ability. These bacteria were isolated from Pawana River, Pune, Maharashtra (18.57305, 73.83121) on St. Nutrient Agar. The screening for BDO production was done using fermentation media with composition of Glucose 50g/L, K_2HPO_4 3g/L, Yeast Extract 10g/L, $MgSO_4.7H_2O$ 0.2 g/L, Peptone 10g/L, Sodium chloride 5g/L, pH 7.0. The bacterial cultures were grown overnight in Nutrient Broth No. 3 (M1902, HIMEDIA) at 37°C for 12 hours and were inoculated in the fermentation media for 24 hours. After the incubation period, the broth was analyzed for producing 2,3-butanediol by HPLC. The column used was Aminex HPX-87H (1250140, BioRad), 5mM H2SO4 was used as a solvent with a column flow of 0.6ml/min, oven temperature of 60°C, and run time of 30 mins with detection using Refractive Index (RI) detector. Standard 2,3-butanediol was procured from Sigma.

5.1.2.2. Molecular characterization, Genome sequencing, and analysis

DNA was extracted from bacterial isolates using a DNeasy Powersoil Pro kit (Qiagen, 47014). The 16S rRNA gene was amplified using 27F and 1492R universal primers and sequenced using a Sanger sequencer. The phylogenetic analysis was carried out using MEGA

v11 software using the Neighbour Joining Method. The genome sequencing was carried out using the protocol described in Yadav et al., 2021, using a MinION sequencer. Briefly, DNA having a purity of 1.7 to 1.9 (260/280) was used for library preparation. The library preparation was initiated with 1.0 µg/48 µl DNA using 1D Ligation sequencing kit SQK-LSK109. Briefly, the following protocol was followed:

a. End-repair was performed using 3.5µl of each NEBNext FFPE repair buffer, Ultra II End-prep buffer, 2µl of FFPE repair mix and 3µl of Ultra II End-prep enzyme mix. The reaction was thermally cycled at 20°C for 10 minutes and 65°C for 10 minutes.

b. 2.5µl of NBD104 and 114, 25µl of Blunt/TA Ligase Master Mix was mixed with 600ng (Qubit estimated) of repaired DNA for barcoding (incubated at 28°C for 10 minutes).

c. Lastly, all the barcoded samples were pooled (each 100ng) and were mixed with 8µl of Adapter mix II (AMII) and 60µl Blunt/TA ligase (incubated at 28°C for 10 minutes). The library was eluted by elution buffer (EB) at 37°C for 10 minutes.

Each step mentioned above was followed by DNA purification using 60µl of AMPure beads and 70% ethanol on a Magnetic Separation Rack (New England BioLabs, #S1509S).

The raw reads were basecalled and demultiplexed using Guppy software (V 5.0.11) with fast basecalling mode, followed by sequence filtering using NanoFilt. Flye tool was used for the assembly of the fastq reads, followed by polishing using the Medaka tool. RAST (Rapid Annotation Server) was used for genome mining for 2,3-BDO metabolism genes.

5.1.2.3. Optimization of 2,3-BD production in synthetic medium

The One Factor at a Time (OFAT) approach was used for the optimization of media and conditions for the maximum production of BDO. For all the optimization, the seed medium consisted of Yeast Extract 5g/L, Peptone 5g/L, NaCl 5g/L, Beef extract 5g/L, Glucose 20g/l, and pH 7.0. A colony was inoculated into a seed medium at 37°C till O.D. 600 reached 8.0.

Further, 10 % of inoculum (inoculum concentration was further optimized) was inoculated into a fermentation medium to produce 2,3-BDO at 180 rpm at 28°C. The initial unoptimized fermentation media consisted of 50g/L Glucose, 10 g/L Yeast Extract and Peptone each, NaCl 5g/L, K_2HPO_4 0.3%, $MgSO_4.7H_2O$ 0.02% and pH 7.0. All the experiments were either carried out in duplicates or triplicates.

5.1.2.4. 2,3-butanediol production using Rotten Tomatoes

Rotten tomatoes were collected from Pune's Pashan market (18.53718, 73.79206). The tomatoes were quickly transferred to the laboratory and ground using a home grinder. A muslin cloth was used to filter the resulting juice. The juice was separated from the peel and seeds, which were autoclaved and discarded. A rota evaporator was used to enhance the sugar concentration to 2X, 3X, and 4X times. "X" denotes the multiple (times) concentration. So the 2X signifies twice the concentration of sugars and other juice ingredients by reducing the amount of water by half. Similarly, 3X denotes removing three times the original volume of water via rota evaporation. These concentrates were then utilized to produce BDO either alone or with the addition of supplementation of optimised media components (Fig. 5.6.).

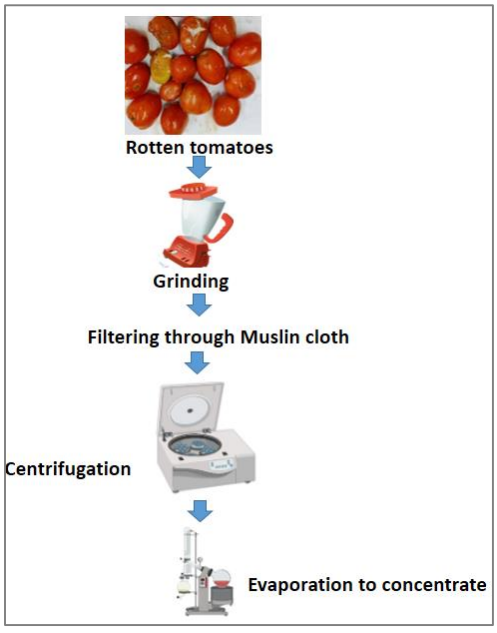

Fig. 5.6. Schematic of 2,3-BDO production from Rotten Tomato juice

5.1.2.5. Analytical analysis

HPLC and Gas chromatography was employed for the detection of metabolites. HPLC conditions included: Column- Aminex HPX-87H (1250140, BioRad), Solvent- 5mM H_2SO_4, Flow rate- 0.6ml/min, Detector- RI, runtime- 30 minutes, HPLC (UHPLC, Ultimate 3000, ThermoScientific). The GC conditions were Column name: FFAP (30mx0.25mmx0.25µm), Detector and injector temperature of 300°C, sample volume 1 ul, and following oven conditions, Gas Chromatography (ThermoScientific, Trace 1300).

Table 5.3. Gas chromatography conditions

Rate °C	Temp°C	Hold Time (min)
	80°C	2
40°C	200°C	10
	Total run time	15

5.1.2.6. Culture and Sequence Deposition

The bacterial isolate *Bacillus halotolerans* EX5-6 is deposited in NCIM at CSIR-National Chemical Laboratory (NCL), Pune, India as *Bacillus halotolerans* (EX5-6) with culture ID NCIM 5826. The culture is also submitted in IDA facility of NCMR at National Center for Cell Sciences (NCCS), Pune, India with IDA accession ID MCC 0265. The NCBI accession number for 16S rRNA sequence is OR263467.

5.1.3. Results and Discussion

5.1.3.1. Screening, taxonomic characterization, and Genome analysis

Previously isolated 4 bacterial cultures (WHEC4, WHEC3, UK1, and EX5-6) from Polycyclic Aromatic Hydrocarbon (PAH) enriched Pawana River sediments and Water hyacinth roots were screened for 2,3-butanediol production. WHEC4, WHEC3, and UK1 were

isolated from water hyacinth roots enriched for 7 days in 2% cellulose (Cellulose from cotton linter) along with Bushnell-Hass medium. EX5-6 was isolated from Anthracene (PAH compound) enriched river sediment8s. 2,3-BDO screening was carried out at flask level using glucose as a carbon source along with Yeast Extract and peptone as a nitrogen source. Three isolates were positive for the production of BDO. The 16S rRNA sequence analysis confirmed the identity of the isolate (Table 5.4.). Since there are multiple reports of BDO production from *Bacillus licheniformis* (Petrov and Petrova, 2021) and no reports from *Bacillus halotolerans*, we selected EX5-6 isolate for optimization of BDO production.

Table 5.4. 2,3-butanediol screening results and molecular characterization of the bacterial isolates.

Sr.No.	Isolate ID	2,3-BDO Production	EZ-taxon-based Identification	Similarity
1	EX5-6	Positive	*Bacillus halotolerans*	99.86%
2	WHEC3	Negative	*Anoxybacillus rupiensis*	100%
3	WHEC4	Positive	*Bacillus licheniformis*	99.85%
4	UK1	Positive	*Bacillus licheniformis*	99%

The 16S rRNA sequence (1425bp) of EX5-6 was analyzed and identified on EZtaxon. The top-hit strain was *Bacillus halotolerans* ATCC 25096 with 99.86% similarity and 95.2% completeness. Further, phylogenic investigation using Neighbour Joining method with bootstrap of 1000, suggested close phylogeny to Bacillus Halotolerans (Fig. 5.7). Further, Average Nucleotide Analysis (ANI) analysis value of 98 was observed to *Bacillus haoltolerans*. The genomic length was found to be 4.22 Mb (Table 5.5.).

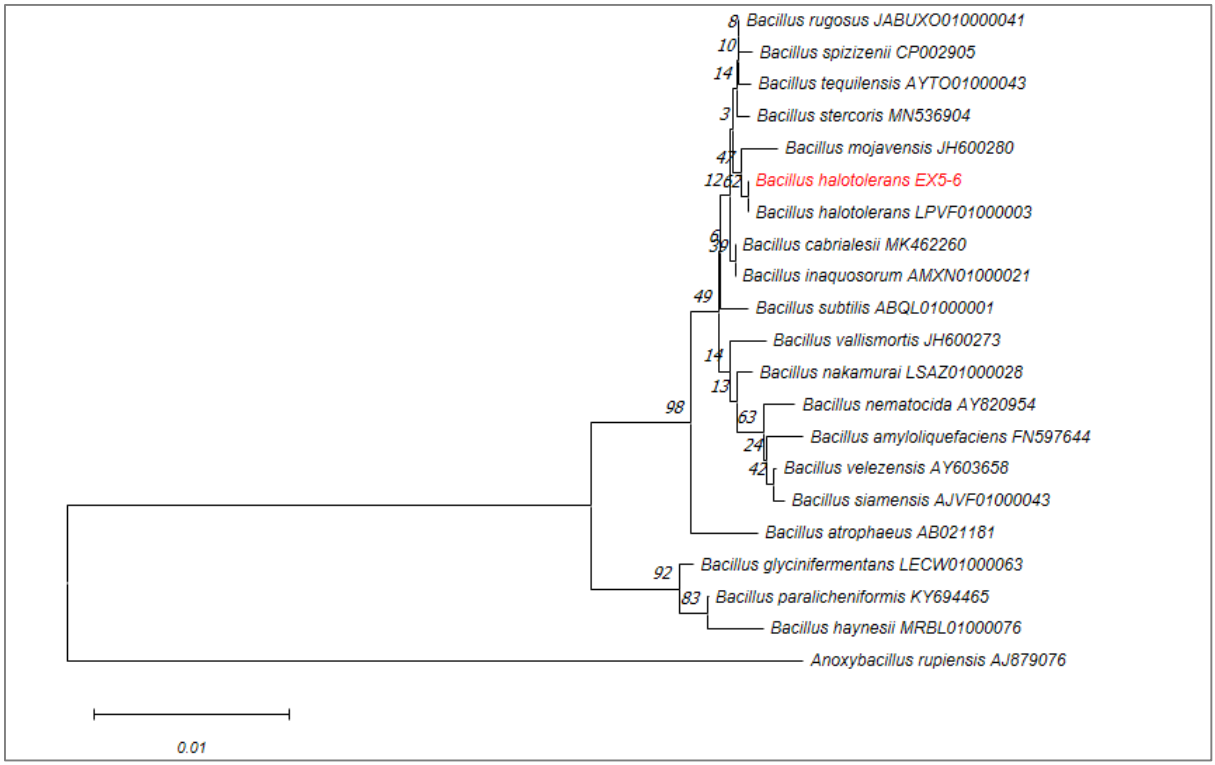

Fig.5.7. Molecular Phylogeny. 16S rRNA gene-based molecular phylogeny analysis using Neighbour Joining method. Red highlighted strain is the bacterial isolate used in the present study.

Table 5.5. Genome statistics of *Bacillus halotolerans* EX5-6

Sr. No.	***B. halotolerans* EX5-6**
1.	Total Sequence Length (bp):4226035
2.	Number of Sequences:4
3.	Longest Sequences (bp):4143713
4.	N50 (bp):
5.	Gap Ratio (%):0.000000
6.	GCcontent (%):43.5
7.	Number of CDSs:4251
8.	Average Protein Length:289.4
9.	Coding Ratio (%):87.3
10.	Number of rRNAs:17
11.	Number of tRNAs:72
12.	Number of CRISPRs:0

The genome analysis using RAST server indicated the presence of following enzymes invoved in the synthesis of 2,3-BDO. The findings further substantiated the ability of EX5-6 to produce 2,3-BDO (Table 5.6).

Table 5.6. 2,3-butanediol genes/enzymes mining using RAST server

Sr. No.	Enzymes/Genes	Encoded functions
1.	Transcriptional regulator of alpha-acetolactate operon AlsR	Regulation of promoter of alsSD operon responsible for acetoin formation
2.	Acetolactate synthase	Conversion of pyruvate to alpha-acetolactate
3.	2,3-butanediol dehydrogenase, R-alcohol forming, (R-) and (S-)-acetoin specific	Reduce diacetyl to acetoin and then to 2,3-butanediol
4.	Transcriptional activator of acetoin dehydrogenase operon *AcoR*	Positive transcriptional regulator (Acetoin to diacetyl)
5.	Alpha acetolactate decarboxylase	Catalyzes conversion of alpha-acetolactate to acetoin
6.	2,3-butanediol dehydrogenase, S-alcohol forming, (S-)-acetoin specific	Reduce diacetyl to acetoin and then to 2,3-butanediol

5.1.3.2. Optimization of 2,3-BD production using *Bacillus halotolerans* EX5-6

The optimization of media (carbon source and nitrogen source) and process parameters (temperature, pH, and time) for the production of 2,3-butanediol were conducted using the One Factor at a Time (OFAT) strategy using *Bacillus halotolerans* EX5-6. The initial medium consisted of 50 g/L Glucose, 10 g/L Yeast Extract and Peptone each, NaCl 5g/L, K2HPO4 0.3%, and MgSO4.7H2O 0.02%. Media with different pH (4.0, 5.0, 6.0, 7.0, and 8.0) was utilized for 2,3-BDO production.

pH optimization

The pH of the medium was adjusted using 0.1M HCL and 0.5N NaOH. The highest BDO titer (g/L) was obtained at pH 5.0 (Fig. 5.8). As the pH was increased, we observed a

drop in BDO production titer. At lower pH 4.0, the growth was slow, and we did not observe BDO production in 20 hours. The pH optimization suggested *B.halotolerans* EX5-6 prefers a range of pH 5.0 to 6.0 for 2,3-BDO production. The pH is a critical factor for microbial production of metabolites. As per the previous studies, the optimal pH for *Bacillus* species ranges from 6 to 7.0, predominantly at 6.5 (Tsigoriyna et al., 2021; Raspoet et al., 1991, Maina et al., 2022; Petrov and Petrova, 2021). Further, a study by Bao et al. 2019, indicates that pH optima for butanediol dehydrogenase is different for its oxidation (pH 8.5) and reduction activities (pH 6.5). The study also suggested that at higher pH, BDO is predominantly converted into acetoin. It has been reported at pH 7.0, enzymes such as lactate dehydrogenase, which are involved in organic acid synthesis, show higher activity (Hakizimana et al., 2020). The strain in the present study produces maximum titer at pH 5.0, essential for reducing acidic byproducts.

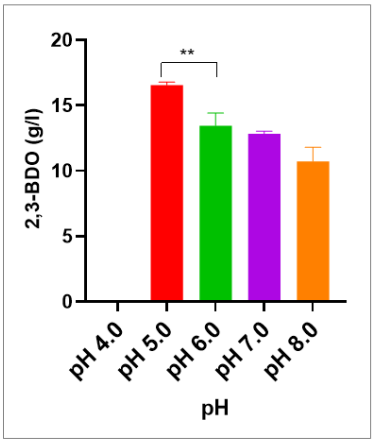

Fig. 5.8. Optimization of pH for 2,3-BDO production. Statistical tests were performed using One-way Anova with Tukey's multiple comparison test

Effect of different carbon sources

We tested the effect of 9 different carbon source on 2,3-BDO production by *B.halotolerans* EX5-6. These included polysaccharides (xylan, cellulose, and starch), Dissacharides (sucrose, galactose), xylose, glucose, fructose, and glycerol. We observed

production of 2,3-BDO in each of the sugars, with highest in glucose, followed by fructose, and sucrose in 24 hours from 50g/L of sugars. The production of BDO xylan and cellulose, indicates the potential of EX5-6 in utilising complex polysaccharides without any pre-treatment (Fig 5.9).

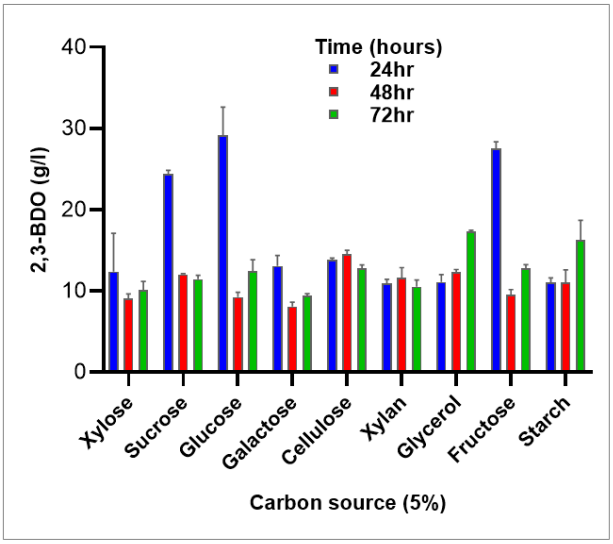

Fig. 5.9. Effect of different carbon source on the production of BDO by *Bacillus halotolerans* EX5-6

Effect of different Nitrogen sources

Further, the influence of different inorganic and organic nitrogen sources (1 % of Yeast extract, Peptone, Malt extract, Beef extract, Urea, and Ammonium chloride) was assessed on the production of BDO by EX5-6 strain. We observed a significantly higher BDO titer (12.86 g/L) in 1% YE compared to the other nitrogen sources, where the production titer remained similar to the control fermentation media with no added nitrogen sources. Previous studies for BDO production by *Bacillus* species such as *B. licheniformis, B.paralicheniformis,* and *B.subtilis* have reported Yeast extract as one of the critical factors for BDO production (Białkowska, 2019; Yuan et al., 2017; Maina et al., 2019) (Fig. 5.10).

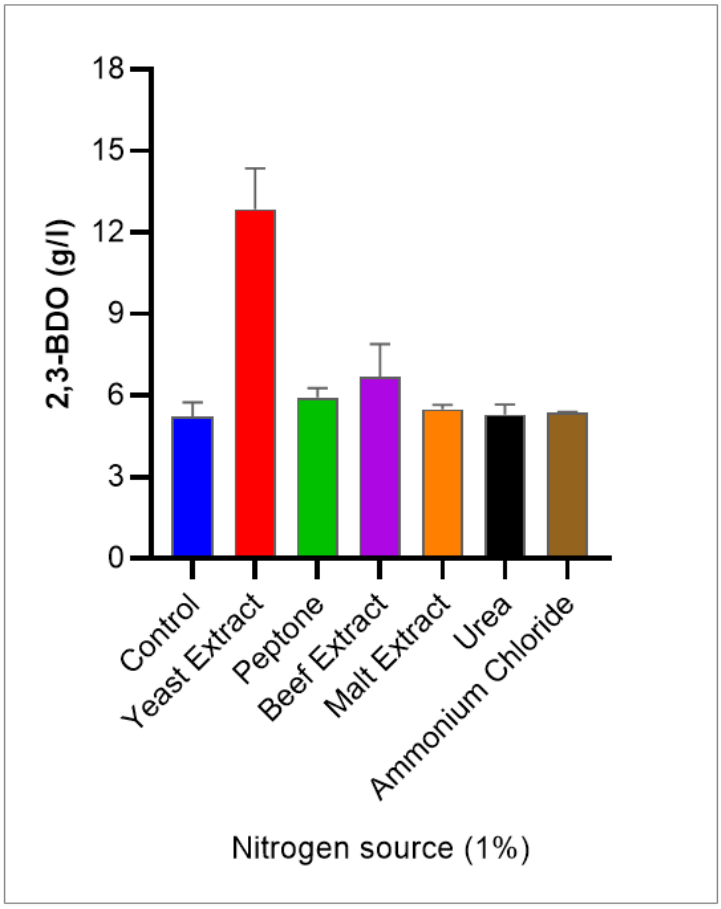

Fig. 5.10. Effect of different carbon source on the production of BDO by *Bacillus halotolerans* EX5-6

Optimization of glucose concentration

Although we did not observe any significant differences in the BDO production titer when glucose or fructose was used as a carbon source, considering the cost, we utilised glucose as a carbon source for further experiments. Glucose concentrations (10%, 15%, and 20%) were optimised for the production of BDO by EX5-6 isolate using 1% YE, 10% inoculum, NaCl 5g/L, K_2HPO_4 0.3%, $MgSO_4.7H_2O$ 0.02%, and pH 5.0 at 28°C at 180 rpm. The highest production titer (21 g/L) was obtained in 15% glucose at 48 hours, followed by 10% glucose. At 20% concentration, we observed an increase in titer at 72 hours; however, it was less than

15% glucose at 48 hours. Considering the productivity and titer, 15% was found to be the optimum concentration for the production of BDO.

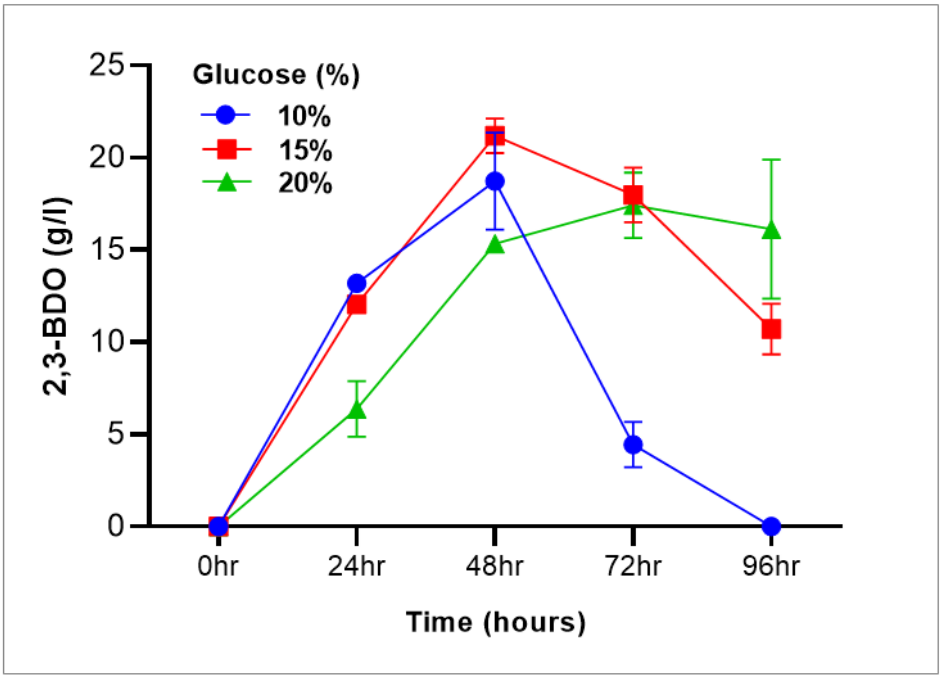

Fig. 5.11. Optimization of Glucose concentration for the production of 2,3-butanediol. Tukey's multiple comparisons test was used for statistical assessment with $p<0.05$

Optimization of Yeast Extract (YE) concentration

Different concentration of YE was supplemented in the media containing 15% glucose, 0.5% NaCl, 0.3% K2HPO4, 0.02% MgSO4.7H2O, and pH 5.0. The effect of YE was clearly observed on BDO production and positively correlated with YE concentration. We observed the highest production titer, 57.26 g/L, in 3% YE; however, not significantly higher than 2.5% YE, which yielded a titer of 53 g/L at 72 hours. A 2.5-fold increase was observed in the BDO titer from the initial medium containing 1% YE. Thus, for further experiments, 2.5% YE concentration was used (Fig. 5.12).

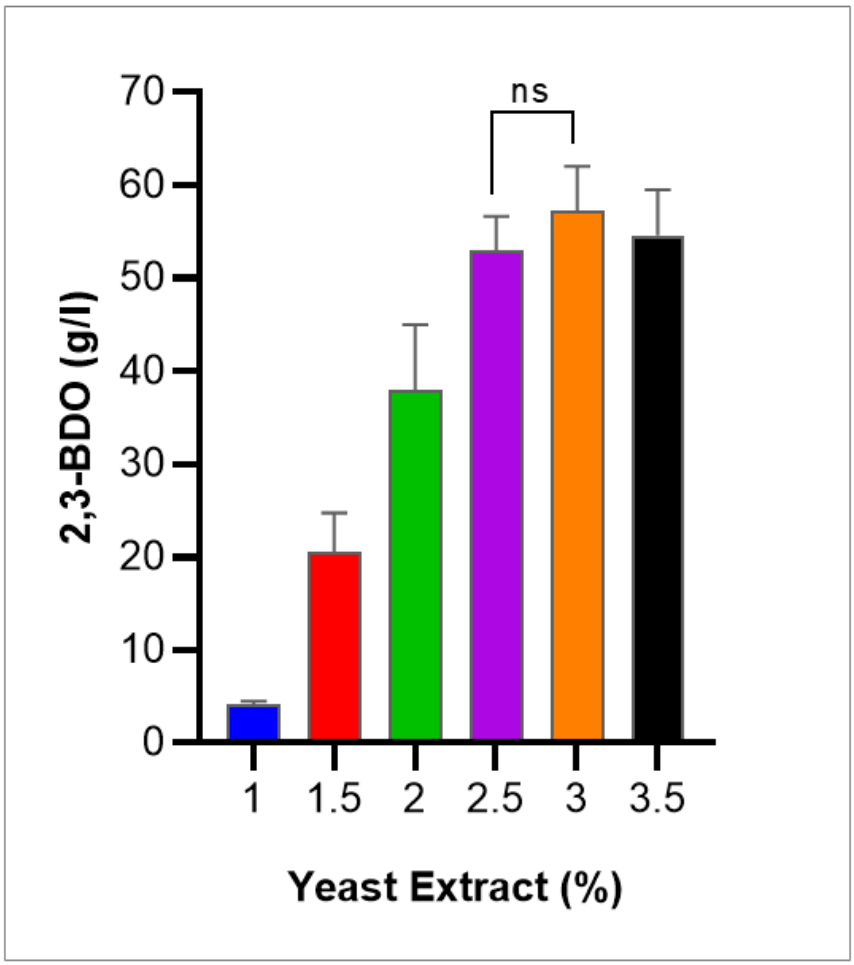

Fig. 5.12. Optimization of Yeast Extract concentration for the production of 2,3-butanediol. One-way ANOVA Tukey's multiple comparisons test was used for statistical assessment with $p<0.05$

Optimization of Inoculum concentration

The inoculum concentration was optimized at different concentrations of 4%, 6%, 8%, and 10% of OD 8.0 culture with 15% glucose, 2.5% YE, 0.5% NaCl , 0.3% K2HPO4, 0.02% MgSO4.7H2O, and pH 7.0. The highest BDO titer was obtained at 8% inoculum concentration with 60 g/L of BDO in 72 hours.

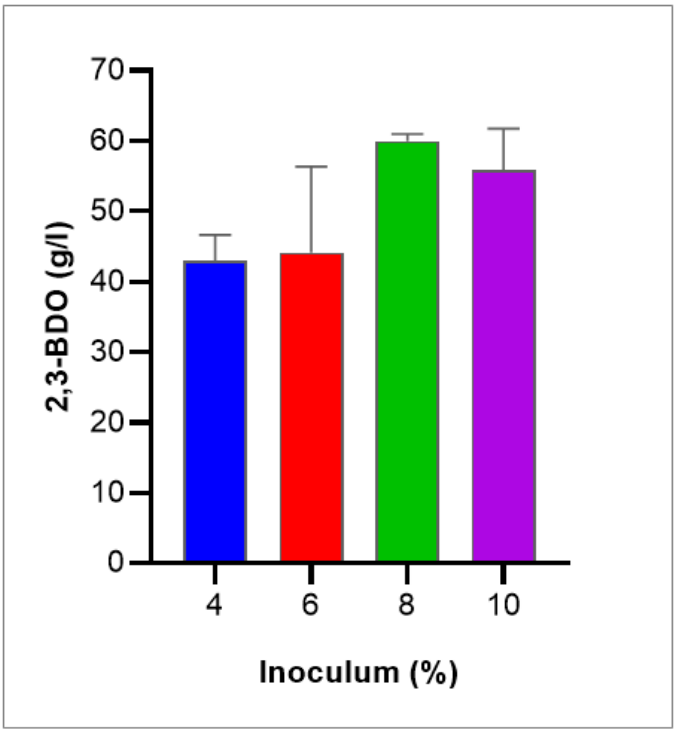

Fig. 5.13. Optimization of bacterial Inoculum concentration. One-way ANOVA Tukey's multiple comparisons test was used for statistical assessment with $p<0.05$

Optimization of Temperature

The temperature optimum for the production of metabolites is strain-dependent. In most cases for Bacillus species, 37°C is reported as the optimal temperature for BDO production. Studies by Ohair et al., 2020 and 2021, utilized higher temperatures for the 2,3-BDO production to optimize the non-sterile fermentation of food waste. The temperature optimization for BDO was performed at 28°C, 37°C, and 45°C with the prior optimized media. We observed non-significant differences in the BDO titer at 28°C and 37°C, 69.11 g/L and 68.14 g/L, respectively. Considering the cost factor at 37°C, 28°C was considered for further experiments (Fig. 5.14).

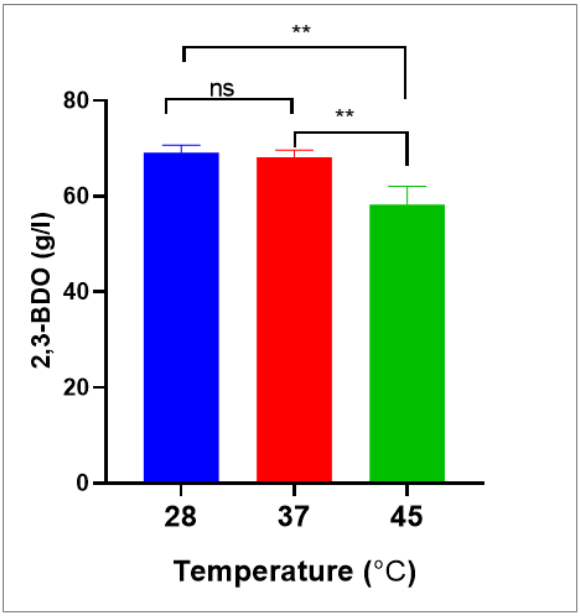

Fig. 5.14. Optimization of temperature for the production of 2,3-BDO. One-way ANOVA Tukey's multiple comparisons test was used for statistical assessment with $p<0.05$

Validation of optimized media and process parameters

The initial fermentation media constituted of 50 g/L Glucose, 10 g/L Yeast Extract and Peptone each, NaCl 5g/L, K2HPO4 0.3%, and MgSO4.7H2O 0.02% with process parameters as temperature of 28°C and pH 7.0. The final optimized media and parameters constituted 150 g/L Glucose, 25 g/L Yeast Extract, NaCl 5g/L, K2HPO4 0.3%, and MgSO4.7H2O 0.02% with 28°C temperature and pH 5.0. We validated the optimized media and conditions to validate the results. Both HPLC and GC were utilized for the detection of metabolites. We observed glucose (150 g/L) consumption by 72 hrs with the highest BDO titer of 68.4 g/L, followed by an increase in acetoin concentration after 72 hours. The GC was performed for the final BDO titer estimation. We obtained 73.19 g/L BDO with yield and productivity of 0.5 g/g and 1.02 g/l/h, respectively (Fig. 5.16). Compared to the unoptimized media and process parameters, we observed a more than 3-fold increase in BDO titer with a simultaneous increase in yield and productivity. Interestingly, *B.halotolerans EX5-6* was able to maintain the low level of acetoin 3.2 g/L, which is generally reported to be a major byproduct in several studies (Fig. 5.15).

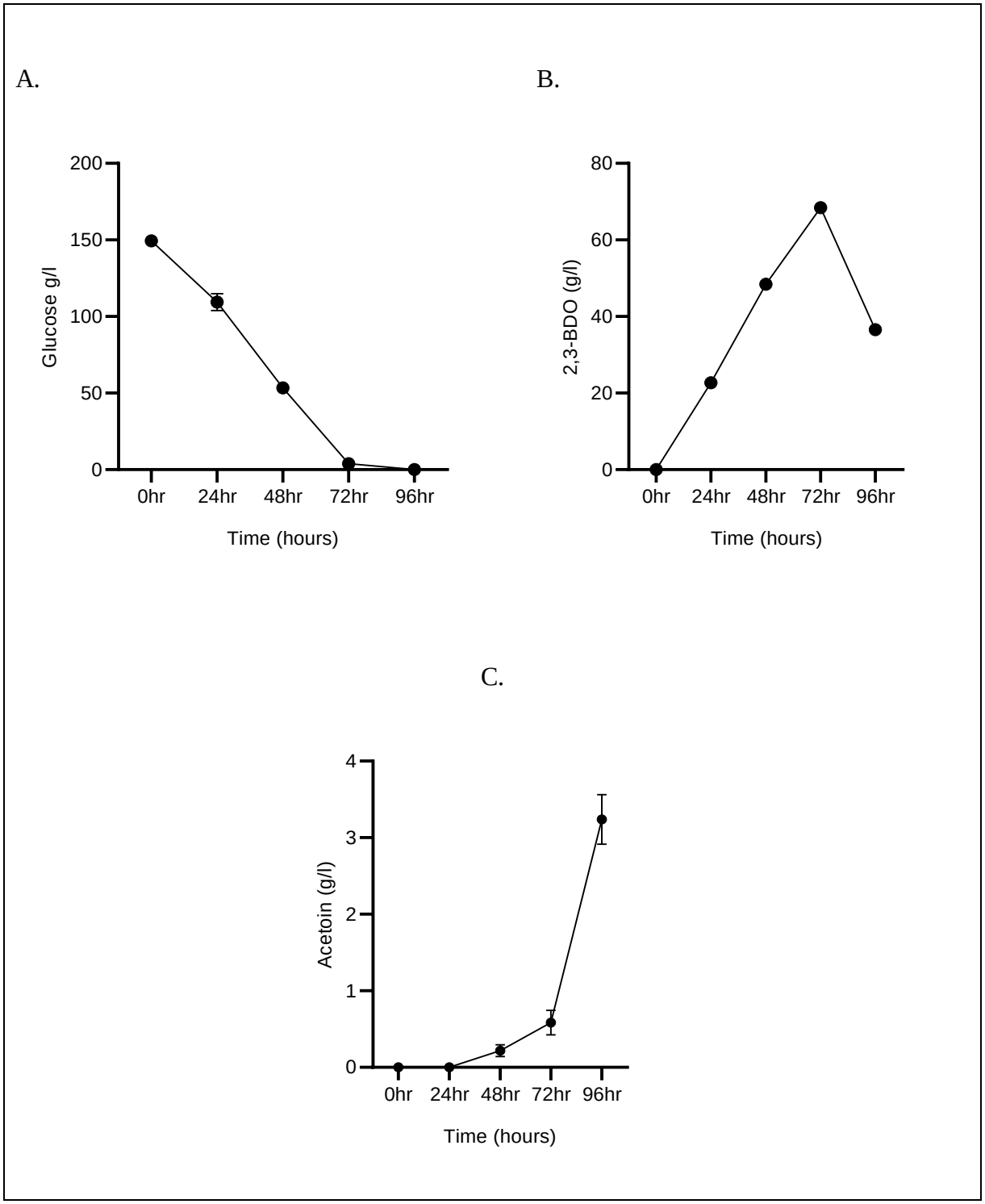

Fig. 5.15. Validation of optimized media using HPLC-based detection. Effect on the production of 2,3-BDO (B), acetoin (C), and consumption of Glucose (A).

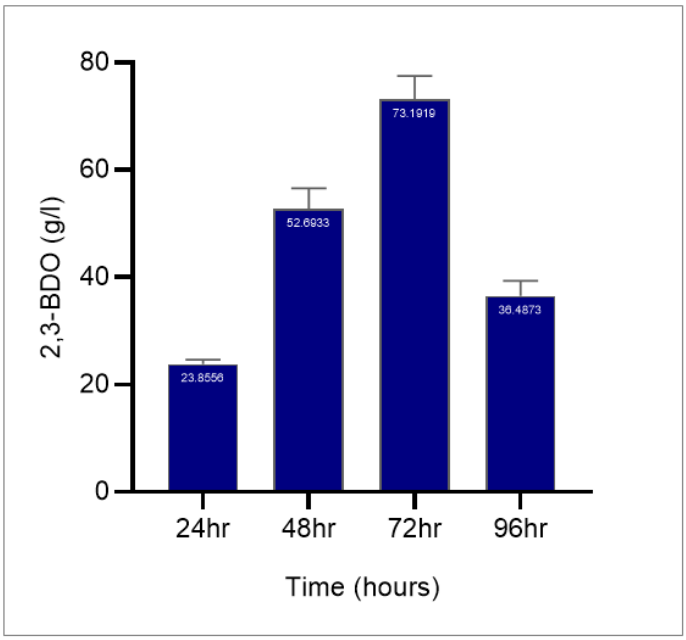

Fig. 5.16. 2,3-BDO production by Bacillus halotolerans EX5-6 using the optimized media and process parameters

High production titre, yield, productivity and lesser by-products at the flask level illustrate the promise of EX5-6 isolate in producing BDO. To our knowledge, this is the first study of BDO production from *Bacillus halotolerans*. In comparison to other *Bacillus* species reported at shake flask level, such as *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus velezensis*, *Bacillus safensis*, and *Bacillus toyonensis*, the isolate used in the present study performed better with high titer and productivity.

Table 5.7. Comparision of 2,3- butanediol production from different *Bacillus* species and *Bacillus halotolerans EX5-6*

Sr. No.	Species	Carbon source	Production titer (g/L)	Yield (g/g)	Fermentation process	References
1.	*B. subtilis*	Glycerol	11.10	0.27	Batch flask	Petrova et al., 2020
2.	*B. velezensis*	Glucose	18.1	-	Batch flask	Petrova et al., 2020

3.	*B.safensis*	Glucose	13.9	-	Batch flask	Petrova et al., 2020
4.	*B. toyonensis*	Glucose	12.0	-	Batch flask	Petrova et al., 2020
5.	*B. licheniformis*	Glucose	144.7	0.4	Fed-batch	Jurchescu et al., 2013
6.	*B. subtilis*	Sucrose	132.4	0.45	Fed-batch	Wang et al., 2021
7.	*B.amyloliquefaciens*	Glucose	92.3	-	Fed-batch	Yang et al., 2011
8.	***B.halotolerans***	**Glucose**	**73.19**	**0.5**	**Batch flask**	**Present study**

5.1.3.3. Production of 2,3-BDO from rotten tomato juice concentrate

The rotten tomatoes were processed as described in the protocol, and the resultant concentrates were employed for 2,3-BDO synthesis under the optimum conditions outlined above. We saw inhibition of *B.halotolerans* EX5-6 at 3X or 4X concentrations. At 2X concentration, however, 16.62 g/L of BDO was produced by EX5-6 at a productivity of 0.69 g/L/h (Fig. 5.17.A). The acetoin was the other major product with a production titre of 2.39 g/L. Because acetoin is easily converted to 2,3-BD, numerous investigations combine its yield with BDO. As a result, the cumulative BDO titre from 2X tomato juice is 19.01 g/L. There was a decrease in BDO production when Tomato concentrate was supplemented with optimal medium ingredients, indicating that tomato concentrate is a comprehensive nutrient substitute for BDO production (Fig. 5.17.B.). It might replace all of the medium components and can prove to be a cheap alternative media. Moreover, our novel process of producing 2,3-BDO from tomatoes resulted in a higher production titer than the earlier reported study by OHair et

al., 2021, where they used mixed food waste (which included tomatoes) and comparable to different lignocellulosic hydrolysates which require several costly pre-treatment processes (Table 5.8.).

5.17.A. **5.17.B.**

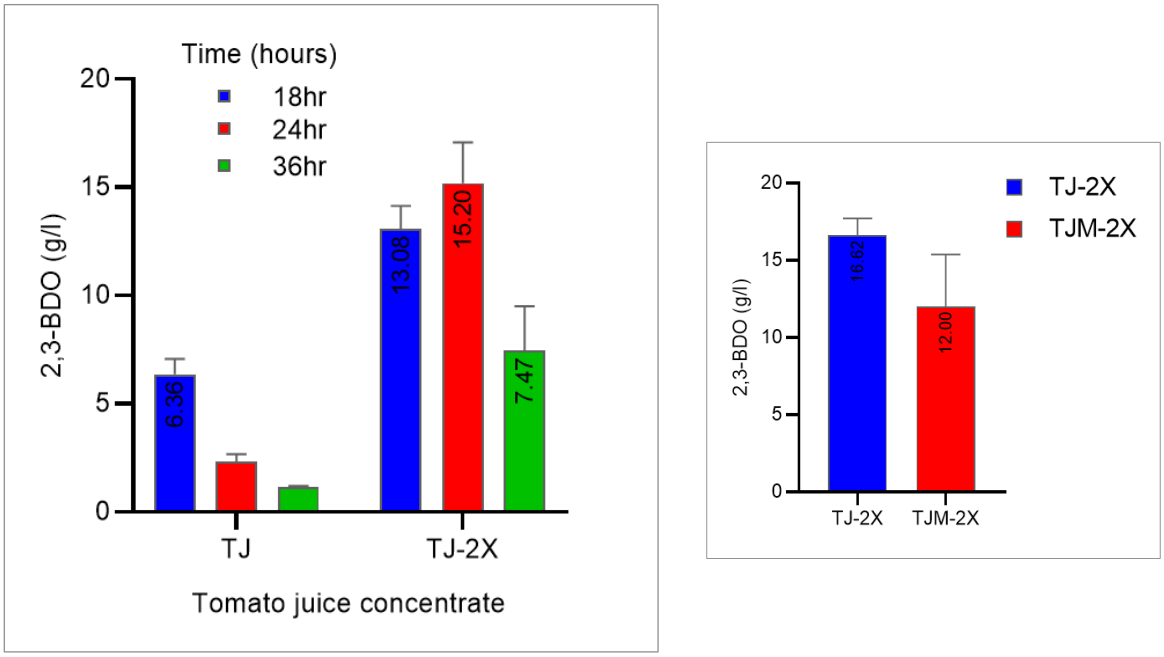

Fig. 5.17. Production of 2,3-BDO from Tomato concentrates. **A.** At different concentration of tomato concentrate, **B.** With 2X concentrate of tomato and with the addition of optimized medium nutrients except carbon source (TJM-2X).

Table 5.8. Production of 2,3-BDO using different types of waste

Sr. No.	Waste	Other Nutrient	*Species*	2,3-BDO (g/L)	Productivity (g/L/h)	Process mode	References
1.	Tomato waste	None	*Bacillus halotolerans EX5-6*	16.62	0.69	Batch flask	Present study

2.	Mixed food waste (containing mixture of potato, pepper, strawberry, tomato, onion, cabbage, and pineapple)	None	*Bacillus licheniformis YNP5-TSU*	4.3	0.18	Batch flask	OHair et al., 2021
3.	Sugarcane bagasse hydrolysate	Yeast Extract and Casamino acids	*Enterobacter aerogenes*	114.3	0.44	Fed-batch	Kim et al., 2020
4.	Bakery waste hydrolysate	Yeast Extract	*Bacillus amyloliquefaciens*	55.2	0.42	Batch Fermentation	Maina et al., 2021
5.	Corn stover hydrolysate	-	*Parnibacillus polymyxa*	18.8	0.31	Continuos feremen tation	Ma et al., 2018
6.	Fruit and vegetable hydrolysate	Yeast ectract, Malt extract, and peptone	*Enterobacter ludwigii*	50	0.4	Fed-batch	Liakou et al., 2018

5.1.4. Conclusion

The current study is the first to report synthesizing 2,3-butanediol from tomato waste. Notably, *Bacillus halotolerans* has never been reported to produce BDO. The optimized synthetic

medium contained Glucose 15%, K2HPO4 0.3%, Yeast Extract 2.5%, Sodium chloride 0.5%, MgSO4.7H2O 0.02%, pH 5.0, and was incubated at 180 rpm @ 28°C. Overall, we found a 2.5-fold improvement in BDO production from a non-optimized medium. We got a concentration of 73.19 g/l of 2,3-BDO, with yield and productivity of 0.5 g/g and 1.02 g/l/h, respectively. Compared to optimal synthetic media, concentrated tomato juice from rotten tomatoes yielded 16.62 g/l of 2,3-BDO with productivity of 0.7g/l/h, which is high compared to many other publications utilizing other lignocellulosic and other wastes. The advantages of using our BDO production process include waste management and green synthesis because the process does not require any pre-treatment steps, unlike other biowastes such as lignocellulose biomass, serves as a complete nutrient media and does not require any external nutrient supplementation, cost-effective because the substrate rotten tomatoes contain carbon and other nutrient sources, thereby eliminating the need for glucose, yeast extract, and ozone. Future studies will focus on scaling it up to the fermenter level.

5.1.5. References

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Chapter 5. To develop the microbial process for conversion of biomass-waste to 2,3-butanediol and Poly-γ-Glutamic Acid

Chapter 5.2. Production of Poly-gamma-glutamic acid by *Bacillus velezensis* WA11

Abstract

Poly-γ-glutamic acid (γ-PGA/PGA) is one of the most promising biopolymers, with applications in food, cosmetics, and a variety of other industries due to its various functional properties. A homopolyamide composed of glutamic acid monomers linked by amide bonds is one of the most expensive polymers chemically. Microbial fermentation is used to produce PGA because it has various advantages over other approaches including chemical and peptide synthesis. However, in order to establish an efficient and cost-effective procedure for the industrial manufacturing of PGA, the cost of the raw substrate required for its production must be considered. PGA is one of the most promising biopolymers and is implicated in a wide range of applications in food, cosmetics, and several other industries owing to its several functional features. Chemically, a homopolyamide comprising glutamic acid monomers linked with amide bonds is one of the costliest polymers. Microbial fermentation is employed for γ-PGA production as it has several advantages over other methods, such as chemical and peptide synthesis. However, for developing an efficient and cost-effective process for the industrial production of γ-PGA, it is essential to reflect on the cost of the raw substrate used for its production. Several studies have optimized PGA production from various synthetic and waste-based media; substrate and pre-treatment expenses remain significant barriers to industrial production. In this investigation, we used untreated sugarcane bagasse to produce PGA with *Bacillus velezensis* WA11. We report 104.3 g/L -PGA synthesis with productivity of 1.09 g/L/h in the optimized synthetic medium containing Maltose as a carbon source, which is 2.9-

fold greater than the earlier study using Maltose as a carbon source. We recovered 11 to 12 g/L of crude dry PGA from bagasse medium compared to synthetic media. The current research aims to provide a sustainable approach for the production of PGA.

5.2.1. Introduction

Polymers are implicated in various applications in food, medicine, and other industries. Owing to the recalcitrant nature of the synthetic polymers, their utility in day-to-day life could be problematic. As a result, there is a need for degradable and non-toxic polymers, which have lesser environmental concerns. Biopolymers, which are synthesized by biological entities, primarily by microbes, are becoming more popular due to their non-toxic, degradability, and several other environmental benefits (Baranwal et al., 2022; Kreyenschulte et al., 2014). Biopolymers are functionally significant for microbes and are involved in storage functions during starvation, defence functionalities such as biofilm formation, and various others. There are various classes of biopolymers depending on their monomeric constituents and chemical bonds, which include polyesters, polysaccharides, polyphosphates, and polyamide types of biopolymers (Rehm et al., 2010). Polyester polymers such as Polyhydroxyalkanoates mainly serve as energy and carbon sources and chemically involve ester bonds. Polyphosphate groups of polymers are negatively charged, containing anhydride-bonded phosphate groups, controlling cell communications and influencing several microbial functions such as energy storing, virulence, and survival (Sehgal et al., 2020). Polysaccharide groups are one of the abundant types of biopolymers, including Cellulose, Pullulan, Xanthan and several others. They are mainly involved in biofilm formation and energy storage (Schmid et al., 2015; Mohammed et al., 2021). Polyamides such as Polyglutamic acid and polylysine are positively and negatively charged and are considered one of the best alternatives implicated in various applications such as flocculants, cosmetics, and antimicrobials (Moradali et al., 2020) (Table 5.9.).

5.2.1.1. Functional attributes and applications

Poly-γ-glutamic acid (γ-PGA/PGA) is a naturally occurring and biodegradable polymer that is chemically an anionic homopolyamide comprised of L- and D-glutamic acid linked by amide

linkages (Luo et al., 2016; Li et al., 2022). The connecting peptide bonds are formed by alpha-amino group of one GA and gamma-carboxylic acids of another glutamic acid molecule (Fig. 5.18). γ-PGA possess variety of properties, which includes water solubility and retention, non-toxicity, biodegradability, edible, antimicrobial activity, and resistance to high heat.

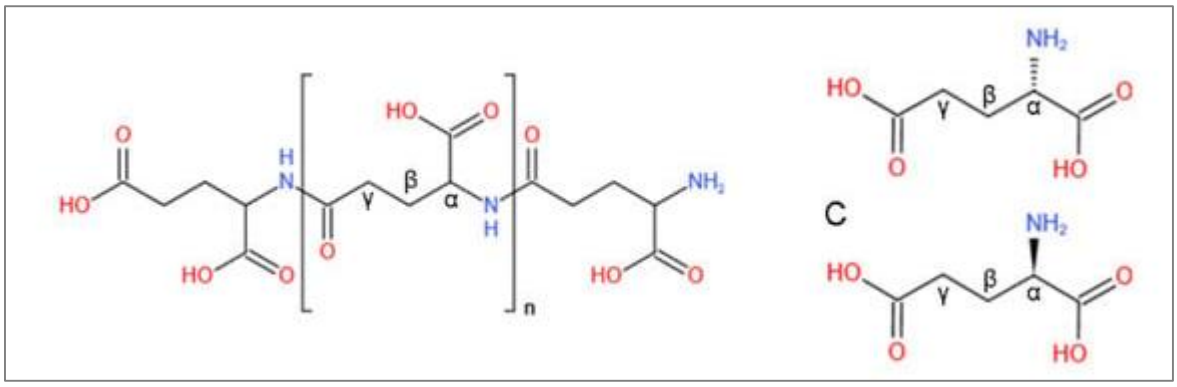


Fig.5.18. Structural formula of γ-PGA and its constituent units. The polymer of γ-PGA (n: repeating units approach at least 10,000) (A) and the L-glutamic acid monomer (B) and D-glutamic acid monomer (C) of γ-PGA (Li et al., 2022).

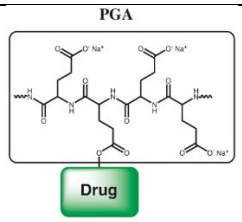
The PGA biopolymer is widely used in various applications due to its unique biological features, including agricultural and food processing, medical treatments, cosmetics, and others (Li et al., 2022) (Table 5.10).

Table 5.9. Comparison of biopolymers (Gheorghita et al., 2021)

Material characteristics	**Biopolymers**	**Synthetic Polymers**
Main Source	Agro-resources	Petroleum and gas
Biodegradability	Yes	No/slow
Structure	Well defined	Stochastic
Chemical Backbone	Carbon, oxygen, and nitrogen	Mostly carbon
Dispersity	Unity	>1
Physicochemical resistance	Low	High

Toxicity	Low	High
Thermal stability	Low	High
Mechanical properties	Low	High
Sustainability	High	Low
Availability	High	Decreasing
Cost	High (Depends on the type)	Low

Table 5.10. Applications of Poly-gamma-glutamic acid in various fields (Information adapted fron Luo et al., 2016 and Nair et al., 2023a)

Field	Applications	Products
Medicine	Metal chelator, Drug carrier, Gene vectors, Tissue engineering	
Bioremediation	Biopolymer flocculant, Metal chelates, Dry removal	
Food industry	Texture enhancer, Food supplement, Oil-reducing agent, Cryoprotectant, Thickener, Animal feed	
Others	Biocontrol agent, agriculture, Moisturizer	

5.2.1.2. Biological functions of gamma-PGA

Because of its functional qualities, the PGA biopolymer is in high demand; nevertheless, its low yield and high production costs limit its implementation (Nair et al., 2023a). It is now produced utilizing four methods: peptide synthesis, biotransformation, chemical synthesis, and microbial-based manufacture. Microbial-based PGA manufacturing is one of the most favourable processes compared to other processes because it has fewer environmental concerns, employs cheaper raw substrates, and has higher production with mild reactions (Luo et al., 2016). Polyglutamic acid has several functional role for microbes. Some microbes produce it for defence mechanism in order to evade antimicrobial agents such as peptides, antibodies, and others use it as a nutrient source. *Bacillus anthracis* produces capsular PGA to to protect itself from phagocytic activity thus evading immune defense system. Morikawa et al in 1992, showed the significance of biofilm formation with the aid of Polygammaglutamate polymer.

5.2.1.3. Microbial biosynthetic pathway

The most crucial component for microbial synthesis of PGA is the availability of either endogenous (alpha-ketoglutarate precursor) or exogenously supplied glutamic acid (GA). The entire synthetic pathway can be divided into four stages: i. Racemization ii. Polymerization iii. Regulation, and iv. Degradation (Fig. 5.19). Both D- or/and L- forms of glutamate can be utilized to synthesize homopolymeric or heteropolymeric gamma-PGA. L-GA can be provided exogenously or can be synthesized by 2-oxoglutarate aminotransferase (Nair et al., 2023a; Li et al., 2022). However, for gamma-PGA, using D-GA residue requires enzyme racemases catalyzing the conversion of D-form to L-form. *RacE/glr* and *yrpC* are the two homologous glutamate racemase genes reported in Bacillus subtilis (Luo et al., 2016). Polyglutamate synthase (encoded by *pgsB, C, A*, and *E* gene in *B.licheniformis* and by *ywsC, ywtAB*, and *capBCA* in *Bacillus subtilis*) is involved in y-PGA polymerization. *Pgs B* and C form the active

catalytic site, whereas *pgsA* removes the polymer chain from the active site. Gamma-PGA regulation involves two signalling mechanisms, which include *ComP* and *A* regulator and *DegQ*, *DegS-U*, and *Swr* system. *DegQ* has been reported to promote the synthesis of PGA along with *SwrA*. *DegU-P* is another activator of gamma-PGA apart from swrA (Osera et al., 2019; Luo et al., 2016). During nutrient limitation, microbes producing PGA secrete peptidases such as Exo-γ-glutamyl peptidase (*Ggt*) and γ-glutamyl transpeptidase (*GGT*) that are capable of cleaving gamma-PGA, which can serve as a source of nutrients.

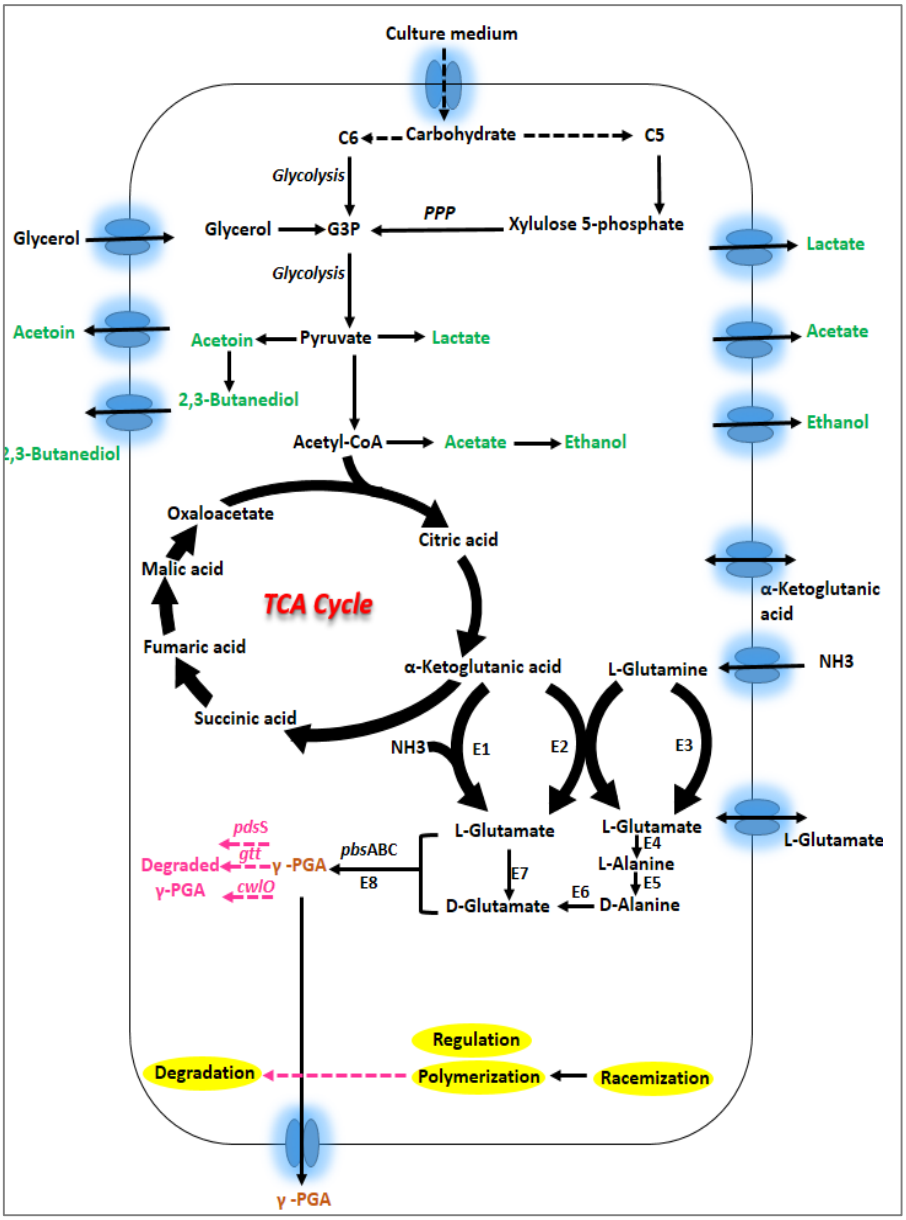


Fig. 5.19. Microbial biosynthesis of γ-PGA [concept adapted from Luo et al, 2016]. Substrates in the culture media were largely a range of biomass materials, cane molasses, and agro-industrial wastes that could be degraded into C6 and C5 compounds before entering the primary carbon metabolism via glycolysis and the pentose-phosphate pathway. Furthermore, glycerol and citrate cycle metabolic intermediates were used as possible substrates. The main byproducts were acetoin and 2,3-butanediol; other byproducts with little production were lactate, ethanol, and acetate. PPP pentose phosphate pathway, G3P glyceraldehyde 3-phosphate, E1 glutamate dehydrogenase (GD), E2 glutamate 2-oxoglutarate aminotransferase, E3 glutamine synthetase (GS), E4 l-glutamic acid: pyruvate aminotransferase, E5 alanine racemase, E6 d-glutamic acid: pyruvate aminotransferase, E7 direction conversion, E8 PGA synthetase.

Since γ-PGA have several applications in food and medicine industries, it becomes important to have a safe microbial producer, which should not produce any immunogenic or toxic substances simultaneously during fermentation process. Hence, *Bacillus* species such as *B. paralicheniformis, B.subtilis, B.licheniformis*, and others, which are generally regarded as safe organisms are used for the production of γ-PGA. These strains can be classified in to two categories: those requiring exogenous supply of glutamic acid are known as glutamic acid dependent and those which can produce without any addition of external GA are known as glutamic acid independent producers. The table 5.10. provide the details of production of γ-PGA by different bacteria.

Table 5.10. State of art for the high γ-PGA production

Strains	Major nutrients	Yield	Productivity (g/L/h)	References
Bacillus paralicheniformis NCIM5769	Sucrose, L-glutamic acid, citric acid, and ammonium nitrate	284 g/L	3.94	Nair et al., 2023b

Bacillus velezensis CAU263	Guar meal, L-glutamic acid	158.5 g/kg	-	Liu et al., 2022
B. subtilis CGMCC1250	Glucose, glutamate, yeast extract	101 g/L	2.19	Huang et al., 2011
B. subtilis NX-2	Glucose, glutamate, $(NH_4)_2SO_4$	71.21 g/L	1.24	Xu et al., 2014
B. subtilis MJ80	L-glutamic acid, glycerol, citric acid, starch,urea	68.7 g/L	0.95	Ju et al., 2014
B. subtilis ZJU-7	Sucrose, tryptone,L-glutamic acid	58.2 g/L	2.42	Shi et al., 2006
B. subtilis ZJU-7	Glutamic acid, glucose, tryptone	54 g/L	0.84	Chen et al., 2010
Bacillus.sp. RKY3 KCTC 10412BP	Glutamic acid, glycerol, citric acid, NH4Cl	48.7 g/L	2.02	Jeong et al., 2010
B. subtilis F-2-01	Veal infusion broth, glucose, L-glutamic acid	48 g/L	0.51	Kubota et al., 1993
B. licheniformis P-104	Glucose, sodium glutamate, sodium citrate	41.6 g/L	1.06	Zhao et al., 2013

5.2.1.4. Problem statetment

Bacillus species are widely recognized to be among the most prolific producers of PGA (Sirisansaneeyakul et al., 2017; Nair et al., 2023a). Most *Bacillus* strains reported for PGA, such as *Bacillus subtilis, Bacillus paralicheniformis,* and *Bacillus licheniformis,* are generally regarded as safe (GRAS) organisms. As a result, they are heavily involved in its manufacture (Nair et al., 2023a; Luo et al., 2016). Even though microbial fermentation for PGA is well-established, with substantial genetic and metabolic route information, cost yield remains a

significant hurdle for industrial production. Microbial strains and culture mediums account for a sizable portion of the cost of PGA manufacturing. As a result, the method requires less expensive feedstock as a carbon or total medium supply and an effective microbial strain for exploiting complicated feedstock for PGA generation.

Researchers worldwide have refined and used various synthetic and waste-based mediums for PGA production, obtaining as much as 158 g/kg of guar gum in solid-state fermentation utilizing *Bacillus velezensis* (Liu et al., 2022). *Bacillus paralicheniformis* produced 284 g/L, *Bacillus subtilis* produced 101 g/L, *Bacillus licheniformis* produced 41.6 g/L, and *Bacillus siamensis* produced 25.22 g/L on synthetic media (Nair et al., 2023a and 2021). Several studies have investigated diverse wastes, ranging from hydrolysate of lignocellulosic biomass to food wastes, including tomato, and reported as high as 100 g/L of PGA (Nair et al., 2023). The requirement of a pretreatment method for releasing monosaccharide sugars from complex polysaccharides such as cellulose and hemicellulose is a fundamental restriction of the waste-based strategy. In this study, we propose producing -PGA directly from sugarcane bagasse, an agro-waste rich in lignocellulosic biomass, without pretreatment utilizing *Bacillus velezensis* isolated from Water Hyacinth.

5.2.2. Experimentals

5.2.2.1. Screening for cellulolytic, ligninolytic, and xylanolytic activities

Thirty bacterial isolates isolated from river sediments and water hyacinth roots were evaluated for lignocellulolytic potentials, as described by Falade et al., 2017. The bacterial isolates were grown in nutrient broth for 12 hours at 37 °C. Following incubation, one loopful of overnight grown culture was spot inoculated for 48 hours at 37 °C in Bushnell Hass agar medium supplemented with different carbon sources (0.5% Carboxymethyl cellulose (CMC), 0.5% Beechwood xylan, 0.2% Veratryl alcohol (VA), and Guaiacol (GA) and yeast extract

(0.1%). The activity was confirmed by monitoring the zone of degradation obtained after the staining procedures were completed. 0.2% Congo Red was applied for 15 minutes for CMC and Xylan-containing plates, followed by two 15-minute washes with 5M Sodium chloride. For VA and GA plates, staining was done for 15 minutes with 1:2 diluted Grams Iodine, followed by washing with distilled water. Himedia and Sigma Chemicals supplied all of the chemicals.

5.2.2.2. Screening for γ-PGA producers, molecular characterization, and Genome sequencing

The bacterial colonies were inoculated in seed media (Glucose 2%, Yeast extract, Peptone, Sodium Chloride, and Beef extract 0.5%, pH 7.0). After overnight incubation at 37 °C, 5% inoculum was added in a fermentation medium (Glucose 5%, L-Glutamic acid monosodium salt monohydrate 4%, Na2HPO4 0.1%, NaH2PO4 0.7%, MgSO4.7H2O 0.02%, Yeast extract 1%, pH 7.0). Following a 24-hour incubation at 28 °C, the broth was centrifuged at 10,000 rpm for 20 minutes. The supernatant was then combined with 4X cooled methanol volume and examined for fibrous precipitate. Using 16S rRNA sequencing, the γ-PGA-producing isolate was molecularly characterized. The 4X chilled methanol was added to the resultant supernatant and was observed for fibrous precipitate. The molecular characterization was carried out for the γ-PGA-producing isolate using the 16S rRNA sequencing. The gene was amplified using 27F and 1492R universal primers and sequencing using a 3500 XL genetic analyzer (Thermofisher, USA). The NCBI accession number for the 16S rRNA sequence is OR263461. The phylogenetic analysis was conducted using MEGA v11 software using the Neighbour Joining Method.

DNA was extracted from bacterial isolates using a DNeasy Powersoil Pro kit (Qiagen, 47014). The 16S rRNA gene was amplified using 27F and 1492R universal primers and sequenced using a Sanger sequencer. The phylogenetic analysis was carried out using MEGA

v11 software using the Neighbour Joining Method. The genome sequencing was carried out using the protocol described in Yadav et al., 2021, using a MinION sequencer. Briefly, DNA having a purity of 1.7 to 1.9 (260/280) was used for library preparation. The library preparation was initiated with 1.0 µg/48 µl DNA using 1D Ligation sequencing kit SQK-LSK109. The raw reads were basecalled and demultiplexed using Guppy software (V 5.0.11) with fast basecalling mode, followed by sequence filtering using NanoFilt. Flye tool was used for the assembly of the fastq reads, followed by polishing using the Medaka tool. RAST (Rapid Annotation Server) was used for genome mining for Poly-gamma-gluatamic acid biosynthetic genes.

5.2.2.3. Optimization of γ-PGA production and characterization

The One Factor At a Time (OFAT) approach was used to optimize medium components, their concentration, and process parameters (pH, Temperature, and rotation speed). The seed medium composition was (Glucose 2%, Yeast extract, Peptone, Sodium Chloride, and Beef extract 0.5%, pH 7.0) for all the optimization experiments. The initial fermentation medium consisted of Glucose 5%, L-Glutamic acid monosodium salt monohydrate 4%, Na2HPO4 0.1%, NaH2PO4 0.7%, MgSO4.7H2O 0.02%, Yeast extract 1%, pH 7.0. The produced crude γ-PGA was characterized using Thin Layer Chromatography (TLC) using solvents n-Butanol: Acetic Acid: H2O in the ratio of 12:5:3, Fourier Transform Infrared (FTIR) Spectroscopy (ATR mode using a Bruker Tensor II spectrophotometer equipped with a diamond crystal probe detector. Absorbance spectra recorded at 500-4000/cm with resoultion of 4 cm–11), and Nuclear Magnetic Resonance (1H NMR, 10mg/mL sample was dissolved in D2O (deuterium oxide) and recorded 1H NMR spectra using Bruker AV 500 MHz). All the statistical analysis was performed in GraphPad PRISM v8.

5.2.2.4. Poly- γ-glutamic acid production from Bagasse

The sugarcane bagasse was collected from a local shop in Pune, India, and was immediately transferred to the laboratory. Further, the Bagasse was washed twice with tap

water to remove dirt, followed by Oven drying at 50°C until it was dried completely. The dried Bagasse was ground to coarse powder form using a home-based grinder. The resultant Bagasse was added with an optimized medium (except maltose as a carbon source) and incubated further for γ-PGA production (Fig. 5.20).

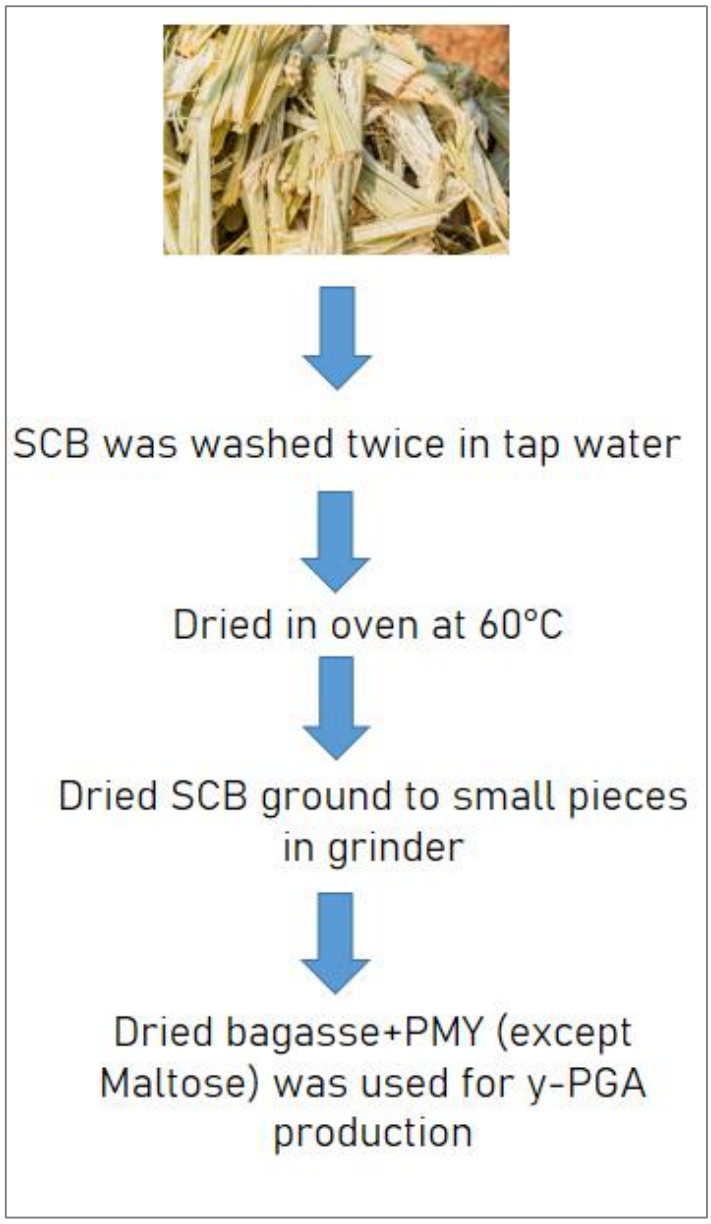


Fig. 5.20. Schematic of Poly-gamma-glutamic acid production from sugarcane bagasse

5.2.2.5. Culture and sequence deposition

The bacterial isolate *Bacillus velezensis* WA11 is deposited in NCIM at CSIR-National Chemical Laboratory (NCL), Pune, India as *Bacillus velezensis* (WA11) with culture ID NCIM 5825. The culture is also submitted in IDA facility of NCMR at National Center for Cell Sciences (NCCS), Pune, India with IDA accession ID MCC 0264. The NCBI accession number for 16S rRNA sequence is OR263461.

5.2.3. Results and Discussion

5.2.3.1. Screening of Ligninocellulolytic and xylanolytic activities and molecular characterization

We observed cellulolytic, ligninolytic, and xylanolytic activities in bacterial isolate WA11 and EX5-6 (Fig. 5.21.). Further, screening suggested WA11 positive for γ-PGA production. Therefore further optimization was carried out for γ-PGA using WA11 bacterial isolate (Fig. 5.22). Further thin-layer chromatography (TLC) confirmed that produced polymer consisted of glutamic acid monomers, indicting production of γ-PGA by WA11 bacterial isolate.

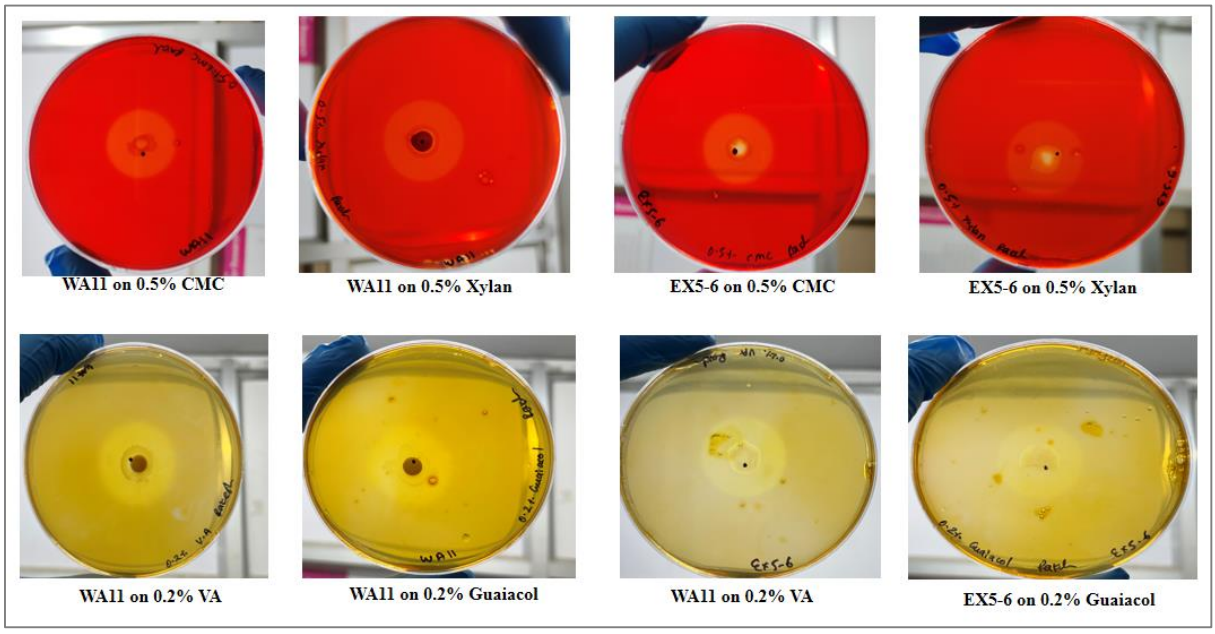


Fig. 5.21. Screening for Ligninocellulolytic activities. CMC: Carboxymethyl cellulose (low viscocity), VA is Veratryl alcohol. VA and Guaicaol are the chemical analogues for Lignin

The lignocellulolytic activity becomes important for the production of from lignocelluosic biomass (LCB) such as sugarcane bagasse. Since, this was a qualitative analysis, further genome mining was carried out to delineate Carbohydrate active enzymes (CAZymes) reported to act on LCB components (Table 5.11). The findings indicated a wide variety of enzymes reported for activity on different LCB substrates including cellulose and Xylan (CAZymes. http://www.cazy.org).

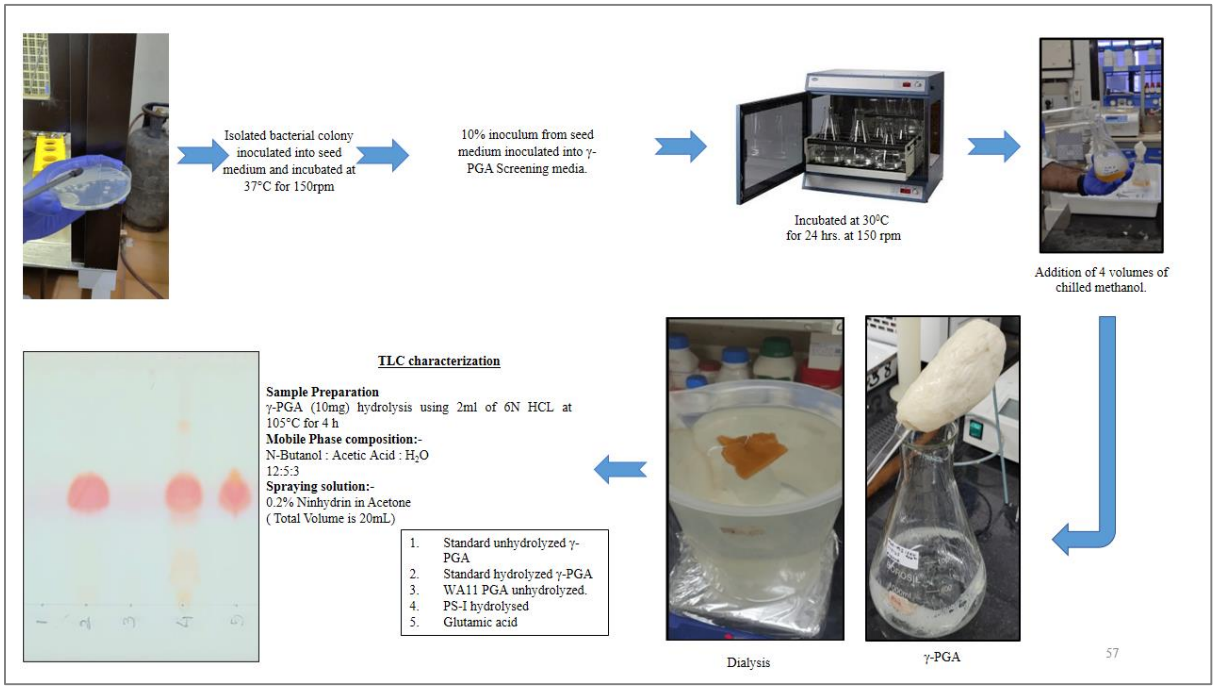


Fig. 5.22. Screening for γ-PGA production

Table 5.11. Genome mining for CAZyme families in bacterial isolate WA11

CAZyme Families	Substrates
AA10 (Lytic polysaccharide monooxygenases (LPMOs)	Recalcitrant polysaccharides

CBM3	Cellulose
CBM6	Cellulose
GH1	Cellulose
GH11	Xylan
GH30	Xylan
GH23	Chitin
GH26	Xylan and Cellulose
GH43	Xylan
GH51	Xylan and Cellulose
GH73	Chitin

The 16S rRNA sequence (1412 bp) was analyzed for closest type strain similarity using EZtaxon. The top-hit strain was *Bacillus velezensis* CR-502(T), with 100% similarity and 95.4% completeness. Further, phylogenic investigation using the Neighbour Joining method with 1000 bootstrap suggested close phylogeny to *Bacillus velezensis* (Fig. 5.23). Further, the ANI analysis using the genome sequence confirmed the identity as Bacillus velezensis with ANI value of more than 98 and genome size of 4.19 Mb.

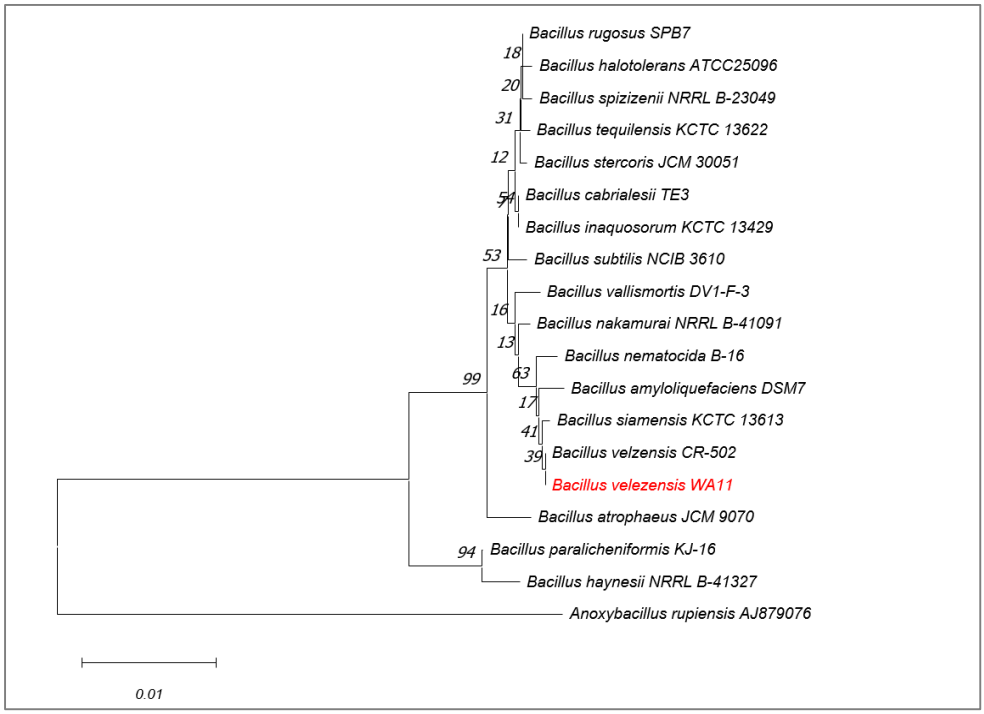


Fig. 5.23. Molecular Phylogeny. 16S rRNA gene-based molecular phylogeny analysis using Neighbour Joining method. Red highlighted strain is the bacterial isolate used in the present study.

5.2.3.2. Optimization for γ-PGA production from synthetic and bagasse containing medium

We utilized the OFAT strategy to optimize the media and physical parameters for γ-PGA production. Higher production was obtained at 28°C and pH 7.0 (16.6 g/L) (Fig 5.23 A and B). The medium's pH is a critical factor for producing γ-PGA, as the glutamate uptake system is reported to be pH-dependent. Earlier reports have suggested pH 6.5 to 7.0 as the optimum pH for most *Bacillus* species (Cromwick et al., 1996; Richard et al., 2003; Luo et al., 2016).

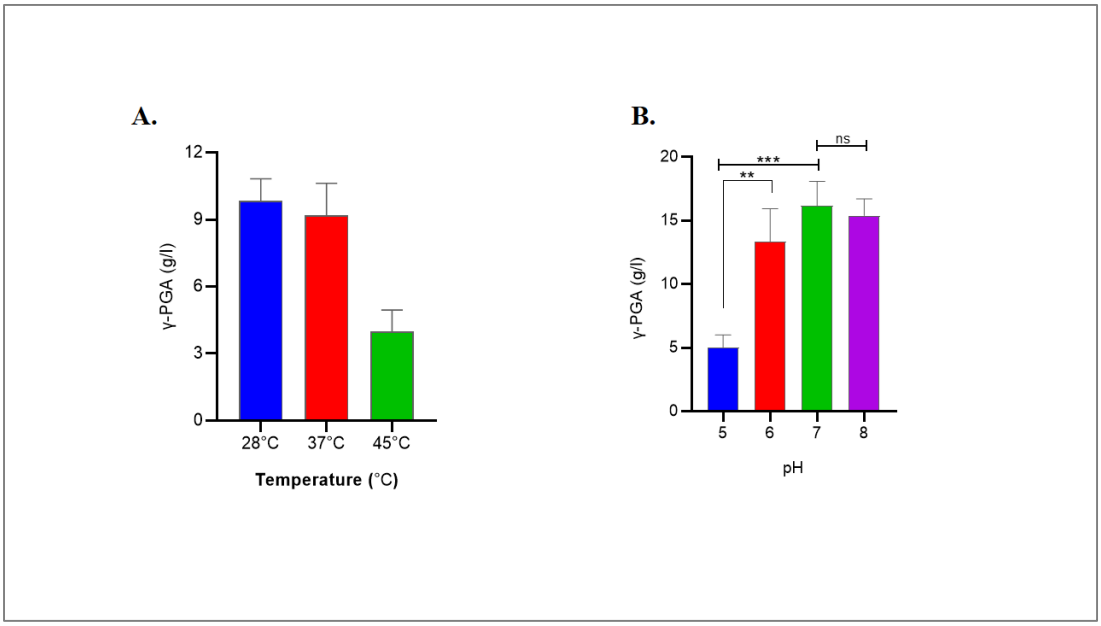


Fig. 5.23. Optimization of Temperature and pH for γ-PGA production in 24 hours. Statistical significance was assessed with One-way Anova with Tukey's multiple comparison test

Further, carbon source optimization revealed significantly higher production of poly-gamma-glutamic acid in maltose sugar (22.53 g/L), two-fold higher than the second-best sugar sucrose (11.39 g/L) (Fig. 5.24A.). Yeast extract was found to be the favourable nitrogen source, yielding 20 g/L than other organic and inorganic nitrogen sources (Fig. 5.24B).

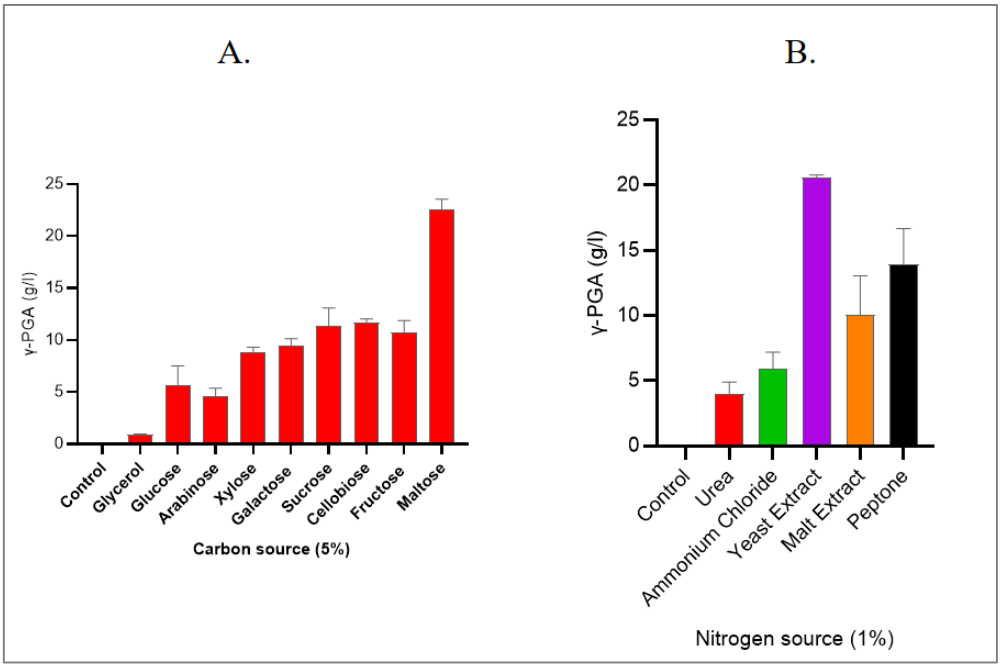


Fig. 5.24. Influence of different Carbon **(A)** and Nitrogen source **(B)** on the production of γ-PGA production in 24 hours by *Bacillus velezensis* WA11. One-way Anova with Tukey's multiple comparison test

Furthermore, the yeast extract concentration was optimized. We did not observe any significant influence of increasing yeast extract concentration on y-PGA production; 1% was considered to be optimum, yielding 20.7 g/L of poly-gamma-glutamic acid. Further, concentration optimization suggested 25% and 6% as the optimum maltose and L-glutamate concentrations for γ-PGA production by *Bacillus velezensis* WA11 (Fig. 5.25.). Further, inoculum concentration, production time, and RPM was optimized (Fig. 5.26). Upon final validation, we could obtain 104.3 g/L of dried crude γ-PGA with a productivity of 1.09 g/L/h from synthetic medium, respectively, from the optimized synthetic medium. In comparison to the intial media, after optimization of medium components and process paramters, we could observe more than 5-fold increase in the γ-PGA production by *Bacillus velezensis* WA11. The optimized synthetic media consisted of Maltose- 25%, L-Glutamic acid- 6%, Na_2HPO_4 - 0.1%, NaH_2PO_4- 0.7%, $MgSO_4.7H_2O$- 0.02%, Yeast Extract- 1%, pH- 7.0, RPM of 180. Further, the γ-PGA was characterized using different methods (Fig. 5.27). To the best of our knowledge,

the highest γ-PGA production reported by using maltose as a carbon source from *Bacillus subtilis* was 35 g/l with a productivity of 0.36 g/L/h (Ogawa et al., 1997). In the present study, we report 104.3 g/L γ-PGA production with productivity of 1.09 g/L/h, 2.9-fold higher than the previous study using maltose as a carbon source.

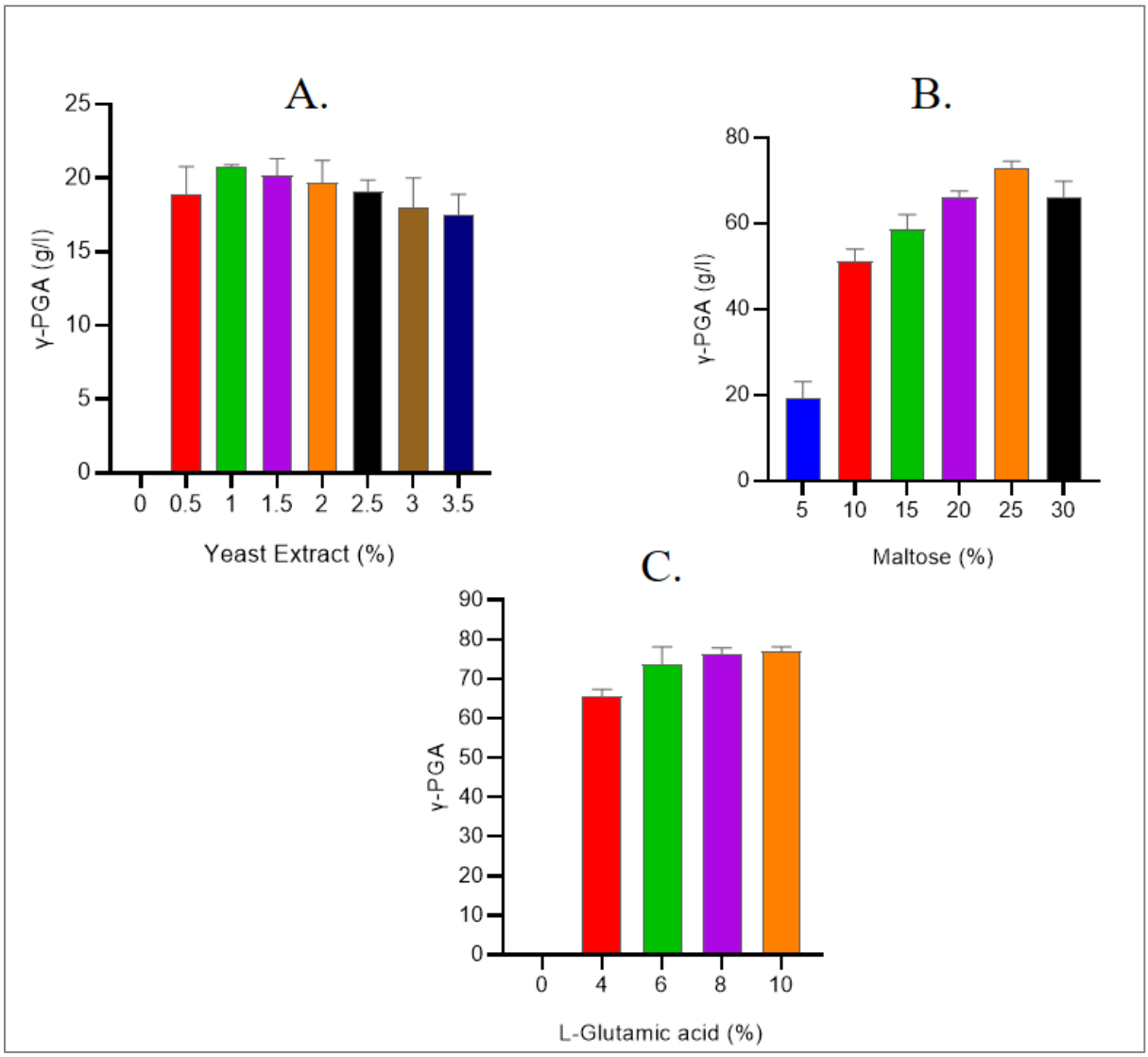


Fig. 5.25. Optimization of the concentration of Yeast Extract (A), Maltose (B), and L-glutamic acid (C), for the production of of γ-PGA production in 24 hours by *Bacillus velezensis* WA11. One-way Anova with Tukey's multiple comparison test

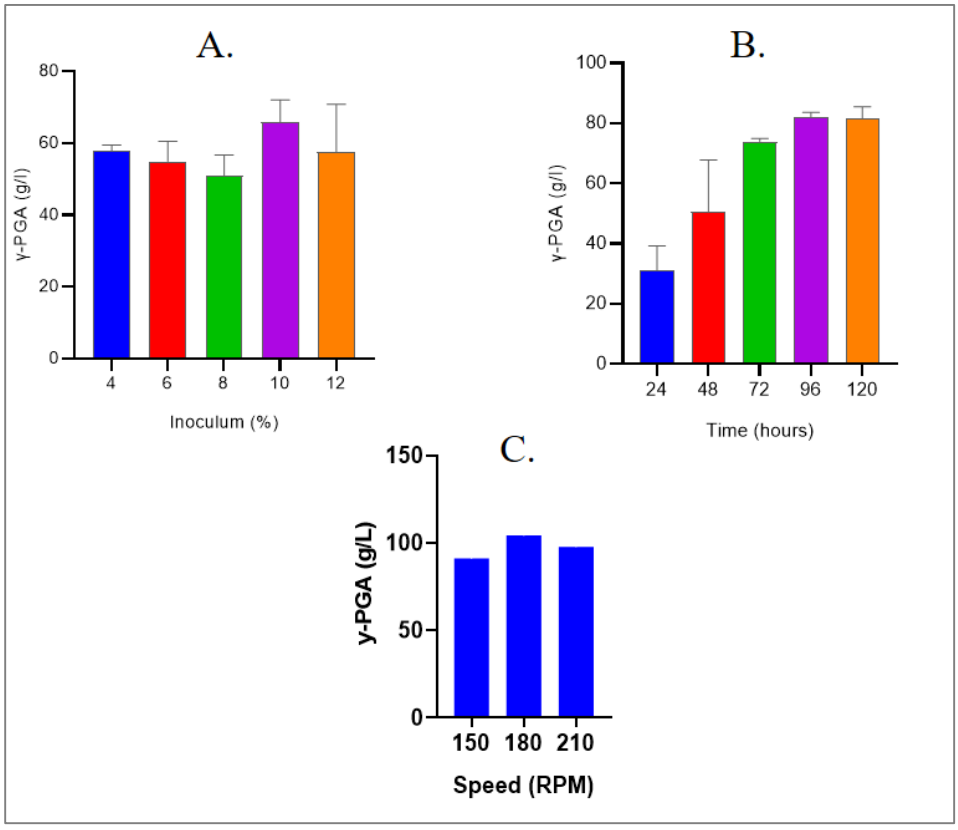


Fig. 5.26. Optimization of Inoculum concentration (A), Time of production (B), and RPM speed (C), for the production of of γ-PGA production in 24 hours by *Bacillus velezensis* WA11. One-way Anova with Tukey's multiple comparison test.

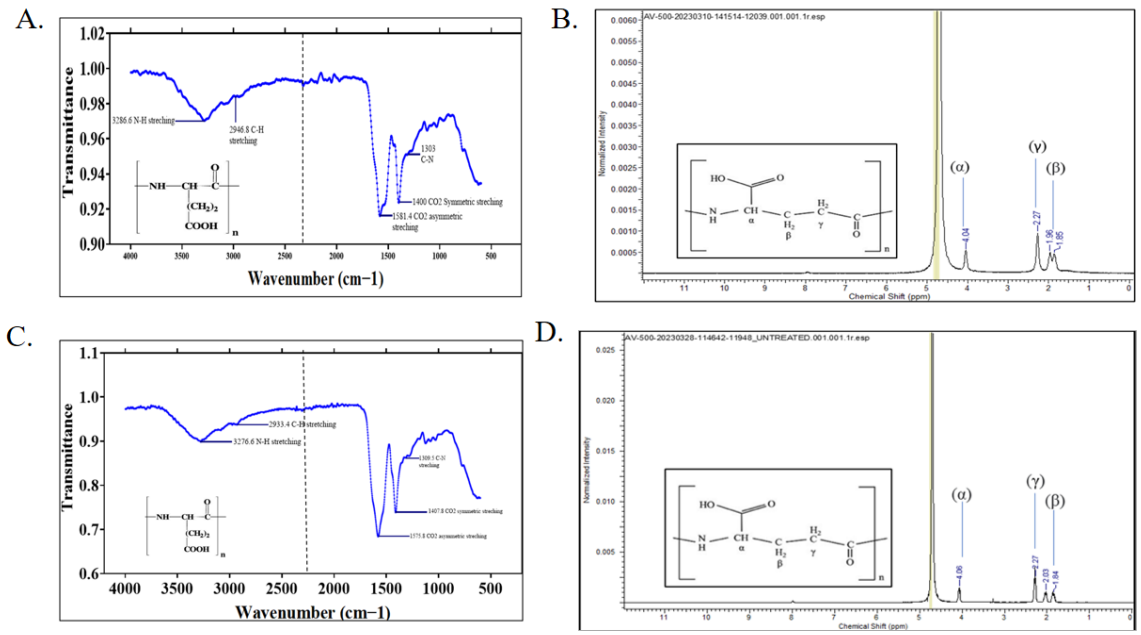


Fig. 5.27. Characterization of γ-PGA produced by *Bacillus velezensis* WA11 using FTIR (A. Standard γ-PGA, C. 48 hours dialyzed γ-PGA), and NMR (B. standard γ-PGA, D. 48 hours dialyzed γ-PGA).

The purity of synthesized γ-PGA was determined by using Fourier transform infrared spectroscopy and Nuclear Magnetic Resonance (NMR) spectroscopy, with comparison to standard γ-PGA obtained from Sigma. The results suggested (Fig. 5.27), the production of pure γ-PGA by *Bacillus velezensis* WA11.

5.2.3.3. γ-PGA production in Bagasse containing medium

Lignocellulosic biomass (LCB) is one of the most abundant and renewable biomass resource that can be applied in generation of biofuels and microbial based biochemicals. LCB mainly includes plant or agricultural-based wastes such as forest residues, sugarcane bagasse, cereal straw, and several others. They are mainly composed of complex polysaccharides (hemicelluloses and celluloses) and lignin (aromatic polymer) (Baruah et al., 2018; Zoghlami and Paës, 2019). The complex assembly of these constituents result in a recalcitrant structure, which is one of the major bottlenecks in utilization of LCB for biobased chemicals, requiring pretreatrment steps, ultimately increasing cost of production and environmental issues. Sugarcane bagasse (SCB) is one such organic LCB residue that is abundantly produced worldwide. The global production is estimated to be 1.6 billion tons of sugarcane and 279 million metric tons of bagasse. India is the second largest producers of sugarcane and produce large amount of SCB. SCB are generally implicated in wide variety of applications ranging from biofuels, electricity, paper, and others (Azala et al., 2021; Hajiha and Sain, 2015) (Fig.5.28).

The dried bagasse was added with optimized medium components (except Maltose C-source) at different concentrations (0%, 1%, 2%, 3%) along with L-Glutamic acid- 6%, Na2HPO4 - 0.1%, NaH2PO4- 0.7%, MgSO4.7H2O- 0.02%, Yeast Extract- 1%, pH- 7.0. Different concentration of bagasse was used to evaluate γ-PGA production. We observed the

highest production of 12.5 g/L in 3% bagasse-containing media. We did not use more than 3% bagasse, as it absorbs most of the water content, because of which the shake flask-based method cannot be used. Further, time optimization suggested 120 hours as the best time for harvesting crude γ-PGA (11.1 g/L) (Fig. 5.29)**.** Despite the recalcitrant nature of SCB, we could obtain γ-PGA production without employing any pre-treatment steps. As compared to other LCB hydrolysates (Table 5.12), our process of utilizing SCB without pretreatment yielded higher γ-PGA titer, indicating reduction in the cost of pretreatment.

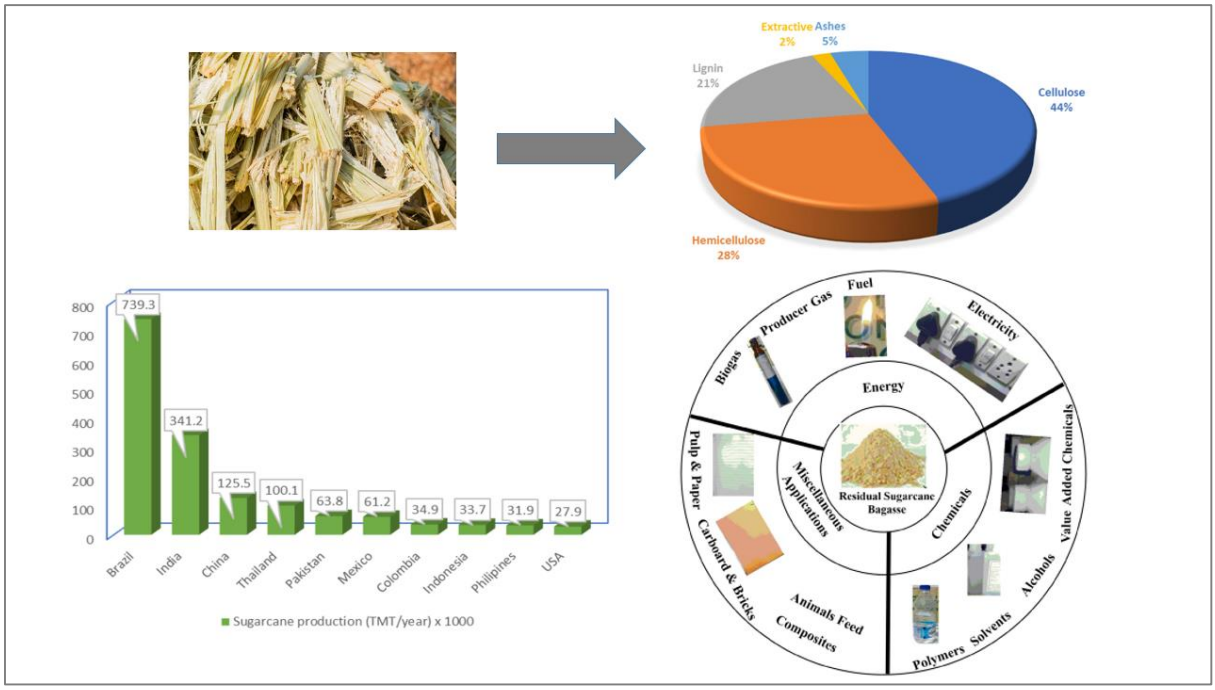


Fig. 5.28. Sugarcane Bagasse production and applications

Table 5.12. Production of γ-PGA from different LCB waste-based media

Bacteria	Wastes	Nutrients	Yield	References
Bacillus velezensis	Sugarcane baggase	L-Glutamic acid, Yeast extract	11 g/L	Present study
B. licheniformis 9945a.	Goose feather hydrolysate	L-glutamate, tri-sodium citrate dihydrate, glycero	5.4 g/L	Atlun 2019

B. licheniformis WX-02	Paper waste hydrolysate	Glucose, sodium glutamate, sodium citrate	6.46 g/L	Scheel et al., 2019
B. subtilisHB-1	Corncob fibrehydrolysate	L-glutamate and yeast extract	24.92 g/L	Zhu et al., 2014

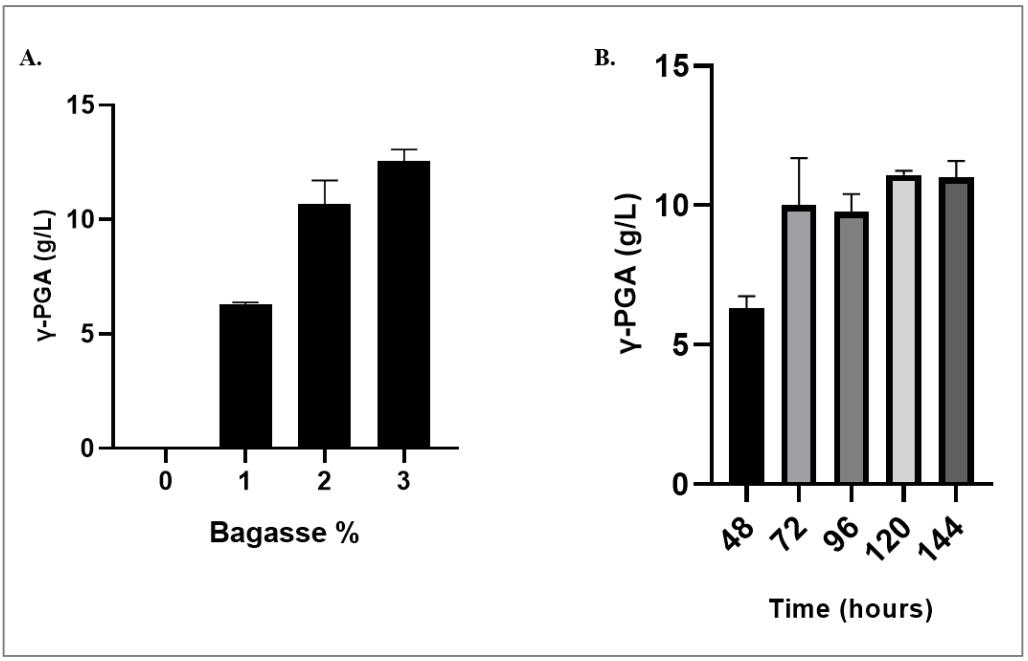


Fig. 5.29. γ-PGA production in Bagasse containing medium using *Bacillus velezensis* WA11.

5.2.4. Conclusion

In the present study, γ-PGA production was carried out using Bacillus velezensis WA11 isolated from Water Hyacinth. γ-PGA is one of the costliest biopolymers involved in various applications. We could obtain 104 g/L of crude dried γ-PGA from the optimized synthetic medium containing maltose as a carbon source with a productivity of 1.09 g/L/h. However, we replaced the synthetic carbon source with sugarcane bagasse to address the feed substrate and its treatment cost. We obtained 11 to 12 g/L of crude dried γ-PGA from sugarcane bagasse. The present study is the first report on producing γ-PGA using lignocellulosic biomass without pre-treatment. We also report 2.5-fold higher γ-PGA production than the previous best-reported

study utilizing maltose as a carbon source. The study shall help reduce costs associated with substrate substrate for γ-PGA production.

5.2.5. References

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Chapter 6. Summary and Future Prospects

Chapter 6. Summary and Future Prospects

Considering the significance of water, it is of utmost importance that we humans are taught to safeguard and preserve it for the future. The dismal state of the rivers reflects the dreadful impact of anthropogenic activities, especially on urban rivers, which largely influence socio-economic developments. Water is a treasured component of our planet connected to several sustainable development goals, crucial for the overall safety and advancement of all humanity, lifeforms, and nature. The microbial communities are the critical component of the ecosystem that have multifarious roles regulating nutrient cycles and productivity, delivering biotechnological products, and echoing environmental changes. Microbes are essential in aquatic environments because they accumulate, convert, and migrate nutrients. Their metabolic potential substantially affects water biogeochemistry, emphasising the necessity to comprehend their involvement in these environments. Due to their high metabolic and growth rates, they are sensitive to physicochemical shifts and are impacted by changes in organic and inorganic components and pollutants in aquatic bodies. As a result, they can serve as bio-indicators of environmental stresses. With the developing NGSTs, it is now possible to include microbes in river biomonitoring by understanding the diversity and functional changes and inferring their biotechnological potentials from polluted environments.

The comprehensive metagenomic study on the Pune riverine system consisting of urban and peri-urban riverine stretches revealed diverse microbial communities in urban and peri-urban sites. The analysis identified pathogenic potentials and faecal indicator microbes in urban rivers, indicating untreated sewage discharge. In contrast, peri-urban river lengths were occupied by ammonium-oxidizing microbes, showing the influence of affected by agricultural runoffs. The diversity research revealed considerable changes in microbial diversity affecting related downstream waterways. The study also identified regional disparities (urban vs. periurban) in ARGs and proposed measures to limit urban resistome inflow into downstream

peri-urban river lengths. The analysis revealed probable xenobiotic bioremediation genes, displaying microbial tolerance to contaminated environments. For pollutant breakdown, functional in-silico mining suggested both aerobic and anaerobic catabolism. Furthermore, the current study underlines the impact of increased anthropogenic stress on riverine systems, emphasizing the importance of appropriate wastewater treatment to reduce environmental stress. The research lays the groundwork for better water body restoration procedures and the investigation of bacteria for biotechnological applications.

The comprehensive study on Pandharpur Wari, a religious event, was performed to reveal the microbial ecological and functional changes in the river ecosystem during such mass bathing. The bathing and post-bathing water samples analysis revealed alterations in the virulence genes, ARGs, and priority pathogens Wari, reflecting the impact of mass anthropogenic stress on rivers. Moreover, the spatiotemporal variations in microbial ecology collectively illustrated the implications of mass bathing. The comparison with post-bathing samples allowed us to conclude the ecological shifts in the river while bathing. We uncovered that post-bathing samples recovered rather well (lower prevalence of ARGs, virulence, and increased bacterial diversity compared to bathing samples); however, additional detailed studies are required to converge the present findings.

Water hyacinth (WH) is a free-floating intrusive aquatic plant with a prolific reproductive and dispersal rate, significantly affecting overall biogeochemistry. The analysis revealed diverse microbial communities linked with water hyacinth roots. Higher statistical values showed water body types and time were likely explanatory variables for the observed diversity of microbes in water hyacinths. The findings suggested different species in WH versus water, such as methane and sulfur-metabolizing microbes. Also, consistent with previous research, our results point to methane metabolism as one of the significant energy-generating pathways in water hyacinth. A more considerable prevalence of sulfur-metabolizing

microbial species was discovered in the core microbiome of WH, showing that sulfur metabolism is likely the other primary energy source. Our predictive functional analysis linked several genes in the WH microbiota to xenobiotic and pollutant-degrading processes. Identifying many genes known for their roles in metal and biocide resistance in the WH metagenome revealed that WH-associated microorganisms may have a role in remediation.

The biotechnological potential of native bacteria from contaminated rivers and water hyacinths for synthesizing 2,3-butanediol and poly-gamma-glutamic acid was investigated. The current work is the first to describe the production of 2,3-butanediol from tomato waste. Furthermore, BDO production by *Bacillus halotolerans* has never been documented. Overall, we observed a 2.5-fold boost in BDO production from an unoptimized medium, yielding 73.19 g/l of 2,3-BDO with yield and productivity of 0.5 g/g and 1.02 g/l/h, respectively. Our unique approach of using concentrated tomato juice from rotten tomatoes produced 16.62 g/l of 2,3-BDO with a productivity of 0.7 g/L/h, which is high compared to many other studies using different lignocellulosic and other wastes. Further, γ-PGA production was carried out using *Bacillus velezensis* WA11 isolated from Water Hyacinth. We could obtain 104 g/L of crude dried γ-PGA from the optimized synthetic medium containing maltose as a carbon source with a productivity of 1.09 g/L/h. We obtained 11 to 12 g/L of crude dried γ-PGA from sugarcane bagasse. The present study is the first report on producing γ-PGA using lignocellulosic biomass without pre-treatment.

Overall, the present thesis unravels the importance of microbial monitoring in polluted rivers to understand the influence of anthropogenic activities and rapid urbanization. Further, the work highlighted the need to explore the native microbial flora for their biotechnological potential. Future studies should examine the physicochemical parameters and correlate them with microbial flora for in-depth longitudinal studies of such systems. In addition, it will be interesting to comprehend the molecular mechanism of selectivity of water hyacinth

rhizobiome in urban waters. Constant periodic surveillance and exhaustive sampling are required for mass bathing events to track river health. The up-scaling of 2,3-BDO production from rotten tomato concentrate is needed, especially at the fermenter level, to address production costs. Through this study, we seek to advance scientific knowledge concerning microbial dynamics from the riverine system, their resilience and adaptability by employing a modern approach, and contribute to developing innovative solutions to pressing environmental and biotechnological challenges.

ABSTRACT

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Title of the thesis: Comprehensive insights into microbial ecology and harnessing biomass conversion potential of culturable bacteria from riverine system

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The microbial communities are the vital element of the ecosystem that have diverse roles regulating nutrient cycles and productivity, offering biotechnological products, and echoing environmental changes. The comprehensive metagenomic study on the Pune riverine system consisting of urban and peri-urban riverine stretches revealed diverse microbial communities in urban and peri-urban sites. The study also identified regional disparities (urban vs. periurban) in ARGs. The analysis revealed probable xenobiotic bioremediation genes, displaying microbial tolerance to contaminated environments. The bathing and post-bathing water samples analysis revealed alterations in the virulence genes, ARGs, and priority pathogens, reflecting the impact of mass anthropogenic stress on rivers. Moreover, the spatiotemporal variations in microbial ecology collectively illustrated the implications of mass bathing. The analysis revealed diverse microbial communities linked with water hyacinth roots. Higher statistical values showed water body types and time were likely explanatory variables for the observed diversity of microbes in water hyacinths. The biotechnological potential of native bacteria from contaminated rivers and water hyacinths for synthesizing 2,3-butanediol and poly-gamma-glutamic acid was investigated. Overall, we observed a 2.5-fold boost in BDO production from *Bacillus halotolerans* EX5-6 from an non-optimized medium, yielding 73.19 g/l of 2,3-BDO with yield and productivity of 0.5 g/g and 1.02 g/l/h, respectively. Our unique approach of using concentrated tomato juice from rotten tomatoes produced 16.62 g/l of 2,3-BDO with a productivity of 0.7 g/L/h, which is high compared to many other studies using different lignocellulosic and other wastes. Further, γ-PGA production was carried out using *Bacillus velezensis* WA11 isolated from Water Hyacinth. We could obtain 104 g/L of crude dried γ-PGA from the optimized synthetic medium containing maltose as a carbon source with a productivity of 1.09 g/L/h. We obtained 11 to 12 g/L of crude dried γ-PGA from sugarcane bagasse. Overall, the present thesis unravels the importance of microbial monitoring in polluted rivers to understand the influence of anthropogenic activities and rapid urbanization and highlighted the need to explore the native microbial flora for their biotechnological potential.

List of Publications

1. **Yadav, R.,** & Dharne, M. (2024). Utility of metagenomics for bioremediation: a comprehensive review on bioremediation mechanisms and microbial dynamics of river ecosystem. Environmental Science and Pollution Research, 1-13.

2. Rajput, V., **Yadav, R.,** & Dharne, M. S. (2023). Correction to: Metagenomic exploration reveals a differential patterning of antibiotic resistance genes in urban and peri-urban stretches of a riverine system. *Environmental Science and Pollution Research*, *30*(46), 103361-103361.

3. **Yadav, R.,** Rajput, V., & Dharne, M. (2023). Corrigendum to" Functional metagenomic landscape of polluted river reveals potential genes involved in degradation of xenobiotic pollutants"[Vol 192, 110332]. Environmental research, 231(Pt 1), 116128.

4. **Yadav, R.,** Rajput, V., & Dharne, M. (2023). Corrigendum to" Metagenomic analysis of a mega-city river network reveals microbial compositional heterogeneity among urban and peri-urban river stretch"[Sci. Total Environ. 783 (2021) 146960]. The Science of the total environment, 892, 164740.

5. **Yadav, R.,** Rajput, V., Gohil, K., Khairnar, K., & Dharne, M. (2020). Comprehensive metagenomic insights into a unique mass gathering and bathing event reveals transient influence on a riverine ecosystem. Ecotoxicology and Environmental Safety, 202, 110938.

List of poster and oral presentations

1. Oral and Poster presentation at International Conference on Biotechnology for Resource Efficiency, Energy, Environment, Chemicals, and Health (BREEECH 2021) entitled “Metagenomic mining of the polluted river reveals potential genes involved in degradation of xenobiotic pollutants”.

Environmental Science and Pollution Research
https://doi.org/10.1007/s11356-024-32373-3

REVIEW ARTICLE

Utility of metagenomics for bioremediation: a comprehensive review on bioremediation mechanisms and microbial dynamics of river ecosystem

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Received: 12 October 2023 / Accepted: 4 February 2024

Abstract
Global industrialization has contributed substantial amounts of chemical pollutants in rivers, resulting in an uninhabitable state and impacting different life forms. Moreover, water macrophytes, such as water hyacinths, are abundantly present in polluted rivers, significantly affecting the overall water biogeochemistry. Bioremediation involves utilizing microbial metabolic machinery and is one of the most viable approaches for removing toxic pollutants. Conventional techniques generate limited information on the indigenous microbial population and their xenobiotic metabolism, failing the bioremediation process. Metagenomics can overcome these limitations by providing in-depth details of microbial taxa and functionality-related information required for successful biostimulation and augmentation. An in-depth summary of the findings related to pollutant metabolizing genes and enzymes in rivers still needs to be collated. The present study details bioremediation genes and enzymes functionally mined from polluted river ecosystems worldwide using a metagenomic approach. Several studies reported a wide variety of pollutant-degrading enzymes involved in the metabolism of dyes, plastics, persistent organic pollutants, and aromatic hydrocarbons. Additionally, few studies also noted a shift in the microbiome of the rivers upon exposure to contaminants, crucially affecting the ecological determinant processes. Furthermore, minimal studies have focused on the role of water-hyacinth–associated microbes in the bioremediation potentials, suggesting the need for the bioprospecting of these lesser-studied microbes. Overall, our study summarizes the prospects and utilities of the metagenomic approach and proposes the need to employ it for efficient bioremediation.

Keywords Bioremediation · Metagenomics · Rivers · Xenobiotic · Water hyacinth

Introduction

Water is indispensable for all life forms, and its importance is immeasurable. However, most of the water bodies on Earth are now in a dismal state, and the contamination of freshwater bodies is of global concern. The increase in anthropogenic activities, urbanization, and industrialization has led to the sad state of rivers, lakes, and other surface water bodies. Access to clean water is necessary and is one of the vital sustainable goals laid by the United Nations General Assembly (UNGA) (SDGs n.d).

Industrialization and urbanization have produced countless xenobiotic chemicals, and their uncontrolled dumping into natural ecosystems such as water bodies has resulted in unpleasant consequences (Yadav et al. 2021a). Various organic and inorganic pollutants often pollute the surface water from numerous point and non-point sources, including industrial effluents, hospital and domestic sewage, agrochemicals, stormwater runoff, municipal waste, and others (Md Anawar and Chowdhury 2020). Globally, several studies have reported pollution of urban rivers by various persistent organic pollutants (POPs) such as polychlorinated biphenyls, polybrominated diphenyl ethers, and

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Published online: 17 February 2024

organochlorine pesticides (Bao et al. 2012; Kroeze et al. 2016; Sousa et al. 2018). Others have reported recalcitrant pollutants, including heavy metal pollution, aromatic petroleum hydrocarbons, and microplastics (Suthar et al. 2009; Debnath et al. 2021; Tsering et al. 2021). Several of these pollutants are highly toxic and mutagenic, exacerbating the situation for humans, surroundings, and other life forms (Suthar et al. 2009; Bao et al. 2012).

Remediation of the water bodies is tremendously challenging and involves higher economic expenses. Various methods have been employed to remove contaminants, including dredging, excavation, pyrolysis, and several others (Perelo 2010; Bala et al. 2022). However, these conventional approaches are economically not viable and often result in the generation of toxic end products (Perelo 2010; Azubuike et al. 2016; Pande et al. 2020; Bala et al. 2022). Bioremediation utilizes the wide metabolic versatility of microorganisms to remove various pollutants. It has numerous advantages, including cost-effectiveness, generation of non-toxic products, and complete mineralization of the pollutants. Although it takes a longer time and multiple strategies for microbes to remediate the environment, it is still considered a promising technique for ecosystem restoration. Different strategies are employed within bioremediation, such as natural recovery in which natural potentials of indigenous microbes are utilized; biostimulation wherein the microbial growth is influenced by the addition of essential factors; bioaugmentation, which involves the introduction of microbes with specific degradation abilities; and phytoremediation, using plants to remove the contaminant (Perelo 2010). However, these approaches have their environmental implications and limitations. For instance, nutrient additions in the biostimulation process promote the growth of other heterotrophic microbes that are not involved in remediation, resulting in changes in the microbial ecological dynamics of the environment (Adams et al. 2015). Similarly, there is a high risk of microbial competition and disruption of existing ecological niches during bioaugmentation, where specific microbes are inoculated. Thus, these processes should only be implemented after evaluating cause and effect. Nonetheless, all of these techniques rely on the metabolic potential of the microbes attributed to enzymes capable of degrading and transforming the recalcitrant pollutants.

Microbial-based bioremediation is a sustainable process for transforming and removing hazardous pollutants. However, bioremediation requires deep insights into their metabolic machinery, such as enzymes and transporters. Thus, practical bioremediation is a research-intensive process that requires preliminary information on habitats, culture conditions, and microbe's genomic and functional traits (Yadav et al. 2021a). With the development of next-generation sequencing technologies (NGS), it is now possible to infer an overall genetic and functional makeup of microorganisms, facilitating the effective designing of remediation strategies (Chakraborty et al. 2020; Mohapatra et al. 2021). Several studies reported pollutant-degrading genes and enzymes from rivers using NGS and conventional techniques (Collins-Fairclough et al. 2018; Behera et al. 2020); however, collective information on bioremediation still needs to be documented. Moreover, the present review also gathers collective information on river microbial shifts amid exposure to various pollutants retrieved from metagenomic explorations. Water macrophytes, such as water hyacinths, are crucial for river ecology and biogeochemistry, thus, need to be included in the study of river ecosystems. Water hyacinths are well documented for their potential to remove hazardous chemicals; however, there is a lacuna in the linkage of their rhizobiome in assisting the bioremediation process (Xu et al. 2022). Pharmaceutical pollution in aquatic bodies and the resultant antibiotic resistance are a global threat with negative consequences for the ecosystem and human health. However, in the present review, we have excluded active pharmaceutical ingredient (API) pollutants and global antimicrobial resistance, as several recent review articles have elaborately summarized their influence on urban rivers (Wilkinson et al. 2022; Reddy et al. 2022).

The present review aims to endow global details of the bioremediation genes/enzymes reported in the rivers, impacts on the microbial diversity changes, and comprehend the role of water hyacinth rhizobiome in facilitating the remediation process. Eventually, this structured information shall provide baseline information for future research explorations in the bioremediation of aquatic bodies.

Pollutant-mediated shifts in the river microbiome

Microbes are the key drivers of the ecosystem, with vital roles in biogeochemical cycling and ecosystem functioning. Any environmental change caused by hazardous and xenobiotic chemicals directly correlate with the alterations in the microbial population. The complex microbial populations respond to such selective environmental pressure through changes in their metabolic functions and overall diversity (Zhang et al. 2020a; Sagova-Mareckova et al. 2021; Gao et al. 2022). We searched electronic media with keywords and brief statements such as microbial shifts in rivers, alterations in microbial diversity upon exposure to pollutants, river metagenomics, river microbiome, river microbial ecology, river pollutants, and metagenomics (till May 2023). Several studies have reported notable alterations in microbial diversity and functionality upon exposure to different pollutants (Table 1).

Table 1 The following table summarizes the metagenomic studies concluding the influence of the hazardous pollutants on the microbial community in rivers worldwide

Pollutants	Type of pollutants	General use	Effect on microbial population of aquatic system	Type of study	Reference
Parabens (methylparaben and propylparaben)	Organic pollutants (emerging organic pollutants EOPs)	Antimicrobials and food preservative	Suppressed gram positive bacteria, promoted oxidative stress tolerant bacteria, altered ecological process	16S rRNA gene high-throughput sequencing	Liu et al. (2021a)
Paper mill waste	Multiple pollutants (chlorinated bisphenol A, resin acids, lignin)	Devoted for making papers	Significant enrichment of certain bacteria such as *Fusibacter*, *Desulfobulbus*; enrichment of carbohydrate-active enzymes in rivers	Denaturing gradient gel electrophoresis (DGGE) fingerprinting and 16S rRNA gene high-throughput sequencing; MinION-based shotgun sequencing	Guo et al. (2016); Rajput et al. (2022)
Polycyclic aromatic hydrocarbons (PAHs) and phthalates (PAEs)	Organic aromatic contaminants (priority pollutants)	Plasticizers, pigments, pesticides	Negative influence on nitrogen and phosphorous metabolism and promotion of sulphur metabolism	16S rRNA gene high-throughput sequencing and qPCR	Liu et al. (2021b)
Terrestrial pollutants	General pollutants, humus, heavy metals, Nitrogen	-	Significant microbial differences on the basis of land use, enrichment of PAHs and bisphenol pathway in industrial area	16S rRNA gene high-throughput sequencing	Qiu et al. (2020)
Pharmaceutical contaminants, personal care products (sucralose and climbazole)	Emerging pollutants	Medicines, cosmetics etc	Microbial community structure associated with high pharmaceutical loads	Denaturing gradient gel electrophoresis (DGGE) fingerprinting, 16S rRNA gene high-throughput sequencing	Chonova et al. (2018), Liu et al. (2021a)

Enrichment of specific microbial groups and alterations in their diversity

Liu et al. (2021a) reported parabens, an emerging organic pollutant, induced microbial enrichment in Jinsha River, China. The *Flavobacterium*, *Prevotella*, and *Hydrogenophaga* were significantly prevalent in industrial area riverine sites with higher pollutant levels. Furthermore, they also reported different pollutants induced enrichment of taxa such as Candidatus Protochlamydia positively correlated with different pharmaceuticals and personal care products, *Yonghaparkia* with caffeine, and *Thiovirga* with methylparaben (MeP) and propyl paraben (PrP). Furthermore, the same study also observed a decrease in the prevalence of Gram-positive bacteria as an effect of parabens, whereas the growth of oxidative stress-tolerant bacteria was promoted. Another study by Guo and the group in 2016 revealed the impact of a paper mill on the nearby river. These bacterial genera are reported for thiosulfate reduction, acidogenesisor syntrophic, acid degradation, and polychlorinated biphenyl (PCB) degradation (Agrawal et al. 2010; Shin et al. 2010). Similar observations were reported by Yuan et al. (2023), wherein *Pseudomonas* and *Ruminococcus* (fecal and sewage indicator bacteria) were correlated with Cu, Zn, and NH_4+-N, and heavy metals were the vital factors for influencing diversity in an anthropogenically disturbed urban river. Furthermore, a metagenomic study by Qiu et al. (2020) showed that the terrestrial pollutants overrode the influence of seasonal impact in shaping the microbial community structure of river sediments. These observed changes in the microbial communities amid the increasing pollutants suggested the employment of a metagenomic approach for assessing microbial bioindicators.

Ecological and functional changes

Deterministic process (homogeneous and heterogeneous selection by similar environmental conditions and distinct selection pressure, respectively) and stochastic process (random death, birth, and reproduction drift) are the two critical ecological processes involved in the microbial assemblage of the ecosystem (Trego et al. 2021). Liu et al. (2021a) showed that amid the increasing paraben concentration along the rural–urban gradient of the river, the stochastic process outclassed the deterministic process and became the vital ecological driver of microbial assembly in the residential and industrial areas. Furthermore, Liu et al. (2021b) revealed the functional influence of polycyclic aromatic hydrocarbons (PAHs) and phthalates (PAEs) on the river microbial ecology. These contaminants were shown to impact phosphorous negatively and nitrogen metabolism and positively affect sulfur metabolism, eventually leading to the blackening and odorization of urban rivers. Altogether, such dynamic and coordinated shifts in the microbial population and functions

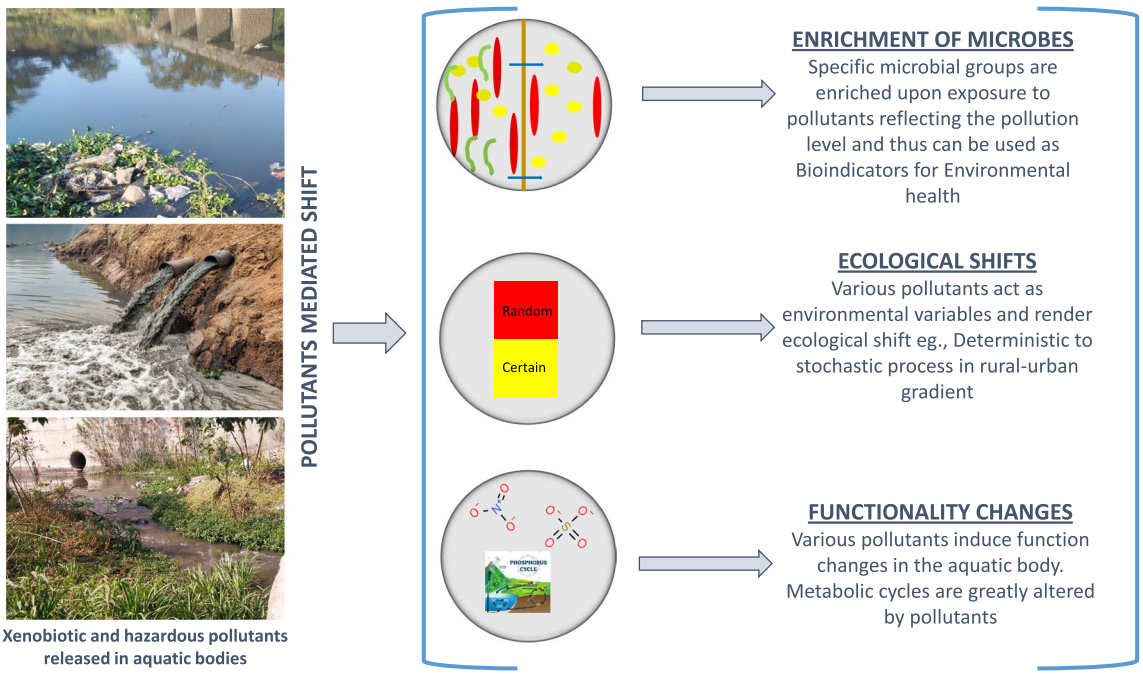

Fig. 1 The illustration depicts the overall shift in the microbial community and ecological processes due to influx of pollutants in rivers

(Fig. 1) reflect the pollution level in the aquatic ecosystem. Also, such microbiological shifts could be used as a potential indicator for evaluating and monitoring ecosystem health (Sagova-Mareckova et al. 2021).

A metagenomic approach to combat xenobiotic pollutants in rivers

Microbes run every process in the biosphere through their endless metabolic potentials. Most of the functions carried out by microbes in an ecosystem result from interacting complex communities. The knowledge of such intricate interactions is often missed when studying a single microorganism in pure culture. As per the definition, metagenomics (targeted/amplicon or shotgun based) is the science of understanding the genetic content of the microbial communities present in a sample as a complete unit (Thomas et al. 2012). Metagenomics could be a powerful tool for studying microbial communities in various contaminated environments, providing a comprehensive insight into the functional potentials of the microbes. This modern approach can provide preliminary information about the pollutant-degrading genes, microbial diversity, and their changing dynamics amid different pollutants and interactions. It serves as a starting point for further environmental remediation studies (Offiong et al. 2023; Kumar et al. 2022; Nawaz and Wang 2022). With the advent of newer, faster, and cost-effective sequencing technologies and bioinformatics tools, it is now possible to infer the genetic and functional makeup and microbial interactions in aquatic environments (Zhu et al. 2022) (Table 2).

Role of metagenomics in bioremediation

The ever-increasing demands of humans for modernization in all aspects of life have led to the emission of xenobiotics in the environment. The water bodies act as a sink for these recalcitrant and hazardous chemicals, which are often emptied through various means resulting in the deterioration of freshwater bodies. Bioremediation, a microbial-driven process, with its superiority over the other methods, is now gaining attention for removing hazardous pollutants. A successful detoxification process depends on multiple factors, including the indigenous microbial communities, type of contaminant, and environmental conditions. Conventional culturing techniques practiced over the years to isolate specific microorganisms for the metabolism of pollutants perform excellently in laboratory conditions; however, their utility in the field is discouraging. The failure to mimic in the field could be attributed to various reasons: (a) scarcity in the knowledge of the catabolic capabilities and field-level performance of pollutant-degrading bacteria; (b) as cultivable microorganisms represent a minute fraction of actual diversity, there is a paucity of information in the complex interactions (often mutualistic) of the microbial communities (Terrón-González et al. 2016).

Based on the intensity of the intervention, the three categories of bioremediation approaches are natural attenuation, biostimulation, and bioaugmentation (Techtmann and Hazen 2016). Natural attenuation involves the detoxification of the contaminants by the native microbial population. Although this process involves indigenous flora and no external additives influencing ecosystem ecology, the process is severely slow. As a result, the contaminant sustains for a longer time in the environment exhibiting its toxic effects on life forms. Biostimulation is an extension to a natural attenuation where the indigenous population is supplemented with the limiting nutrients to expedite the degradation process. However, in some settings, it still results in a slower rate owing to the incapability of the native microbes to degrade the pollutants (Dellagnezze et al. 2014; Techtmann and Hazen 2016). To address such metabolic limitations, another process known as bioaugmentation is used. In this process, the non-native specialist is added to the ecosystem with the metabolic potential to degrade the specific pollutants. Bioaugmentation proves to be more efficient than other methods; however, in some cases, it fails for various reasons. The incapability of the organism to survive in newer environmental conditions and competitive interactions with other communities results in the failure of bioaugmentation process. Furthermore, the augmented organism might also have undesired ecological alterations in the collective microbial ecology even after removing pollutants (Dellagnezze et al. 2014; Techtmann and Hazen 2016). Nonetheless, crucial preliminary information about the genetic and functionality of the microbial communities in the ecosystem is required to understand and apply any of these processes practically. Metagenomics can play a vital role in delineating the information required to apply bioremediation efficiently (Kachienga et al. 2018). For instance, metagenomics would provide all the essential information on the overall microbial community structure and functions, enabling optimization of nutrients to be added and the revealing correlation of the non-native organism with the native populations. It also unveils the repertoire of remediating genes already existing in the environment, which can be cloned and applied for detoxification. Over the past decade, there has been growing interest in metagenome-based mining for bioremediation genes and enzymes, and thus, different databases have been developed (Fig. 2). MetaRouter tool containing information on bioremediation was designed to assess the environmental pollutant's fate and their remediation strategies (Pazos et al. 2005). The University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD) was also developed to provide curated

Table 2 The following table summarizes findings of the metagenomic studies (targeted and shotgun-based) for mining pollutant degrading genes and enzymes in rivers worldwide

Target pollutants	Genes detected	Enzymes/protein	Methodology	River	Reference
Para-nitrophenol	*pnpA*, *mar*	4-nitrophenol 4-monooxygenase, maleylacetate reductase	16SrRNA gene-based TRFLP and DNA-SIP with [13C] PNP	River Dene, UK	Kowalczyk et al. (2015)
Arsenic	*ars*, *acr*, *arr*	Arsenite methyl transferase, arsenate respiratory reductase, arsenite efflux pump	Amplified clone library	Reigous and Amous Rivers, France	Desoeuvre et al. (2016)
Broad mining for multiple pollutants	Biocides and metal resistance genes	-	Shotgun metagenomic	Duhaney River, Jamaica	Collins-Fairclough et al. (2018)
Broad mining for multiple pollutants	*acn*, *pstB*, *arsA*, *recG*, *ruvB*, *czrA*, *czcA*, *mdtB*, *cnrA*, *copA*, *actP*, *arsB*, *copB*, *acrD*, *silP*, *merA*, *arsM*	Multiple metal resistance proteins	Shotgun metagenomic	River Ganga, India	Reddy and Dubey (2019)
Metal tolerance	271 MRGs	Multiple metal resistance proteins	Targeted and shotgun metagenomic	River Yamuna, India	Mittal et al. (2019)
Multiple xenobiotic pollutants	15 functional genes related to xenobiotics biodegradation	-	16S rRNA gene sequencing, PICRUSt-based	Qingliu River, China	Zhang et al. (2020b)
Azo dye	Multiple genes such as *azoR*, *nahA*, *C,D,P,F*	Laccase, tyrosinase, azoreductase	16S rRNA gene sequencing, PICRUSt-based	Coastal areas of Rongjiang and Hanjiang Rivers, China	Zhuang et al. (2020)
Broad mining for multiple pollutants	Multiple genes encoding for different transporters, *urt* gene	Metal transporting ATPase, transporter proteins	Shotgun metagenomic	River Ganga and Yamuna	Behera et al. (2020)
Metal tolerance	MRGs	-	Shotgun metagenomic and 16S rRNA gene sequencing	Cooum river	Aishwarya et al. (2021)
Variety of xenobiotic pollutants, plastic and dye metabolism	*ben*, *cat*, *pca*, *bad*, *dsr*, *nem*, *cat*, *tfd*, *abm*, *sty*	Benzoyl-CoA ligase, catalase peroxidase, arylsulfatase and others	MinION-based shotgun sequencing	Mula, Mutha, Mula-Mutha, Ramnadi, Pawana, and Bhima eivers, India	
Nitrate	Genes involved in nitrate reduction pathway	Multiple enzymes	Metagenomic study	Huangshui River, China	Hu et al., (2022)
Lignocellulose	Carbohydrate active enzymes (CAZyme) encoding various genes	Glycoside hydrolases, polysaccharide lyases, carbohydrate esterases and others	MinION-based shotgun sequencing	Ganga and Yamuna rivers	Rajput et al. (2022)
Micropolyethylene (Microplastics)	*alk*, *alm*	Laccase, alkane 1-monooxygenase, flavin-binding monooxygenase	Full length 16S rRNA sequencing, and shotgun metagenome	Pearl River, China	Sun et al. (2022)
Variety of xenobiotic pollutants, plastic and dye metabolism	Multiple genes	4-hydroxybenzoate 3-monooxygenase, catalase-peroxidase, and altronate hydrolase	Shotgun metagenome	River Ganga	Rout et al. (2022)

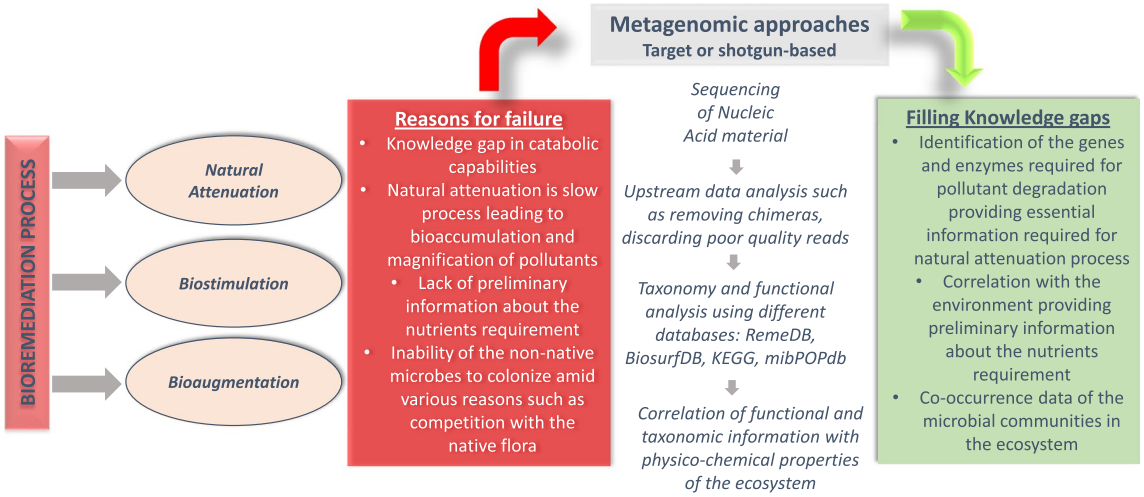

Fig. 2 The illustration summarizes the utilities and potential of metagenomics in carrying out successful bioremediation

information on catabolic genes for environmental pollutants (Ellis et al. 2003). BacMet database is a widely used comprehensive database for antibacterial biocide and metal resistance genes (MRGs) (Pal et al. 2014). Some recently developed databases and tools include BioSurfDB (Oliveira et al. 2015), RemeDB (Sankara Subramanian et al. 2020), and mibPOPdb (Ngara et al. 2022). Most recently developed tools contain detailed information on pollutant-degrading genes and pathways. The KEGG: Kyoto Encyclopedia of Genes and Genomes database also contains information on xenobiotic metabolism (Kanehisa 2002; Aoki and Kanehisa 2005; Kanehisa et al. 2017). With these recent developments, metagenomics can be efficiently employed for finding newer pollutant-degrading enzymes, microbes, and pathways for successful bioremediation (Fig. 2).

Functional metagenomics of rivers: a link to metabolic potentials for bioremediation

A detailed characterization of the functional diversity is required to understand the intricacy of bioremediation in aquatic bodies such as rivers. Several studies worldwide have employed functional metagenomics to unravel the different xenobiotic degrading genes and enzymes in polluted rivers. Table 2 briefly summarizes the findings of the metagenomics study (targeted or shotgun-based) related to mining bioremediation genes and enzymes in rivers. To have a comprehensive perception, we used different keywords such as "river metagenome remediation," "pollutant degrading genes in the river," "river microbial remediation," "urban rivers bioremediation," and several others to search in NCBI PubMed and Web of Science collections. Altogether, there are several studies on the overall microbiome of rivers, but very few focused on the bioremediation aspects (Table 2). Most studies on bioremediation gene mining in rivers were from China and India (Table 2). The urban rivers in India (estimation of 70% of surface water is polluted) and China are severely polluted due to the unprecedented growth rate of both countries, inflicting tremendous pressure on the aquatic body, mainly on urban and peri-urban rivers (Water pollution n.d; Chen et al. 2019; Yadav et al. 2020, 2021b; Samson et al. 2023; Wang et al. 2023).

Metabolism of various xenobiotic pollutants in rivers

Kowalczyk et al., in 2015, reported genes and enzymes for para-nitrophenol (PNP) degradation in River Dene of the UK by combining terminal restriction fragment length polymorphism and SIP-pyrosequencing 16S rRNA techniques (Table 2). PNP is a persistent and toxic nitroaromatic contaminant for producing medicines, pesticides, and preservatives (Kowalczyk et al. 2015; Xu et al. 2021). The study reported 4-nitrophenol 4-monooxygenase encoded by *pnp* and maleylacetate reductase encoded by mar in the water. Upon targeted microbial isolation, they revealed *Pseudomonas* species encoded these enzymes for PNP metabolization. Behera et al., in 2020, conducted a detailed investigation to understand the bioremediation potentials of the microbiome in the River Ganga and Yamuna. Apart from metal-tolerating genes, the study reported cytochrome 450 (haem thiolate protein) and hydrolases (HAD) (Table 2). These proteins play a significant role in the degradation of various environmental toxins and pesticides. Also, results revealed the presence of urea ABC transporter proteins and urea permease for removing urea from rivers. In one of our studies (Yadav

et al. 2021a), we performed MinION-based shotgun sequencing of 24 sediment samples from seven different urban and peri-urban rivers to comprehend the bioremediation potentials and operational metabolic pathways. Our findings revealed many catabolic genes involved in the biodegradation of various xenobiotic pollutants, including the metabolism of benzoate, polycyclic aromatic hydrocarbons (PAH), nitrotoluene, caprolactam, atrazine, DDT, xylene, and various other hydrocarbons. We observed aerobic and anaerobic metabolic pathways for bioremediation converging to the central benzoate degradation cycle. Often, wastewater discharge from paper mills is released directly into River Ganga and Yamuna, generating carbohydrate-rich wastes (Rajput et al. 2022). In our other study, we reported the prevalence of various carbohydrate active enzymes (CAZymes) for the first time in river water that are active against such carbohydrate-rich wastes (Rajput et al. 2022) (Table 2). We reported an abundance of GH3 family enzymes encoding for xylanase, cellobiohydrolase, β-glucosidase, endoglucanases, and glucosylceramidase involved in cellulose and hemicellulose degradation. Increasing anthropogenic activities, such as excessive use of fertilizers, have resulted in increased nutrients in aquatic bodies due to runoffs. Nitrogen and its other forms, such as nitrate, are pretty common pollutants in the river these days. The abundance of nitrate-reducing microbes in rivers, especially from the effluents of wastewater treatment plants, can have negative implications due to antibiotic resistance. Hu et al. (2022) revealed the prevalence of nitrate reduction genes (NRG) such as *nir*, *nas*, *nos*, *nap*, and *nrf* in Huangshai River. Rivers polluted with wastewater are reported to have higher concentration of nitrogen along with excess antibiotic ingredients (Hong et al. 2020). Such environments provide the favorable conditions for the co-selection of ARGs with NRG. The study observed a correlation of nitrate reduction genes with antibiotic resistance genes, suggesting nitrate-reducing organisms in a polluted environment could act as a carrier of drug resistance (Table 2). The study further revealed the ability of *Acinetobacter* and *Hafnia* impacting the NRG-ARG relationship and suggesting their utility in bioremediation.

Abundance of MRGs in rivers

Due to its environmental accumulation, toxicity, and abundance, metal contaminants in aquatic bodies have become a global problem. Removing heavy metals is troublesome compared to other pollutants, as they adhere and settle in the river sediments. Several studies reported the presence of heavy metal tolerance genes to cope with the toxicity of metals. Desoeuvre et al. reported arsenic-tolerating genes such as *arsB*, *acr3.1*, *acr3.2*, arsenic methylating genes *arsM*, and respiration gene *arrA* in a zone of confluence between arsenic-rich drainage and the pristine river in France (Table 2). ArsB and Acr3p families are known to encode for arsenic efflux pumps, and *arsM* encodes arsenite methyl transferases which catalyze the transformation of toxic arsenite into less toxic form (Desoeuvre et al. 2016). Similarly, Collins-Fairclough et al. (2018), Reddy and Dubey (2019), Mittal et al. (2019), and Aishwarya et al. (2021) reported the prevalence of metal-tolerating mechanisms in different rivers worldwide. Collins-Fairclough and his group, in 2018, revealed the influence of waste leachate in the Duhaney River. They reported that 91 metal resistance proteins and only 26% were shared between leachate and river microbial populations. Further findings revealed enhanced resistance of cadmium and copper in rivers metagenomic assembled genomes (MAGs) than in leachate MAGs. The BacMet database analysis identified 23 different MRGs conferring resistance to mercury, copper, arsenic, nickel, cadmium, and others in the highly revered River Ganga, India (Reddy and Dubey 2019). The metal resistance genes *acn*, *pstB*, *arsA*, *recG*, *ruvB*, *czrA*, *czcA*, *mdtB*, *cnrA*, and *mexR* were detected in water samples of the river, whereas *copA*, *actP*, *arsB*, *copB*, *acrD*, *silP*, *merA*, *arsM*, and *pbrA* in the river sediments (Table 2). The river Yamuna, one of the most important rivers of India, is highly polluted, and efforts are ongoing to restore it. Mittal et al. (2019), using the BacMet database, predicted the presence of 271 MRGs conferring resistance against 47 different metal categories. The resistance against copper, zinc, and nickel was highest in the Yamuna. The Central Pollution Control Board (CPCB) reported the highest biochemical oxygen demand (BOD) of 345 mg/l in the river Cooum, termed it the most polluted river in India (Cooum River n.d). Aishwarya et al., in 2021, carried out metal resistome profiling of the Cooum River and observed higher copper resistance (29.6%), followed by resistance against tellurium and selenium. Most of these studies have also reported the co-occurrence of antibiotic resistance genes (ARGs) with MRGs. The co-selection of ARGs and MRGs in the environment is reported in many studies, mainly attributed to physical location plasmids and pollutant-induced horizontal transfer. Nevertheless, these tolerant metabolic properties increase the persistence of the resistant organisms, providing opportunities for further propagation and dissemination of antibiotic resistance.

Plastic and dye-degrading enzymes in rivers

The release of textiles and other dyes is a nuisance for water bodies worldwide. These dyes are highly carcinogenic and toxic. Several microbial enzymes, laccase, azoreductase, and peroxidase, have been reported for the

degradation of these dyes (Bilal et al. 2019; Zhuang et al. 2020). Like dyes, surface water receives copious amounts of plastic, which are highly recalcitrant. Furthermore, these plastics undergo weathering and are broken down into smaller pieces of a few microns, known as microplastics (MPs). Owing to their smaller size, these tend to accumulate in the food chain, eventually affecting all life forms (Sun et al. 2022). Zhuang et al. (2020) revealed the presence of an *azoR* gene encoding for the azoreductase gene in the Hanjiang River (Table 2). Apart from the *azoR* gene, they also detected laccase and naphthalene-degrading enzymes in the study. Naphthalene is a thermally stable functional group of the intermediates of the dye degradation process. Our study (Yadav et al. 2021a) and Rout et al. (2022) also reported several dye-degrading enzymes such as peroxidases, aryl sulfatase, and manganese catalase from Indian rivers. We also observed the plastic-degrading enzyme phenylacetaldehyde dehydrogenase (styD gene) in our study of the Pune riverine system (Yadav et al. 2021a) (Table 2). Furthermore, Sun et al. (2022) reported the presence of microplastic degrading enzymes laccase and alkane monooxygenase in Mycobacteriaceae MAGs from Pearl River, China. This study provided evidence of indigenous microplastic biodegraders that can be harnessed to remediate urban rivers. Water bodies act as environmental sink for plastic pollutants and forever chemicals such as per- and poly-fluoroalkyl substances (PFASs), which are resistant to degradation and accumulate in the environment. The limitations in their biodegradation include the complexity and diversity of these contaminants and a lesser understanding of the microbial metabolic processes and interactions for removal (Shilpa et al. 2022; He et al. 2023; Berhanu et al 2023). Therefore, the metagenomics approach can be employed as a starting point to infer the microbial co-occurrences, their interactions, and metabolic machinery for efficient bioremediation.

Utility of water hyacinth microbiome in remediation: needs for metagenomic interference

The growing evidence suggests that aquatic plants play a crucial role in structuring microbial communities of water bodies (DeWolf et al. 2022). One of the most prevalent and ubiquitously found water macrophytes are *Eichhornia crassipes*, commonly known as water hyacinth (WH) (DeWolf et al. 2022; Harun et al. 2021). It is a rapidly growing notorious weed, ubiquitously found in aquatic bodies worldwide (Harun et al. 2021). Owing to its prolific growth rate, the WH affects the overall water biogeochemistry, resulting in alterations in dissolved oxygen levels and depleting essential nutrients, thus affecting other life forms (Guna et al. 2017; Ting et al. 2018; Xu et al. 2022). Several methods have been employed to control WH growth, including physical removal and herbicides (Galgali et al. 2023). However, these processes are labor-intensive, inefficient, and expensive. Nevertheless, due to the wide availability of WH, several studies have explored its potential for value-added applications (Madikizela 2021). Some applications include using water hyacinth for biogas and ethanol production, in composites, and as adsorbents for removing pollutants such as dyes, heavy metals, and others. Structurally, the water hyacinth comprises cellulose and hemicellulose packed in a lignin structure. WH's roots contain various functional groups, C = O, C–H, OH–, and phosphate, which are instrumental in the adsorbent properties of water hyacinth (Madikizela 2021). Therefore, water hyacinths are used widely in the bioremediation of water for removing pollutants (Emam et al. 2020; Salahuddin et al. 2021).

Although several studies have reported the utility of water hyacinths in removing pollutants from water, limited studies have implicated the role of water hyacinth–associated microorganisms in bioremediation (Pramanic et al. 2023)

Table 3 The following table describes the findings of the enhancements of bioremediation by water hyacinth-associated microbes and microbial communities of water hyacinths

Purpose of the study	Associated and dominant microbes	References
Mercury removal	Isolation sulfate-reducing bacteria	Achá et al. (2005)
Removal of chlorpyrifos	Isolation-based study, *Acinetobacte*r strain	Anudechakul et al. (2015)
Meta-transcriptome study to understand crosstalk of fungi and water hyacinth in heavy metal removal	De novo meta-transcriptomic analysis Upregulation of *Fusarium*	Luo et al. (2015)
Water hyacinth microbiome analysis for nutrient cycling	Profiling of water hyacinth rhizobiome *Deltaproteobacteria* and *Sphingobacteriia* were dominant	Ávila et al. (2019)
Mitigation of soil acidity using water hyacinth derived biochar	16S rRNA sequencing study. High abundance of *Bacillus* with biochar amendment	Jutakanoke et al. (2023)
Metagenome of water hyacinth for microbiome and functionality aspects	140 pollutant degrading enzymes were reported	Yadav et al. (2023) (bioRxiv study)

(Table 3). Achá et al. (2005) correlated the sulfate-reducing bacterial (SRB) group with mercury removal by water macrophytes. They observed a reduction in mercury methylation upon inhibition of SRB metabolism, indicating the potential role of rhizospheric microbes of macrophytes in bioremediation. The presence of rhizosphere-associated *Acinetobacter* strain significantly enhanced the removal of Chlorpyrifos, an organophosphate commonly employed for tackling nematode plant parasites in agriculture.(Anudechakul et al. 2015). Our recent research article published in bioRxiv (Yadav et al. 2023) gave an account of the overall microbiome of water hyacinth with particular emphasis on the functional potential for bioremediation. Over 140 PDEs were detected in the microbiome of water hyacinth along with multiple metal and biocide resistance genes. Various dye-degrading enzymes such as peroxidases, hydrocarbon-metabolizing enzymes such as mono and di-oxygenases, and plastic-degrading enzymes catalase, phenyl acetate dehydrogenase, and hydrolases were detected in water hyacinth rhizobiome. Furthermore, Pramanic et al. (2023) have comprehensively elaborated on the fungal endophytes associated with water hyacinth in their recently published review article. These findings suggested the vital role of water hyacinth-associated microorganisms in the bioremediation of rivers. Due to its importance in the overall ecology of rivers, future studies shall be directed towards bioprospecting water hyacinths for valuable microbes.

Conclusion and future research needs

Global industrialization and the increase in anthropogenic activities have led to the generation of a multiplex mixture of chemical contaminants that are recalcitrant, highly toxic, and possess the highest level of threats to environmental sustainability. Eventually, these xenobiotic chemical contaminants are dumped into freshwater bodies resulting in a menace to all life forms on Earth. Due to its economic feasibility, environmentally friendly, and sustainability, bioremediation is considered one of the best viable approaches for the environmental cleanup of chemical pollutants. Bioremediation utilizes the complex metabolic machinery of microorganisms for the catabolism of recalcitrant pollutants. The present review comprehensively summarized the findings about the application and utility of metagenomics in fetching baseline information on pollutant-degrading enzymes and microbial populations. These pieces of information from metagenomic mining can be systematically used to carry out efficient biostimulation and bioaugmentation processes (Fig. 2; Table 2). Well-organized information is provided on the dynamic changes in microbial communities and ecological determinants upon exposure to chemical pollutants in the river water (Fig. 1; Table 1). Furthermore, water macrophytes, such as water hyacinths prominent in polluted rivers, are widely reported for various applications, including bioremediation; however, the associating link of the water hyacinth-associated microbiome is missing. As the NGS cost continues to decrease, the utility of various sequencing technologies will increase, providing detailed information on the bioremediation process by microbes in polluted rivers and their macrophytes. While the metagenomic approach is promising, there are some limitations to it. It gives an account of the metabolic potentials. However, the actual expression of pollutant-degrading enzymes can be evaluated only after combining a metagenomic approach with culture-based strategies. Furthermore, metagenomic studies shall undertake deep sequencing, generating a large amount of sequencing yield for each survey to cover the entire genome efficiently. The sequence database is another limitation which results in missing out on novel strains and genes. Nevertheless, combining metagenomics with conventional culture-based approaches will ultimately facilitate an understanding of the taxa, genes, pathways, and bioprospecting required for successful bioremediation.

Acknowledgements The authors are grateful to the Director, CSIR-NCL, for providing the facilities, infrastructure, and support. RKY would also acknowledge University Grants Commission (UGC) for fellowship.

Author contribution Rakeshkumar Yadav performed literature research and data analysis, manuscript writing; Mahesh Dharne contributed in supervising, reviewing, and editing.

Data availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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Environmental Science and Pollution Research (2023) 30:103361
https://doi.org/10.1007/s11356-023-29845-3

CORRECTION

Correction to: Metagenomic exploration reveals a differential patterning of antibiotic resistance genes in urban and peri-urban stretches of a riverine system

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Published online: 13 September 2023

Correction to: Environmental Science and Pollution Research (2021) 28:66477–66484
https://doi.org/10.1007/s11356-021-16910-y

The correct details of affiliation 2 is shown in this paper.

The original article can be found online at https://doi.org/10.1007/s11356-021-16910-y.

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Environmental Science and Pollution Research (2021) 28:66477–66484
https://doi.org/10.1007/s11356-021-16910-y

SHORT RESEARCH AND DISCUSSION ARTICLE

Metagenomic exploration reveals a differential patterning of antibiotic resistance genes in urban and peri-urban stretches of a riverine system

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Received: 30 June 2021 / Accepted: 2 October 2021 / Published online: 13 October 2021

Abstract
Antimicrobial resistance in the riverine ecosystem of urban areas is an alarming concern worldwide, indicating the importance of molecular monitoring to understand their patterning in urban and peri-urban areas. In the present study, we evaluated the influence of urban rivers on the connected peri-urban rivers of a riverine system of India in the context of antibiotic resistance genes. The rivers traversing through urban (Mula, Mutha, Pawana, and Ramnadi) and peri-urban stretches (Bhima and Indrayani) form the riverine system of Pune district in Maharashtra, India. The MinION-based shotgun metagenomic analysis revealed the resistome against 26 classes of antibiotics, including the last line of antibiotics. In total, we observed 278 ARG subtypes conferring resistance against multiple drugs (40%), bacitracin (10%), aminoglycoside (7.5%), tetracycline (7%), and glycopeptide (5%). Further, the alpha diversity analysis suggested relatively higher ARG diversity in the urban stretches than peri-urban stretches of the riverine system. The NMDS (non-metric multidimensional scaling) analysis revealed significant differences with overlapping similarities (stress value = 0.14, p-value = 0.004, ANOSIM statistic R: 0.2328). These similarities were reasoned by assessing the influence of downstream sites (sites at the outskirts of Pune city; however, directly impacted), which revealed significant differences in the ARG contents of urban and peri-urban stretches (stress value = 0.14, p-value = 0.001, ANOSIM statistic R: 0.6137). Overall, we detected the dissemination of antibiotic resistance genes from the polluted urban rivers into the peri-urban rivers located downstream in the connected riverine system potentially driven by anthropogenic activities.

Keywords MinION · Peri-urban rivers · Urbanization · Antibiotic resistance

Introduction

Contamination of the natural environment with active pharmaceutical ingredients (antibiotics) is rapidly increasing amid its unrestricted and widespread use. The release of contaminated wastewaters from various industries, hospitals, agriculture, animal production, and aquaculture into the aquatic bodies facilitates the emergence and evolution and potentially triggers the dissemination of antibiotic-resistant genes (ARGs) (Singh et al. 2019). Globally, India is one of the largest consumers of antibiotics and shows higher irrational usage of antibiotics (Marathe et al. 2017). Consequently, there appears to be a higher incidence of antibiotic resistance, which is reflected in the water bodies of India, resulting in their dismal conditions (Singh et al. 2019, Ahammad et al. 2014, Marathe et al. 2017, Yadav et al. 2021, Water Quality Database, Borthakur and Singh 2016). Several studies have revealed the enormity of ARGs in important and highly revered Indian rivers (Reddy and Dubey 2019, Diwan et al. 2017, Yadav et al. 2020, Samson et al. 2019). Reddy and Dubey, in 2019, reported 23 different ARG types in River Ganga, whereas Yadav et al. (2020) reported 29 different ARG types in the river Bhima and Indrayani (142 and 333 ARG subtypes, respectively).

Responsible Editor: Robert Duran

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The peri-urban rivers possess hybrid urban–rural features and are irreversibly crucial as they provide water for various purposes, including drinking water and agriculture irrigation (Yadav et al. 2021; Zheng et al. 2018; Chen et al. 2019a, 2019b). Despite their significance, these rivers are subjected to uncontrolled wastewater discharges from various sources. Several reports worldwide have revealed diverse ARGs in these peri-urban river ecosystems (Zheng et al. 2018; Chen et al. 2019a, 2019b; Zhang et al. 2020).

Pune city is the ninth largest city in India, located in Maharashtra, with a population near 6 million. The rivers Mula, Mutha, Mula-Mutha (formed after the confluence of Mula and Mutha River), Ramnadi, Pawana, Indrayani, and Bhima are the major rivers flowing through the Pune district and form a unique riverine system. The rivers Mula, Mutha, Mula-Mutha, Ramnadi, and Pawana primarily traverse through the highly populated and polluted urban settlements of Pune city. The Ramnadi and Pawana are the tributaries of Mula River, meeting at the right and left bank, respectively, before Mula River meets Mutha River at the heart of Pune city to form Mula-Mutha River (Fig. 1). Further, the Mula-Mutha River flows downstream (away from Pune city) and unloads its content into Bhima River (mostly flowing through peri-urban and agriculture dominant regions) at the outskirts of Pune city; eventually, Bhima River flows towards southern India and meets Krishna River, which empties itself in the Bay of Bengal.

In the last decade, the rivers of Pune have been reported for harboring diverse ARGs and ARBs, including the resistance to the last line of antibiotics (Marathe et al. 2017; Yewale et al. 2019; Razavi et al. 2017). However, these studies were limited to fewer stretches of the riverine system and did not address urban and peri-urban aspects. To the best of our knowledge, few efforts have systematically explored the ARGs of the interconnected urban and peri-urban rivers (Zheng et al. 2018; Chen et al. 2019a, 2019b; Zhang et al. 2020). River sediments harbor the maximum living biomass and are hugely responsible for the metabolic cycles (Gibbons et al. 2014). We recently elaborated microbial diversity differences in the urban and peri-urban stretches, reflecting the differential influence of urbanization and industrialization that accelerated anthropogenic activities (Yadav et al. 2021). Here, we hypothesized dissimilarity in the composition of ARGs in the interconnected urban and peri-urban stretch amid the differential level of urbanization. The MinION-based shotgun sequencing of river sediments was performed to examine the differences of ARGs in the urban and peri-urban stretch and understand the overall ARG structure in the riverine system.

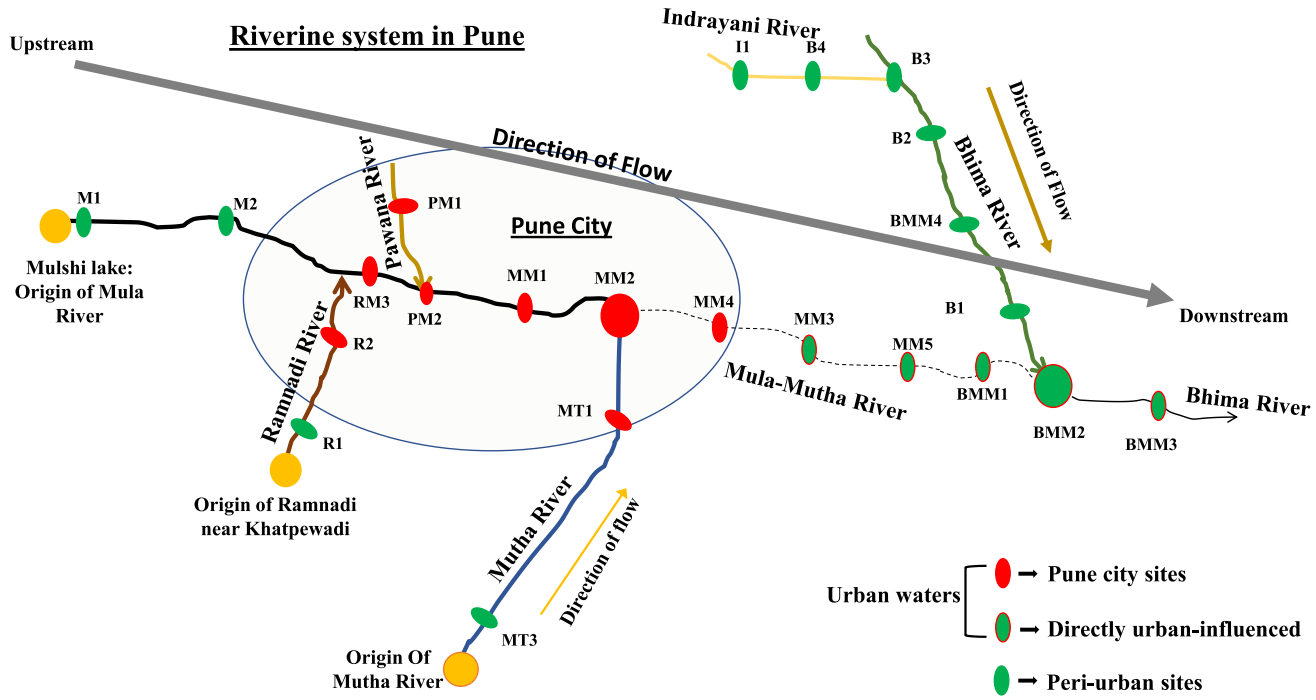

Fig. 1 Schematic of riverine system. The illustration displays the sampling sites and describes the groupings of sampling sites

Materials and methods

Sample site description and sample collection

Based on geographic region, population density, and previous studies, we divided the riverine system into urban waters (sites in the Pune city and downstream sites of Mula-Mutha River) and peri-urban waters (sites upstream of Mula River, sites of River Indrayani and River Bhima (except BMM3)) (Dhawde et al. 2018, Census India 2011, Hui and Wescoat Jr 2019) (Online Resource 1, 2 and Fig. 1). River sediment samples (10–15 cm from the top) were collected during December 2018 in sterile 250 ml containers and stored at 4 °C until further processing. Details of sampling sites and coordinates are given in Online Resource 1. Urban sites ($n = 13$) include MM3 MM4, MM5, BMM1, BMM2, BMM3, MT1, R2, RM3, PM1, PM2, MM1, and MM2, and peri-urban sites ($n = 10$) include M1, M2, R1, MT3, I1, B1, B2, B3, B4, and BMM4.

MinION library preparation and sequencing

Metagenomic DNA was extracted from 400 mg of river sediment using DNeasy PowerSoil Pro Kit (Qiagen). The quality and quantity of DNA were assessed by NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific) and Qubit fluorometer (Thermo Fisher Scientific) using broad range (BR) high sensitivity (HS) assay kit (Thermo Fisher Scientific, Q32853 and Q32851, respectively). The library preparation was carried out using 1D ligation sequencing kit SQK-LSK109, and barcoding was done with native barcoding EXP-NBD104 and NBD114. Around 500 ng of the library was loaded on FLO-MIN 106D R9 Flow cells. The data is submitted in NCBI Sequence Read Archive (SRA) repository (Online Resource 1).

Data processing and analysis

The reads were basecalled using Albacore (v2.3.4), followed by demultiplexing and trimming with qcat (v1.0.1) and porechop (v0.2.4). The antibiotic resistance genes and mobile genetic elements were predicted using a long-read specific NanoARG tool (Arango-Argoty et al. 2019) with default parameters. NanoARG tool uses deep learning-based approach to predict ARGs. Further, stringency was put by selecting only those ARG reads, which showed a minimum bit score of 60 and identity of 30 (Pearson 2013). The alpha diversity and non-metric multidimensional scaling (NMDS) was constructed (using Bray–Curtis dissimilarity measure) using R packages phyloseq and microbiome (McMurdie and Homes 2013, Lahti and Shetty 2017).

Statistical analysis

The alpha diversity was performed using the unfiltered gene data, as we observed only 3X difference between the minimum and the maximum number of ARG reads per sample. The significance of alpha diversity was assessed using the Welch two-sample *t*-test. The variable sequence depth was adjusted using the DESEQ2 package (Love et al. 2014). ANOSIM (p-value threshold of 0.05, permutation of 999) was used to interpret the ordination analysis statistically. Beta-disper (p-value threshold of 0.05, permutation of 999) was used to assess the homogeneity. Fold change analysis was performed using METAGENassist (Arndt et al. 2012).

Results and discussion

Antibiotic resistance genes are now considered emerging environmental pollutants. They can freely disseminate and evolve independently of their host via various transfer routes, such as mobile genetic elements (Ouyang et al. 2015). Urbanization affects the movement of people in the urban surroundings, resulting in an increase in population density and industrialization to facilitate economic activities (Peng et al. 2020; Neiderud 2015). Various studies have implicated the correlation of urbanization with the increased prevalence of antibiotic resistance. Human activities such as irrational use of antibiotics, use of additives in animal and fish feedings, and release of partially or untreated wastewater are the multiple factors of urbanization that affect the riverine systems (Ouyang et al. 2015). These direct impacts of increasing anthropogenic activities on the urban rivers have a chain effect, polluting the connected downstream rivers in the outskirts of cities. To understand such a phenomenon concerning antibiotic resistance in the rivers, we used a metagenomic approach to study the differences in the resistome of a riverine system composed of urban and peri-urban stretches. Overall, the nanopore sequencing generated 3.64 million reads yielding around 18.9 Gbases with an average quality score and sequence length of 8.86 and 5.1 kb, respectively.

Occurrence and distribution of ARGs

The aquatic environment provides a suitable ecological niche for the emergence and dissemination of antibiotic-resistant microbes due to ARG transfer between autochthonous and allochthonous bacteria (Almakki et al. 2019). The analysis revealed ARGs against 26 categories of antibiotics,

including the last resort antibiotics such as polymyxin in the riverine system. Overall, 40% of the ARGs were classified to *multidrug* resistance (MDR) genes, followed by 10% conferring resistance to *bacitracin*, 7.5% to *aminoglycoside*, 7% to *tetracycline*, and 5% to *glycopeptide* (Fig. 2). The other highly prevalent ARGs (0.1%) were detected against the categories such as *macrolides*, *lincosamides*, and *streptogramines* (MLS), *fosmidomycin*, *fluoroquinolone*, *beta-lactam*, *peptide*, *rifamycin*, *sulfonamide*, *phenicol*, *diaminopyrimidine*, *polymyxin*, *triclosan*, *aminocoumarin*, *nucleoside*, *pleuromutilin*, and *fosfomycin* (Fig. 2). The fold change analysis revealed a significant increase of ARGs against

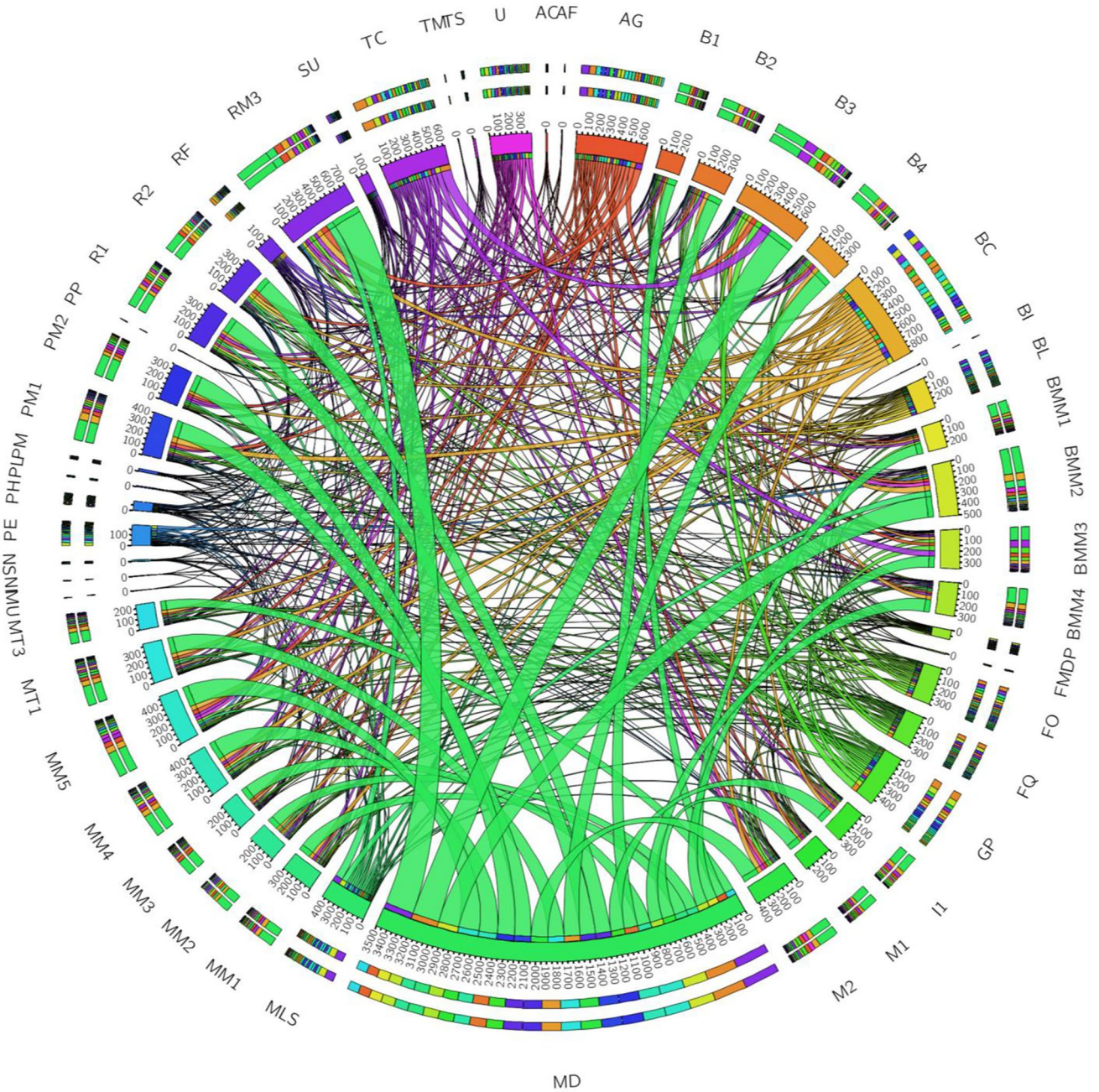

Fig. 2 Resistance against different categories of antibiotics. The circos plot illustrates the detection of different antibiotic resistance genes against 26 categories of antibiotics. GP, glycopeptide; BL, beta-lactam; PP, polyamine and polypeptide; MD, multidrug; SU, sulfonamide; AG, aminoglycoside; FQ, fluoroquinolone; BC, bacitracin; PE, peptide; TC, tetracycline; FO, fosmidomycin; U, unclassified; MLS, macrolides, lincosamides, and streptogramines; RF, rifamycin; PH, phenicol; DP, diaminopyrimidine; AC, aminocoumarin; PM, polymyxin; FM, fosfomycin; TM, tetracenomycin C; NS, nucleoside; PL, pleuromutilin; TS, triclosan; NI, nitroimidazole; M, mupirocin; BI, bicyclomycin; AF, antibacterial free fatty acids

fosfomycin, *nitroimidazole*, *sulfonamide* (*t*-test with FDR correction, $p < 0.05$), and *tetracenomycin C* in the urban and urban-influenced sites (Online Resource 3). At the same time, the peri-urban region showed enrichment of ARGs against polyamine and polypeptide group of antibiotics. In all, the total ARGs (against antibiotic classes) were more extensive in this riverine system as compared to the other polluted rivers of India, such as the River Ganges and River Yamuna (Das et al. 2020, Reddy and Dubey et al. 2018), and were comparable to urban and peri-urban rivers across the world (Chen et al. 2019c, 2019d; Zheng et al. 2018; Singh et al. 2019). Relatively, the ARGs against six categories, including *antibacterial free fatty acids*, *tetracenomycin*, *polyamine/polypeptide*, *nitroimidazole*, *mupiromycin*, and *bicyclomycin*, were scarcely identified (Fig. 2).

Altogether, the analysis revealed 278 different ARG subtypes in the riverine system. The previous study by Marathe et al. (2017) on Mutha River identified 175 ARGs, the majority of them getting mapped to city samples. The ARGs such as *ompR*, *rpoB2*, *bcrA*, *bacA*, *tetA(48)*, *kdpE*, *vanR*, *multidrug ABC transporter*, *mtrA*, *rosB*, *oprM*, and *patA* were among the most dominant ARG (≥ 2%) subtypes in the riverine system (Fig. 3). The *ompR*, a regulator gene involved in significant upregulation of numerous MDR drug exporter genes (Hirakawa et al. 2003), was the most dominant ARGs, followed by *rpoB2* RNA polymerase variant, which evades the action of rifamycin (CARD database). Further, most of the detected ARGs such as *mtr*, *acr*, *ade*, *cme*, *mds*, *mdt*, *mex*, and *sme* (Online Resource 4) encoding resistance to multiple drugs provide antibiotic efflux as the central mechanism of resistance (CARD ontology and CARD database). We observed numerous genes for various beta-lactamases that efficiently hydrolyze many third-generation antibiotics. Several members of *VEB beta-lactamases* (also known as Vietnamese extended-spectrum beta-lactamases) were observed, such as *VEB-1*, *VEB-2*, *VEB-3*, and *VEB-7* (Online Resource 4). These extended-spectrum beta-lactamases (ESBLs) are known to inactivate broad-spectrum *cephalosporins* and *aztreonam* (CARD database). The analysis also revealed several other important beta-lactamases, which include *tem* gene that confers resistance against *cephalosporin*, *OXA beta-lactamases* that are reported in pathogens such as *Acinetobacter baumannii* and *Enterobacter*, and *NmcR beta-lactamase* commonly found in *Enterobacter* species (Palzkill 2013, McArthur et al. 2013). Previously, the mobile

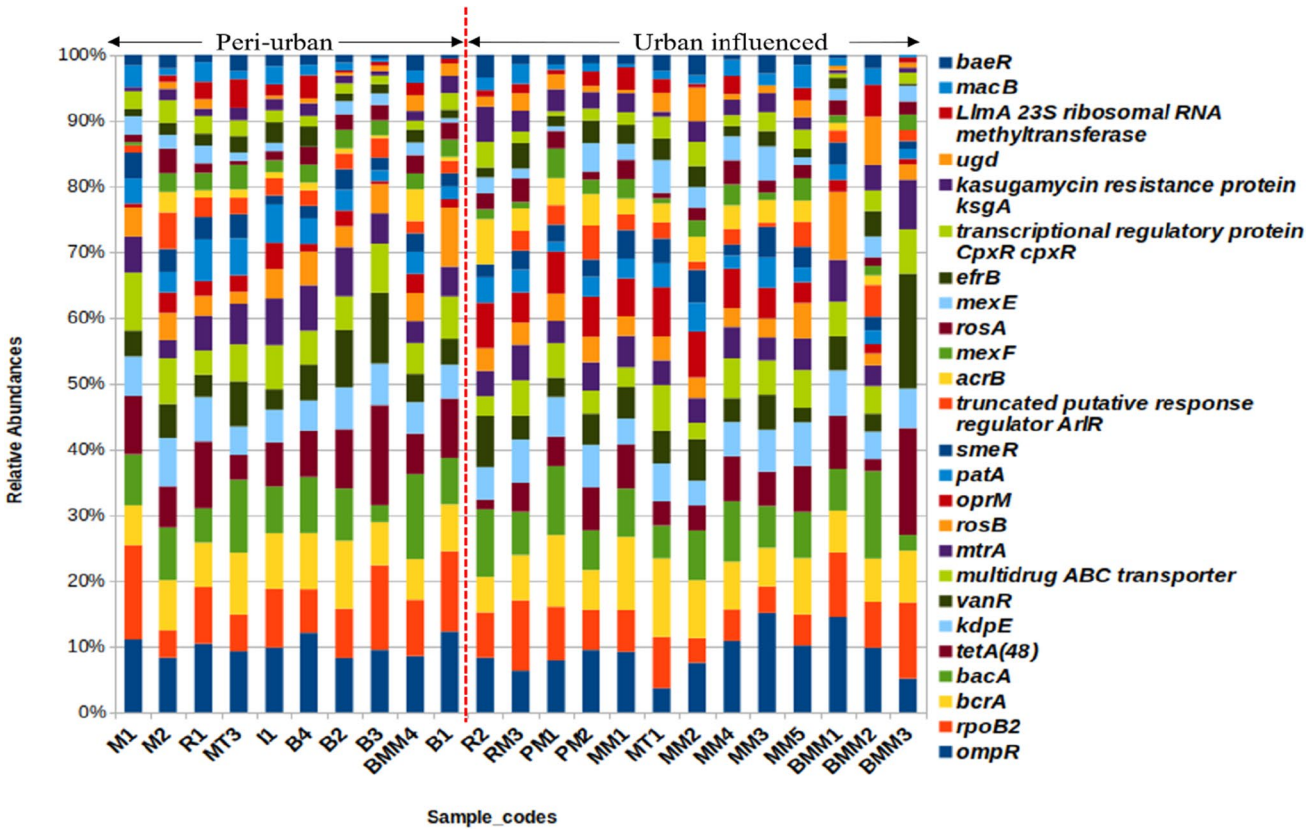

Fig. 3 Distribution of Antibiotic resistance genes. The relative abundance stacked bar plot illustrates the distribution of ARGs (≥ 1%) in the riverine system

colistin resistance gene *(mcr1)* conferring resistance to the last resort drug polymyxin was reported in Mutha River by Marathe et al. (2017). These *mcr* genes confer resistance against drugs such as colistin belonging to polymyxin, which is often considered the last line of antibiotics. The analysis revealed the polymyxin inactivating *mcr5* and *arnA* gene in the riverine system. Overall, the results indicated a higher prevalence of different ARG subtypes conferred against almost all major clinically relevant antibiotics.

Spatial diversity of ARGs in the riverine system and mobilome

Alpha and beta diversity analysis was performed to understand the overall differences in the ARGs of the urban and peri-urban stretch of the riverine system. We observed relatively higher alpha diversity (Shannon and Chao1 indices) of the ARGs in the urban stretch compared to the peri-urban stretch (Online Resource 5); however, it was not significant. The NMDS analysis, along with ANOSIM statistic, indicated significant diversity differences (p-value = 0.004, ANOSIM statistic R: 0.2328); however, the lower R value indicated the overlapping similarities (Fig. 4). This could probably be attributed to the similar climate conditions and the downstream sites MM3, MM5, BMM1, BMM2, and BMM3. We confirmed this by taking "rivers" as a variable. We observed no significant influence of rivers on the observed diversity differences of ARGs (p-value = 0.301, ANOSIM statistic R: 0.05679). Although geographically, the downstream sites MM3, MM5, BMM1, BMM2, and BMM3 are at the outskirts of Pune city, these sites are directly influenced by the urban waters, unlike the other peri-urban sites, and thus, these sites were grouped into urban waters. Further, the comparison of the urban waters (excluding the sites MM3, MM5, BMM1, BMM2, and BMM3) with the peri-urban sites revealed significant differences (p-value = 0.001) with higher ANOSIM statistic R of 0.6137. These observations strongly indicate the following. (i) These sites are potentially responsible for the overlapping similarities observed due to their dual characteristics of urban and peri-urban. (ii) We observed clear demarcation in the ARG diversity between the Pune city and peri-urban sites.

The mobile genetic elements (MGEs) (Online Resource 6) are responsible for disseminating ARGs, leading to an increase in multidrug resistance. We observed the dominance of transposases, integrases, and recombinases at all the sites. The MGEs such as insertion sequences (IS) and transposons are DNA segments carrying different ARGs that

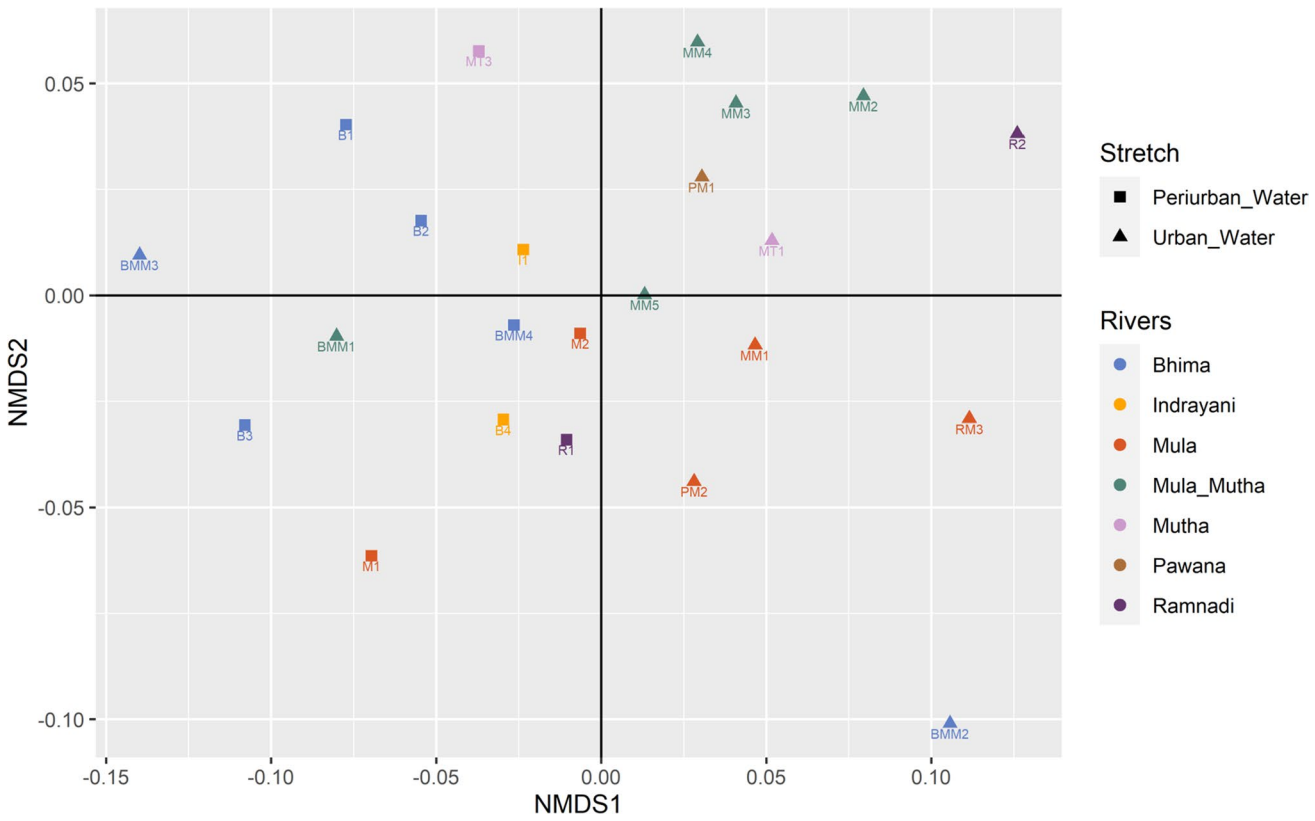

Fig. 4 Beta diversity of ARGs. The beta diversity was assessed using NMDS (stress value = 0.14) with the Bray–Curtis dissimilarity matrix. The statistical significance was estimated using ANOSIM (p-value = 0.004, ANOSIM statistic R: 0.2328)

can move themselves to random locations with the help of enzymatic machinery that involves transposases and recombinases. The other elements, such as integrons with the help of integrases and various recombinases, carry out site-specific recombination to spread resistance (Frost et al. 2005). The class 1 integron (intI1) detected in all sediment samples is one of the major MGEs present in various environmental microorganisms (Chen et al. 2020). Together these observations suggested the considerable impact of the urban waters on the connected downstream sites at the outskirts of city in the interconnected rivers.

Conclusion

In conclusion, we observed a relatively higher diversity of ARGs in the urban waters than the peri-urban stretches indicating the differential influence of urbanization on the stretches of a riverine system. Nonetheless, the NMDS analysis also suggested the impact of the urban waters on the connected downstream stretch implicating the cumulative impact of the urbanization on the riverine stretch. The analysis also reflected that the downstream peri-urban sites MM3, MM5, BMM1, BMM2, and BMM3 were directly impacted by the urban waters and thus exhibited overlapping similarities in the resistome. Further, we also observed ARGs against all the clinically important antibiotics, including the last line of antibiotics such as polymyxin. Overall, this study provides an overview of the ARGs prevalent in the riverine system and delineates the spatial differences of the antibiotic resistance genes. The results discussed in this study emphasize necessary strategies such as appropriate treatment of the wastewater, removal of active pharmaceutical ingredients from the wastewater, adequate planning of city drains, and prevention of unrestricted release of untreated wastewater to curb the inflow of urban resistome entering into the downstream peri-urban river stretches.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-021-16910-y.

Acknowledgements The authors are grateful to the Director, CSIR-NCL, for providing facilities, infrastructure, and support. RKY acknowledges the University Grants Commission (UGC), New Delhi, for fellowship. We acknowledge technical assistance of Mr. Digeshwar Rane during sample collection.

Author contribution All the authors have read and review the manuscript for submission. VR: Analysis and writing original draft. RKY: Editing, sample collection, and sequencing. MD: Supervision, designing study, and reviewing.

Availability of data and materials The data is submitted in NCBI Sequence Read Archive (SRA) repository (Refer to Online Resource 1).

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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Corrigendum to "Functional metagenomic landscape of polluted river reveals potential genes involved in degradation of xenobiotic pollutants" [Vol 192, 110332]

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The authors regret that the printed version of the above article contained a minor error related to the institute affiliation of 1st and last author. "Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, India" to be changed to "Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, **Uttar Pradesh 201002**, India." The authors would like to apologise for any inconvenience caused.

DOI of original article: https://doi.org/10.1016/j.envres.2020.110332.

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https://doi.org/10.1016/j.envres.2023.116128

Available online 13 May 2023

Environmental Research 192 (2021) 110332

Contents lists available at ScienceDirect

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Functional metagenomic landscape of polluted river reveals potential genes involved in degradation of xenobiotic pollutants

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ARTICLE INFO

Keywords:
MinION
Xenobiotics
Benzoate
Riverine system
Bioremediation

ABSTRACT

Rapid industrialization contributes substantially to xenobiotic pollutants in rivers. As a result, most of the rivers traversing urban settlements are in significantly deteriorated conditions. These pollutants are recalcitrant, requiring robust catabolic machinery for their complete transformation into bioavailable and non-toxic by-products. Microbes are versatile dwellers that could adapt to such contaminants by using them as a source of nutrients during growth. However, efficient bioremediation requires an in-depth knowledge of microbial diversity and their metabolism related genes in the polluted niches. We employed MinION shotgun sequencing, to comprehend the biodegradation related genes and their function potential operating in the polluted urban riverine system of Western India. A vast number of catabolic genes were detected for the xenobiotic pollutants such as Benzoate, Nitrotoluene, Aminobenzoate, Drug metabolism, and Polycyclic Aromatic Hydrocarbons. Aerobic, and anaerobic catabolism genes, were mapped for their ability of degradation of xenobiotics. Interestingly, catabolism profiles of multiple aromatic compounds culminated into the Benzoate degradation pathway, suggesting it as a plausible central pathway for the autochthonous bacterial communities. Further mapping with RemeDB database, predicted plastic and dye degrading enzymes. Moreover, the diversity indices for the pollutant degrading enzymes suggested little variations (R^2 value of 18%) between the city and non-city (outskirts of city limits) riverine stretch indicating the impact of industrialization in the outskirts of the city stretch as well. Altogether, this study would serve as a preliminary baseline for future explorations concerning river cleaning programs and also exploiting such microbes for bioremediation applications.

1. Introduction

Conservation and safeguarding of water is an absolute necessity to save all the life forms on planet Earth. Rapid population explosion, urbanization and industrialization along the river basins have severely impacted river health and resulted in its deterioration (Suthar et al., 2009). Undoubtedly, industrialization has provided an immense opportunity and have transformed human life with economic and social developments; however, it has also brought unpleasant environmental pollution with it. This has resulted in generation and dumping of various toxic contaminants and xenobiotics to the water bodies, especially in the rivers traversing the urban settlements (Malla et al., 2018; Mittal et al., 2019).

India has a vast network of rivers. However, most of the rivers are in extremely dismal conditions (Water Quality data, CPCB). Urban rivers have a multitude of benefits for the city dwellers; however, the pollution of the urban river is becoming more critical (Wang et al., 2012; Suthar et al., 2009). The untreated sewage or wastewater is one of the main reasons for their contamination and pollution (Marathe et al., 2017). Pune is among the fastest-growing cities in the western part of India. Mula, Mutha, Pawana, Ramnadi, Indrayani, and Bhima Rivers that flow in Pune district. The Mula-Mutha, Ramnadi, and Pawana rivers mostly traverse urban stretches and are affected by wastewaters from industries, domestic sources and hospitals. The Mula and Mutha join Bhima River in the downstream outside the city stretches. Presently, these rivers are in dismal conditions, and it is progressively worsening (Water Quality data CPCB, Yewale et al., 2019; Nawani et al., 2016).

Xenobiotic pollutants such as Polycyclic aromatic hydrocarbons, azodyes, halogenated compounds, nitroaromatic compounds triazines and many more are recalcitrant and persistent chemicals having adverse and toxic effects (Godheja et al., 2016). Untreated sewage and industrial effluents result in the pollution of the surface water bodies, especially

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https://doi.org/10.1016/j.envres.2020.110332
Received 3 June 2020; Received in revised form 3 September 2020; Accepted 7 October 2020
Available online 15 October 2020
0013-9351/

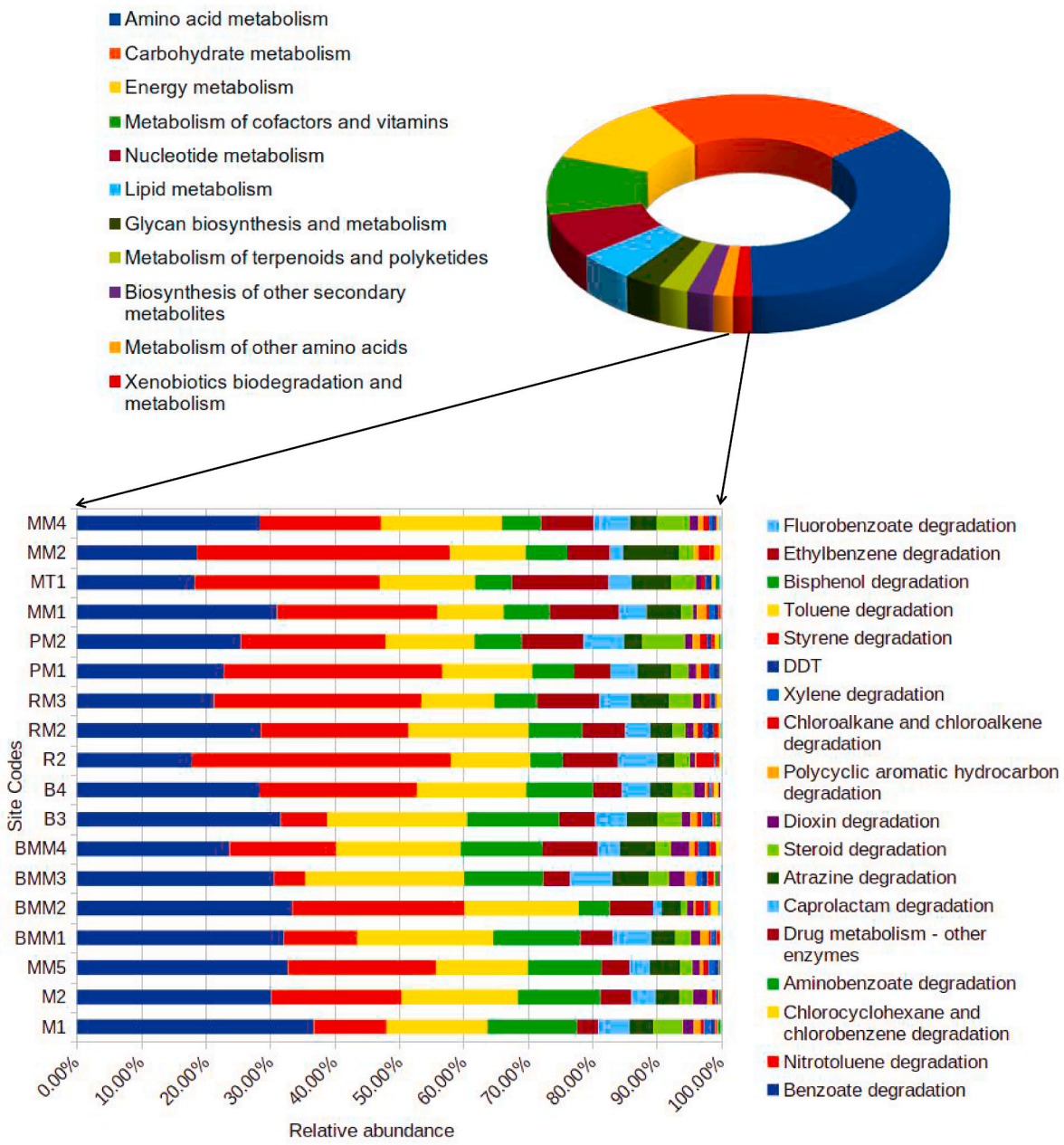

Fig. 1. Metabolism hierarchy and the distribution of degradation genes for various xenobiotic pollutants. Thestacked bar plot describes the relative abundance of the catabolic genes observed for each category of the pollutants across the. length of the riverine system. (City sites: R2, RM2, RM3, PM2, PM1, MM1, MM2, MT1, MM4 and non-city sites M1, M2,MM5, BMM1, BMM2, BMM3, BMM4, B3, B4).

rivers, with various xenobiotic pollutants ranging from petroleum chemicals to heavy metals (Nikanorov and Stradomskaya 2009; Suthar et al., 2009; Guo et al., 2012). While several approaches have been used to reduce xenobiotic pollution, it still remains a grave problem. Microbial based-bioremediation is one of the sustainable ways to transform these recalcitrant pollutants by utilizing their metabolic adaptability (Azubuike et al., 2016). However, two significant questions arise when it comes to the isolation of such microbes. Firstly, their source of isolation and secondly, their diversity and growing conditions. Successful isolation of diverse microbes and their ease of cultivation forms the basis of effective bioremediation. With the advent of Next Generation Sequencing technologies (NGSTs), it is now possible to infer the detailed genomic, metagenomic and functional potential of the microorganisms of any environment. NGSTs studies concerning the bioremediation process provides key preliminary insights of the biodegradative pathways, detoxification mechanism and the diversity of detoxifying microbes (Malla et al., 2018). This information, thus, can be effectively used to strategize the bioremediation process of any environment.

In this comprehensive study, we used MinION-based shotgun sequencing to unravel the complexity and diversity of the xenobiotic degradation mechanism operating in the urban riverine system. The main aim of the study is to; i). examine the mechanism of degradation of various xenobiotic pollutants, ii). understand the diversity of pollutant degrading enzymes in the riverine system. This detailed study will provide an opportunity for future studies to accurately isolate and extract the well-adapted autochthonous microbial community for the bioremediation purpose.

2. Materials and methods

2.1. Details of the study sites and sample collection

The riverine system (formed from Mula, Mutha, Ramnadi, Pawana, Mula-Mutha, and Bhima River) under this study comprises of the riverine stretch (mainly Mutha, upstream of Mula-Mutha, Ramnadi, Pawana, and mainly downstream stretch of Mula River) mostly traversing through a highly urbanized Pune city, Maharashtra, India. The Mula and Mutha Rivers travel around 52 Km and 21 Km respectively, from their origin to meet in the heart of Pune city. Pawana travels around 60 Kms to meet Mula at its left bank, whereas Ramnadi (Stream) approximately originates near Manas lake to meet Mula on its right bank. The Bhima River originates from the Western ghats and joins Mula-Mutha River at the outskirts of Pune city. The Bhima River empties itself into the Krishna River which ends in the Bay of Bengal. Based on the location of the sites concerning Pune city, we broadly divided the riverine system into two parts, namely city (n = 8) and Non-city (n = 14). ('n' denotes the number of sites or samples). The sediment samples (n = 18, depth ~10–15 cm) were collected during December-2018, in gamma-irradiated sterile containers and stored at 4 °C until further processing. The elaborated details of the sample sites are stated in (Supplementary Table 1.). The city sites were named as R2, RM2, RM3, MM1, MM2, MT1, PM1, PM2, and MM4 sites whereas the non-city area includes M1, M2, MM5, BMM1, BMM2, BMM3, BMM4, B3, and B4 sites.

2.2. MinION library preparation and sequencing

The sediment samples (400 mg–600 mg) were subjected to DNA extraction using DNeasy PowerSoil Pro Kit (Qiagen) following the manufacturer's instructions. DNA with the purity of 1.7–1.9 (260/280) assessed by Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific) followed by its qubit analysis on Qubit fluorometer (Thermo Fisher Scientific) using Qubit Broad range (BR) assay kit, and Qubit High sensitivity (HS) assay kit (Thermo Fisher Scientific, Q32853 and Q32851 respectively) was processed for library preparation. The library was prepared with a DNA concentration of 1.1 μg/48 μl using 1D Ligation sequencing kit SQK-LSK109, and the 18 samples were barcoded using Native barcoding EXP-NBD104 and NBD114 as per the manufacturer's instructions. The resultant library was quantified around 500 ng of the library was loaded on FLO-MIN 106D R9 Version.

2.3. Metagenomic data processing and analysis

Base-calling was performed using Albacore (v2.3.4), and the resultant fastq reads were demultiplexed using qcat (v1.0.1). The adapter trimming was carried out by using Porechop (v0.2.4). We used MG-RAST (Keegan et al., 2016). (E-value cut-off = 1 × e−5 and sequence identity of 60% using the KEGG database for the prediction of xenobiotic degradation genes. The taxonomy-based filtering of the xenobiotic gene was carried out by creating a filter for Bacteria only (RefSeq taxonomic annotations) followed by a functional filter of Xenobiotics degradation and metabolism in MG-RAST. We used DIAMOND (Buchfink et al., 2015) for the alignment with a parameter of "diamond blastx -e 0.00001 –id 60 –more-sensitive" for RemeDB analysis (Sankara et al., 2019). We mainly used the R package phyloseq package (McMurdie and Homes 2013) and microbiome (Lahti et al., 2017) for alpha diversity and beta diversity analysis.

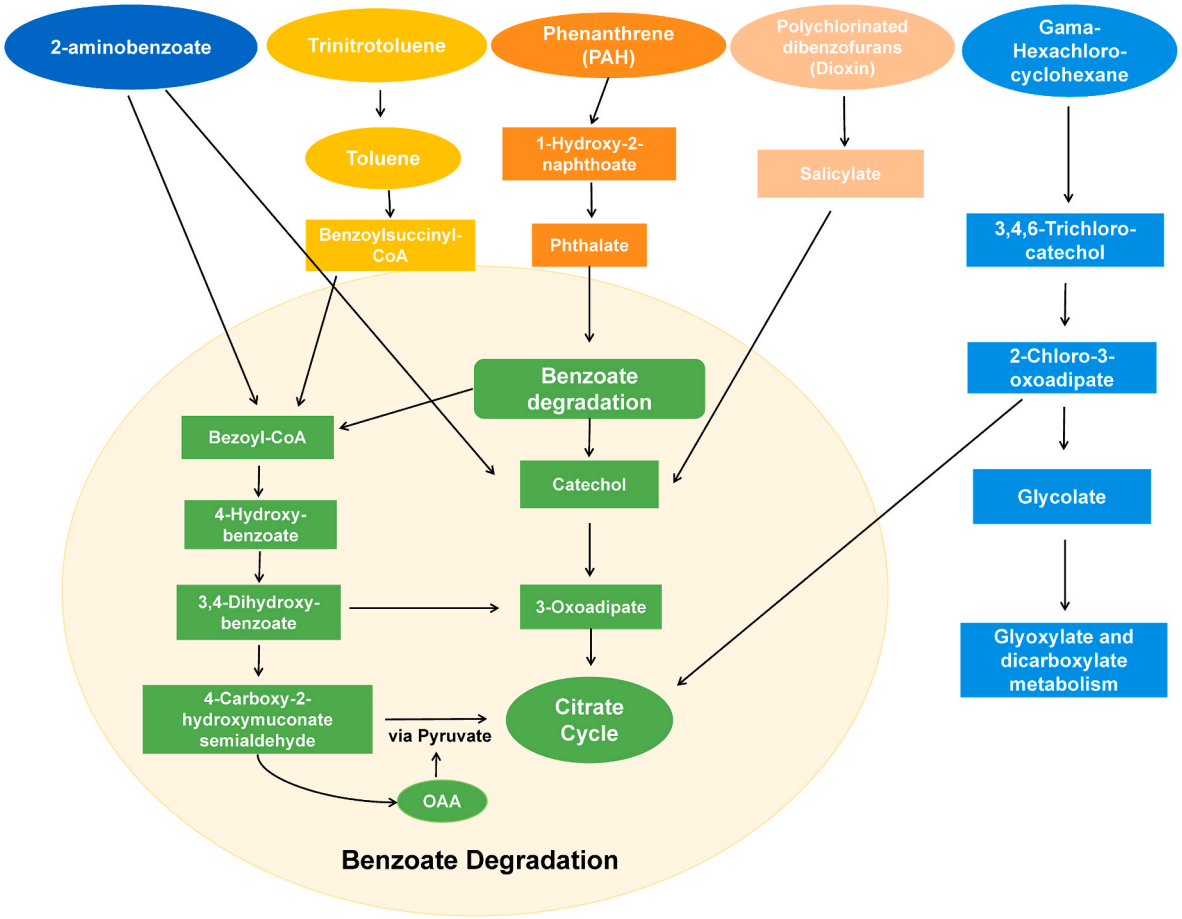

Fig. 2. Predicted composite pathway. The schematic depicts the predicted composite catabolic pathway operational in the riverine system.

2.4. Statistical analysis

Prior to the estimation of alpha and beta diversity, the samples were randomly sampled (set.seed of 100) to lowest denomination. Bray-Curtis dissimilarity measure was used to construct the Principal Coordinate Analysis (PCoA) plot for estimating beta diversity. Permanova (Adonis test, p-value threshold of 0.05). Betadisper test (p-value threshold of 0.05) was done to assess the significance of compositional heterogeneity and homogeneity among the groups. The distribution of data was checked using the Shapiro-Wilk normality test (p-value threshold of 0.05) and Q-Q plot. STAMP (Statistical Analysis of Metagenomic Profiles) (Parks et al., 2014) was used for analyzing significant features between city and non-city samples at level 3 of Xenobiotic category. Welch's *t*-test (p-value threshold of 0.05) along with Benjamini-Hochberg FDR for statistical significance assessment and multiple test corrections was used.

3. Results and discussion

Microbes are metabolically highly diverse, possessing an extensive repertoire of enzymes for the efficient transformation and cycling of complex recalcitrant compounds (Wainwright et al., 1999). Based on multiple physico-chemical parameters, various studies (Borthakur et al., 2016; Yewale et al., 2019) and government report (Water Quality data, CPCB) have indicated the dismal condition of these rivers. The MinION sequencing of the 18 samples generated 14.4 Gbases of the basecalled data. The average sequence length was 4.9 kb and the average quality score was ~8.9. The predictive functional analysis using KEGG database assigned the metagenomic reads to broadly six functional modules at level 1 of classification viz., Metabolism, Genetic Information Processing, Environmental Information Processing, Cellular Processes, Human Diseases, and Organismal Systems. Metabolism is a core process for the survival of any living being, and thus a majority of the reads (~60%) were assigned to Metabolism module. At level 2 of KEGG hierarchy of Metabolism category, the maximum reads were mapped to essential functions such as Amino acid metabolism (35.46%), followed by Carbohydrate metabolism (22.69%), Energy metabolism (10.95%), and other eight categories including Xenobiotics biodegradation and metabolism (1.46%) (Fig. 1.). Furthermore, the analysis using the RemeDB database resulted in the prediction of more than 350 pollutant degrading enzymes involved in hydrocarbon, plastic and dye degradation.

3.1. Predictive composite pathway for xenobiotic pollutant degradation in the riverine system

Aromatic compounds are the second most widely disseminated class of organic compounds and are among the most prevalent and persistent pollutants in nature (Valderrama et al., 2012; Seo et al., 2009). Several genes involved in the degradation of various xenobiotic compounds were predicted at level 3 classification of KEGG orthology. Among these, majority of the reads were mapped to the genes involved in benzoate degradation (28%) (significantly higher in non-city samples (Supplementary Fig. 1.), WT test, BH FDR corrections, p-value<0.05) (Fig. 1.), which is a common human xenobiotic metabolite. Furthermore, higher abundance of the genes involved in degradation and metabolism of major pollutants such as Nitrotoluene (NT) (20.46%), Chlorocyclohexane and Chlorobenzene (CCH: CB) (17.12%), Aminobenzoate (AB) (10.18%), Drug metabolism (DM) (6.43%), Caprolactam (4.51%), Atrazine (4.43%), Steroid (3.10%), and Dioxin (1.48%) were observed. Considerable reads (>0.5%) were also mapped to the genes involved in the degradation of Chloroalkane and chloroalkene, Polycyclic aromatic hydrocarbons (PAH), and Xylene. Notably, the genes involved in the degradation of AB, benzoate, and CCH: CB were significantly higher in non-city samples, whereas PDE for NT and DM were higher in city sites. (Welch's *t*-test, Benjamini-Hochberg FDR corrections, $p < 0.05$) (Supplementary Fig. 1.). Based on the detection of the different xenobiotic degrading enzymes, we propose here a probable composite degradation pathway (Fig. 2.) operational in the riverine system under study.

Table 1
List of the predicted catabolic genes for the predicted composite pathway.

Xenobiotic Pollutant Degradation	Probable Pathways operating in the riverine system	Gene/Enzymes detected in the riverine system
1. Benzoate Degradation	a. Benzoate degradation via 3-Oxoadipate pathway with Catechol as an intermediate b. Benzoate degradation via Benzoyl-CoA c. Other genes	*benB-xylY, benD-xylL, catA, catB, catC, pcaD, pcaI, pcaF.* *badA, hbaBCD, pobA, pcaBC, ligABIJ, oah, badK.* *badDEFGHI, aliA, aliB, benC-xylZ, cmtD, dhbB, dch,hbaA, pcaH,* EC1.14.13.12, EC:1.3.1.62, EC:4.1.1.77, EC:5.3.2.-.
2. Nitrotoluene degradation	a. Trinitrotoluene to Toluene degradation via formation of 2,4,6-Triaminotoluene b. Other genes	E1.12.99.6 L and E1.12.99.6 S, *dsrA, dsrB,* and *nemA. nfnB, nfsB.*
3. Hexachlorocyclohexane (HCH) degradation	a. HCH degradation to either Glycolate and dicarboxylate metabolism or Citrate cycle via Chloroacetate b. Other genes	*catA,* EC3.1.1.45, EC1.3.1.32, EC:3.8.1.2, EC:3.8.1.3. *tfdB, catB, dmpB, bphC, bedC1,* EC1.14.13.7, EC:1.14.13.50, EC:1.14.12.-, EC:1.13.11.37
4.2-Aminobenzoate (ABz) degradation	a. ABz degradation via Benzoyl-CoA b. ABz degradation via Catechol c. Other genes	*abmG, abmA* *antA* *vanAB, antC, desB, mdlC,* EC:3.1.3.41, EC:4.1.1.61, EC:5.1.2.2.
5. Phenanthrene	a. Phenanthrene degradation via Phthalate	*phdF, nidD, phdJ,* EC:1.14.12.7, EC:4.1.1.55

3.1.1. Benzoate degradation

Benzoate is generally used as a model compound for studying the bacterial metabolism of aromatic compounds (Valderrama et al., 2012). We detected multiple vital genes involved in Benzoate degradation. Close inspection of the detected genes suggested a predominance of mainly two pathways (Fig. 2.) for Benzoate degradation in the riverine system. The key genes benB-xylY and benD-xylL encoding Benzoate 1, 2-dioxygenase beta subunit and dihydroxycyclohexadiene carboxylate dehydrogenase, respectively, convert Benzoate to catechol (Table 1). Apart from these two genes, the presence of other essential genes such as catB, catC, pcaD, pcaI, and pcaF genes confirmed the benzoate degradation via an aerobic β-ketoadipate pathway (Table 1) with catechol as an intermediate (Kanehisa and Goto et al., 2000; Kanehisa et al., 2019; Yoon et al., 2007; Valderrama et al., 2012).

Benzoate → Catechol → 3-Oxoadipate-enol-lactone → TCA

Based on gene prediction, the other pathway seemed to be operating in the riverine system was an anaerobic pathway initiated by Benzoate-CoA ligase (badA) (Harwood et al., 1998; Valderrama et al., 2012). The Benzoate-CoA ligase converts Benzoate to Benzoyl-CoA which is converted to 4-Hydroxy-benzoate via an essential enzyme 4-hydroxy benzoyl-CoA reductase. Further, the detection of genes such as pobA, pcaBC suggested catabolism of 4-Hydroxy-benzoate via formation of β-ketoadipate and detection of lig genes, oah and badK indicated its metabolism to Citrate cycle (tricarboxylic acid (TCA) cycle) via formation of 4-Caboxy-2-hydroxymuconate semialdehyde Table 1 (Kanehisa and Goto et al., 2000; Kanehisa et al., 2019).

Benzoate → Benzoyl-CoA →4-hydroxybenzoate →3-Oxoadipate →TCA

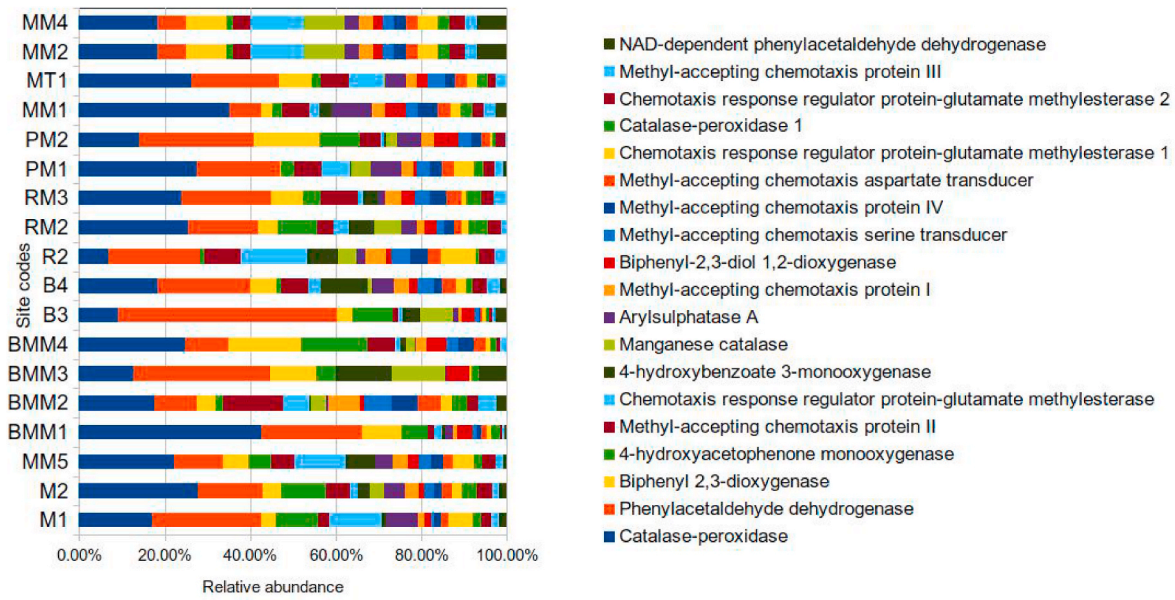

Fig. 3. Pollutant degradating Enzymes (PDE). The stacked bar plot depicts the relative abundance (1%) of the PDE involved in the degradation of various pollutants. RemeDB database was used for the analysis. (City sites: R2, RM2, RM3, PM2, PM1, MM1, MM2, MT1, MM4 and non-city sites M1, M2, MM5, BMM1, BMM2, BMM3, BMM4, B3, B4).

4-Carboxy-2-hydroxymuconate semialdehyde

Altogether, the riverine system harbours both aerobic as well as anaerobic machinery for the metabolism of Benzoate.

3.1.2. *Nitrotoluene transformation*

The stable aromatic ring structure and nitro groups make the degradation of Nitrotoluene very challenging (Serrano-Gonzalez et al., 2018). Prevalence of Nitrotoluene degradation genes such as nemA, dsrA and dsrB (Table 1), and the hydrogenase enzymes were observed. Based on the enzymes detected, the Trinitrotoluene (TNT) was probably transformed via 2,4,6-TNT entering into Toluene degradation. Very less abundance (0.3% of total xenobiotic degradation genes) of Toluene degradation gene was observed in the riverine system. The detection of bbs genes suggested anaerobic degradation of toluene via benzyl-succinate to the subsequent modification to Benzoyl Co-A which ultimately enters Benzoate degradation pathway (Fig. 2.) (Blazquez et al., 2018, Kanehisa and Goto et al., 2000; Kanehisa et al., 2019).

a. TNT → 4-Hydroxylamino-2,6-dinitrotoluene 2,4,6-Triaminotoluene Toluene
b. Toluene → Benzyl-succinate Benzoylsuccinyl-CoA → Benzoyl-Co Benzoate degradation

3.1.3. *Hexachlorocyclohexane (HCH) degradation*

Widely used organochlorine insecticide Lindane (γ-HCH) and its isomers are persistent toxic organic pollutants (Singh and Kuhad, 1999). Despite the detection of a vast number of genes for Lindane degradation, an essential genes (LinA, LinB and LinC) were not present. However, the presence of catechol 1,2-dioxygenase, carboxymethylenebutenolidase, maleylacetate reductase, 2-haloacid dehalogenase, and haloacetate dehalogenase (Table 1) probably indicated γ-HCH degradation via formation of 2-Chloro-3-oxoadipate.

γ-HCH ⇢ 2-Chloro-3-oxoadipate → Chloroacetate → Glycolate → GDM

3.1.4. *2-Aminobenzoate and polycyclic aromatic hydrocarbon (PAH) degradation*

The detection of abmG and antA gene (Table 1) suggested the prevalence of aerobic as well as anaerobic degradation of 2-Aminobenzoate in the riverine system (Lochmeyer et al., 1992). The abmG encodes 2-aminobenzoate-CoA ligase which converts 2-Aminobenzoate to 2-Amino-benzoyl-CoA. The 2-Amino-benzoyl-CoA is transformed into Benzoyl-CoA, ultimately entering in Benzoate degradation pathway (Kanehisa and Goto et al., 2000; Kanehisa et al., 2019). Anthranilate 1, 2-dioxygenase encoded by antA gene converts 2-Aminobenzoate to catechol. PAH are ubiquitous pollutants generated primarily due to incomplete combustion of organic materials (Abdel-Shafy and Mansour 2016). We did not observe an enrichment of genes responsible for the degradation of high molecular weight PAH compounds such as Benzo[a] pyrene. However, genes were detected involved in the degradation of Phenanthrene (Low Molecular weight PAH compound). Prevalence of *phdF, nidD, phdJ,* phthalate 4,5-dioxygenase, and 4,5-dihydroxyphthalate decarboxylase (Table 1) indicated phenanthrene degradation via Benzoate degradation pathway with phthalate as an intermediate (Kanehisa and Goto et al., 2000; Kanehisa et al., 2019).

Phenanthrene ⇢ 1-Hydroxy-2-naphthoate ⇢ Phthalate ⇢Benzoate degradation

Most of the genes involved in the degradation and transformation of other xenobiotic compounds such as Atrazine, Drug metabolism, Bisphenol, Xylene and Steroid were not detected. Thus, the most suitable pathway for their degradation in the riverine system could not be predicted. Furthermore, as most of the catabolic pathway converged to the Benzoate degradation pathway, it appeared to be the central pathway for the aromatic compound degradation in the riverine system.

3.2. *Pollutant degrading enzymes (PDE) in the riverine system*

The analysis using RemeDB database (Fig. 3.) provided extensive information on the PDE. Phenylacetaldehyde dehydrogenase and Catalase peroxidase were the most abundant PDE predicted in the riverine system. Phenylacetaldehyde dehydrogenase, encoded by styD gene, is a component of styABCD operon. This plastic degrading enzyme is involved in the catabolism of styrene, which is a building block of Polystyrene (Mooney et al., 2006). Peroxidases are oxidoreductases

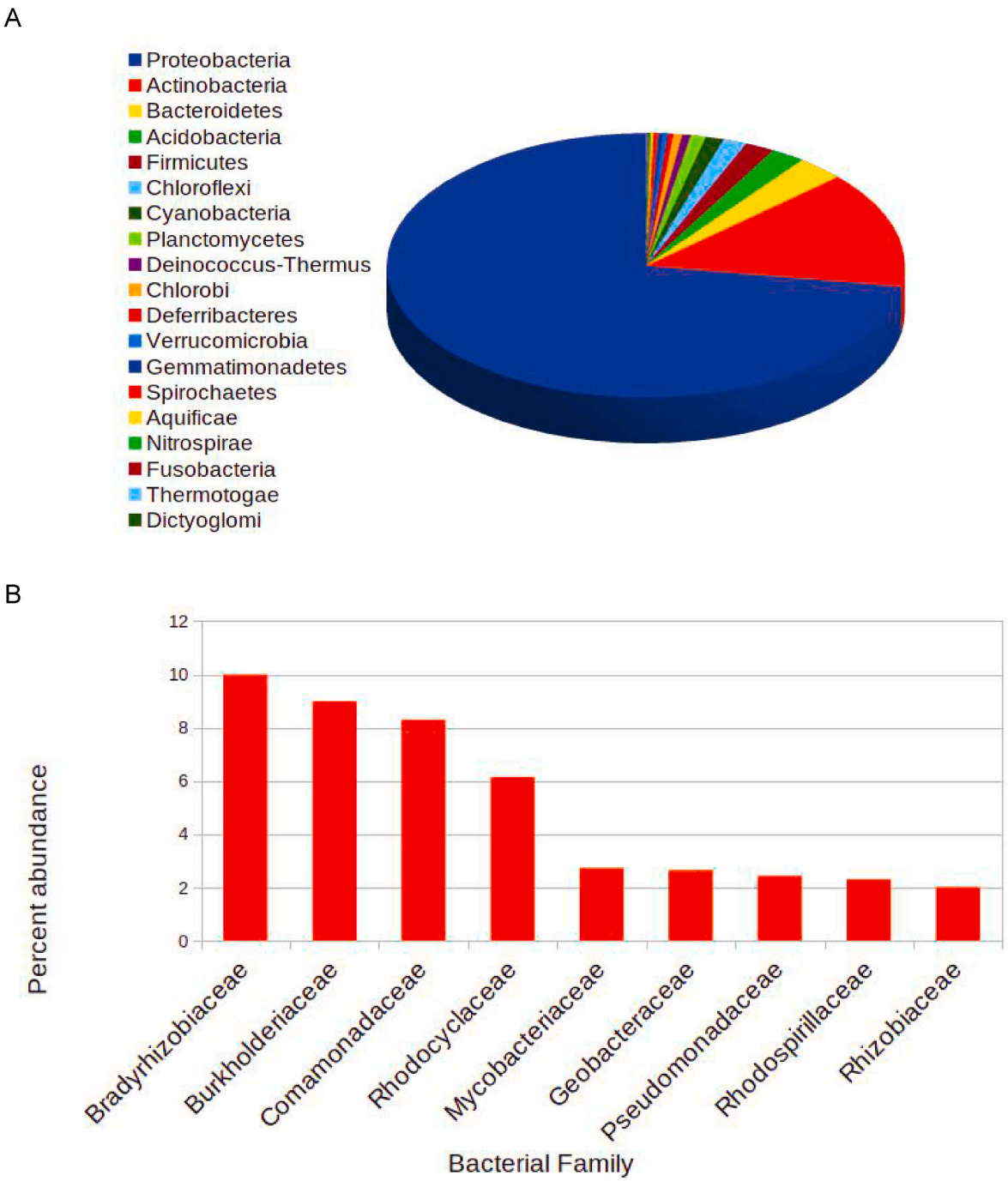

Fig. 4. Abundance for xenobiotic category filtered by taxonomic entities. The stacked bar blot. depicts the relative abundance of xenobiotic gene abundance filtered by bacteria taxa 4 A. at Phylum Level and 4 B. at Family level of classification (Top 10).

involved in the degradation of recalcitrant dyes and other xenobiotic pollutants such as Phenol (Bansal and Kanwar 2013). In addition to these PDE, Biphenyl 2,3-dioxygenase, 4-hydroxybenzoate monooxygenase, Aryl sulphatase A, and Manganese catalase were considerably detected that are involved in hydrocarbons and dye degradation (Subramanian et al., 2020). Enzymes such as Chemotaxis response regulator protein-glutamate methylesterase, Methyl accepting chemotaxis proteins, Methyl accepting chemotaxis serine and aspartate transducer were quite prevalent (>1%) in the samples. Chemotaxis allows microbes to sense environmental cues and react in a positive or negative way towards particular chemicals (Ud-Din and Roujeinikova 2017). It is believed to enhance the bioavailability and biodegradation ability of xenobiotic pollutants (Ahmad et al., 2020). Overall, the analysis indicated the presence of a wide range of PDE for plastic, dye and hydrocarbon degradation in the riverine system.

3.3. *Abundance for xenobiotic category filtered by taxonomic entities*

The taxonomic filtering-based analysis linked xenobiotic degradation genes to 19 bacterial phyla. Maximum degrading genes (72.46) (Fig. 4A.) were related to Proteobacteria, followed by a higher prevalence (>1%) of genes filtered by Actinobacteria, Bacteriodetes, Acidobacteria, Firmicutes, Chloroflexi, and Cyanobacteria. Higher abundance of the xenobiotic degradation reads was filtered by the

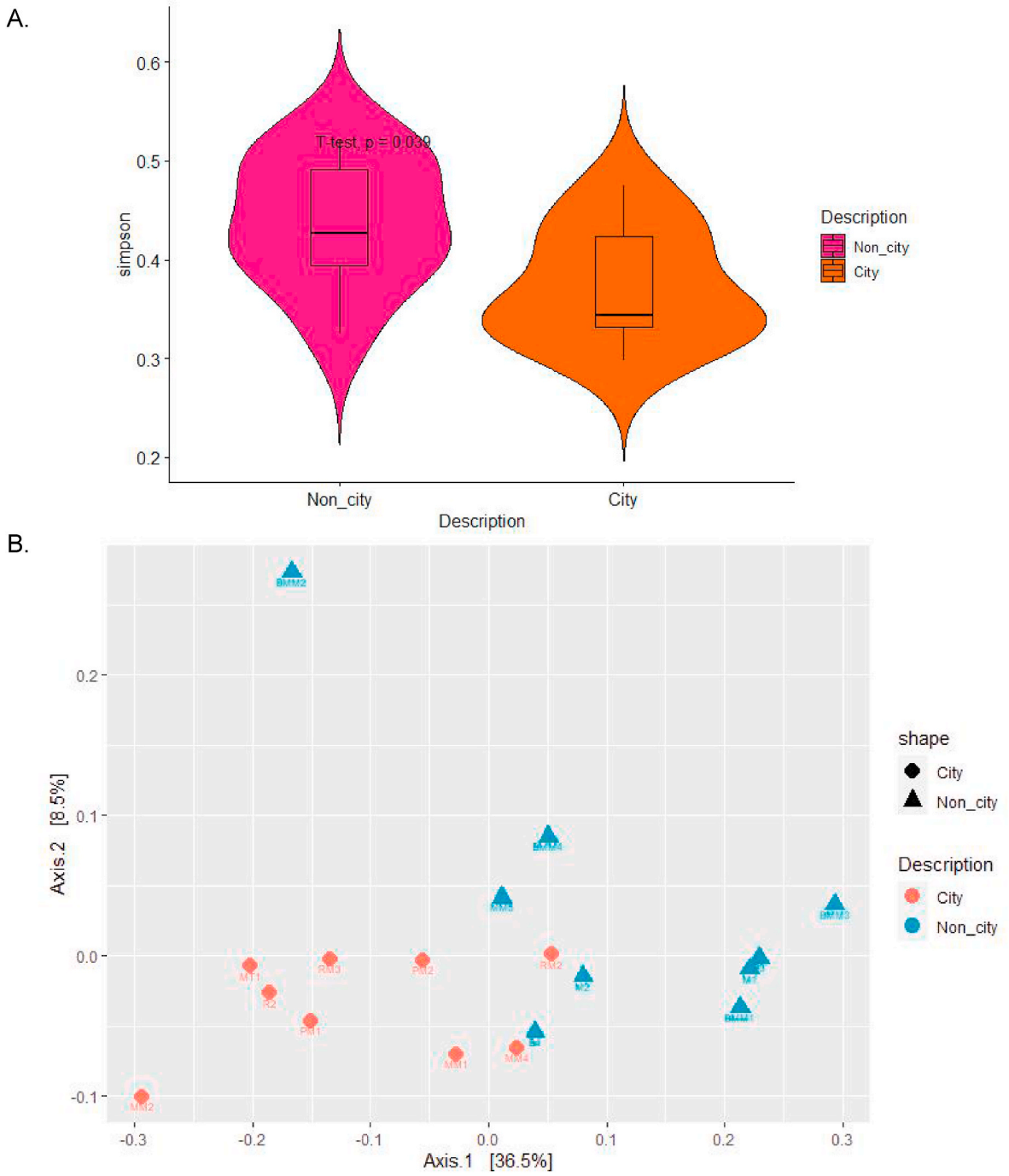

Fig. 5. Diversity of xenobiotic metabolism genes. 5 A. Violin Plot for the assessment of the alpha diversity. using Simpson indices (T-test, $p < 0.05$), 5 B. Beta diversity estimation using PCoA plot with Bray-Curtis dissimilarity measure (Permanova, Adonis test, $p < 0.05$, R2 value 0.18, Beta-disper test-p >0.05).

bacterial family (Fig. 4B.) Bradyrhizobiaceae, Burkholderiaceae, and Comamonadaceae. Rhodopseudomonas, a phototrophic non-sulfur purple bacteria, belonging to *Bradyrhizobaceae*, is a major participant in the degradation of aromatic compounds (Rahalkar et al., 1991; Egland et al., 2001). Burkholderiaceae is a Betaproteobacteria having immense potential for the degradation of a vast array of aromatic compounds (Pérez-Pantoja et al. (2012)). The members of this group such as Burkholderia, Cupriavidus and Ralstonia are versatile degraders, involved in the degradation of pollutants such as aromatic compounds, Nitoaromatic compounds, Trichloroethylene and various others (Xu et al., 2018; Ryan et al., 2007; Lykidis et al., 2010). Members of Comamonadaceae have been reported to have biodegradation potential towards a vast array of aromatic and priority pollutants (Lee et al., 2019). Apart from these taxa, higher abundance of genes was filtered by the members of Mycobacteriaceae, Rhodocyclaceae, Pseudomonadaceae, and Geobacteraceae, which are well-known for their efficiency in pollutant degradation (Singleton 1994; Kleemann and Meckenstock 2011; Galazka et al., 2018), thus indicating their prevalence and involvement in xenobiotic degradation in the riverine system.

3.4. Xenobiotic degrading gene diversity across the riverine stretch

The diversity of xenobiotic degrading genes in the riverine system was assessed at the functional level of the xenobiotic category. The alpha

diversity was analyzed using richness estimators: Chao1 and Observed indices, and diversity estimators: Shannon function and Simpson index (Supplementary Fig. 2, Fig. 5A). The alpha diversity of the non-city stretch was significantly higher as compared to the city samples ($p < 0.05$). The PDE diversity and heterogeneity between the city and non-city stretch was further analyzed by estimating beta-diversity. The PCoA plot (Fig. 5B.) using NMDS matrices suggested significant differences between the city and non-city stretch (riverine stretch) in terms of xenobiotic functional metabolism with lower heterogeneous dispersion among the groups (Permanova, Adonis test $p < 0.05$, R2 value = 0.18; Beta-disper $p > 0.05$). The river type did not have any impact on the diversity of xenobiotic degrading genes (Permanova, Adonis test $p > 0.05$). Despite the significant differences, the lower R2 value (only 17 percent of differences were explained), suggested a very low impact of the riverine stretch. Moreover, the significant difference observed could probably be attributed to the confluence site BMM2 (confluence of Mula-Mutha with Bhima River), which reflected major differences as compared to the other sites. Due to the convergence of different rivers, confluence sites are more prone to changes in their genetic structure (Samson et al., 2019).

4. Conclusion

Shotgun metagenomics-based comprehensive analysis of riverine system revealed potential genes involved in the bioremediation of xenobiotics and also their plausible detoxification mechanisms. Such remediation related genes and enzymatic pathways could aid microbes to thrive in such a polluted and harsh environment. Functional mining indicated utilization of both aerobic and anaerobic catabolism for potential degradation of the pollutants. Benzoate degradation appears to be a central pathway for the catabolism of majority of aromatic compounds. Moreover, the diversity analysis suggested marginal differences in the xenobiotic transformation potential between the city and non-city riverine stretches, indicating the impact of urbanization and the associated pollution in the non-riverine stretch as well. Overall, present investigative study would serve as a fundamental basis for utilizing metagenome-aided functional information for designing improved strategies to remediate such contaminated water bodies and also to explore such microbes for bioremediation applications.

Author contributions

Rakeshkumar Yadav, Data curation, Formal analysis, Investigation, Methodology, Roles/Writing - original draft. Vinay Rajput, Formal analysis, Review and editing. Mahesh Dharne, Conceptualization, Supervision, Resources, Writing - review & editing.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Research data

The datasets supporting the conclusions of this article are available in the MG-RAST. Following are the MG-RAST Ids for the metagenome samples: M1 (mgm4846908.3), RM3 (mgm4846909.3), MM5 (mgm4846910.3), BMM2 (mgm4846911.3), R2 (mgm4846912.3), BMM1 (mgm4846913.3), MM1 (mgm4846914.3), B3 (mgm4846916.3), BMM4 (mgm4846917.3), BMM3 (mgm4846919.3), M2 (mgm4846920.3), PM1 (mgm4846921.3), MM2 (mgm4846922.3), RM2 (mgm4846924.3), PM2 (mgm4846926.3), B4 (mgm4846927.3), MT1 (mgm4846928.3), MM4 (mgm4846930.3).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Authors are grateful to the Director, CSIR-NCL for providing facilities, infrastructure and support. RKY would also acknowledge University Grants Commission (UGC) for fellowship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2020.110332.

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Science of the Total Environment 892 (2023) 164740

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Corrigendum

Corrigendum to "Metagenomic analysis of a mega-city river network reveals microbial compositional heterogeneity among urban and peri-urban river stretch" [Sci. Total Environ. 783 (2021) 146960]

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The authors regret that the printed version of the above article contained an error related to the institute affiliation. The correct and final version follows. The authors would like to apologise for any inconvenience caused.

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DOI of original article: http://dx.doi.org/10.1016/j.scitotenv.2021.146960.
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http://dx.doi.org/10.1016/j.scitotenv.2023.164740

Available online 16 June 2023

Science of the Total Environment 783 (2021) 146960

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Metagenomic analysis of a mega-city river network reveals microbial compositional heterogeneity among urban and peri-urban river stretch

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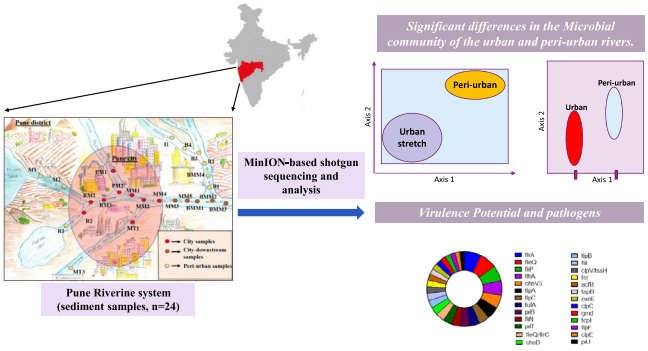
HIGHLIGHTS

- Urban waters influence the microbial ecology of the connected peri-urban rivers.
- Differential microbial enrichment was observed on the basis of riverine stretch.
- Beta diversity suggested significant dissimilarity in urban and peri-urban waters.
- Analysis predicted 295 virulence genes mapping to 38 different pathogenic bacteria.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 26 February 2021
Received in revised form 30 March 2021
Accepted 1 April 2021
Available online 7 April 2021

Editor: Frederic Coulon

Keywords:
Riverine system
Urbanisation
Industrialisation
Virulence factors
Peri-urban

ABSTRACT

The rivers in the megacities face a constant inflow of extremely polluted wastewaters from various sources, and their influence on the connected peri-urban river is still poorly understood.
The riverine system in Pune consists of Rivers Mula, Ramnadi, Pawana, Mutha, and Mula-Mutha, traversing through the urban settlements of Pune before joining River Bhima in the peri-urban region. We used MinION-based metagenomic sequencing to generate a comprehensive understanding of the microbial diversity differences between the urban and peri-urban zones, which has not been explored at the meta scale until date. The taxonomic analysis revealed significant enrichment of pollution indicators microbial taxa (Welsch's *t*-test, $p < 0.05$, Benjamini-Hochberg FDR test) such as *Bacteriodetes*, *Firmicutes*, *Spirochaetes*, *Synergistetes*, *Euryarcheota* in the urban waters as compared to peri-urban waters. Further, the peri-urban waters showed a significantly higher prevalence of ammonium oxidising archaeal groups such as *Nitrososphaeraceae* (Student's *t*-test p-value <0.05 with FDR correction), thereby probably suggesting the influence of agricultural runoffs. Besides, the microbial community diversity assessment also indicated the significant dissimilarity in the microbial community of urban and peri-urban waters. Overall, the analysis predicted 295 virulence genes mapping to 38 different pathogenic bacteria in the riverine system. Moreover, the higher genome coverage (at least 60%) for priority pathogens such as *Pseudomonas*, *Klebsiella*, *Acinetobacter*, *Escherichia*, *Aeromonas* in the sediment metagenome consolidates their dominance in this riverine system. To conclude, our investigation showed that the unrestrained anthropogenic and related activities could potentially contribute to the overall dismal conditions and influence the connected riverine stretches on the outskirts of the city.

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https://doi.org/10.1016/j.scitotenv.2021.146960
0048-9697/

1. Introduction

The improper discharge of sewage, effluents, anthropogenic waste, and storm runoffs are undoubtedly disastrous for river health (Wang et al., 2018; Suthar et al., 2010; Fonseca et al., 2016). The resultant surge in the pollutant leads to the emergence of waterborne pathogens and increases infection rates, thus degrading public health in the fast-growing cities (Abraham, 2010). Multiple studies across the globe have implicated that the urban waters serve as an ecological niche for the emergence and dissemination of antibiotic resistance genes (ARGs) and infectious pathogens (Samson et al., 2019; Almakki et al., 2019; Zhang et al., 2019; Marathe et al., 2017; Xu et al., 2016; Reddy and Dubey, 2019; Zheng et al., 2018; Zhou et al., 2017). India has an extensive network of rivers; however, most of them are in a dismal state (Borthakur and Singh, 2016; Water Quality Database). Unrestricted release of untreated wastewater from industries, hospitals, and agricultural runoffs has exacerbated the quality of these water bodies.

By virtue of their versatile metabolic adaptability, prokaryotic microorganisms can sustain different organic and inorganic pollutants by utilising them as their growth substrate and energy source (Chakraborty and Bhadury, 2015). However, the multi-faceted consequences of this environmental distress can contribute to functional shifts in microbial diversity and health-related factors (Chen et al., 2019; Chakraborty and Bhadury, 2015). The peri-urban rivers are unique symbiotic urban-rural interface, resembling landscapes of hybrid urban and rural features (Zheng et al., 2018; Chen et al., 2019a). These rivers are resourceful as they provide drinking water and are essential for agricultural irrigation and recreational purposes (Zhang et al., 2020). Despite their irrevocable importance, these rivers are greatly impacted by urbanisation. They are subjected to massive waste from different sources such as industries, hospitals, wastewater treatment plants (WWTPs), and other domestic sources. Several studies reported peri-urban rivers to be a significant reservoir of antimicrobial resistance and pathogens (Zheng et al., 2018; Chen et al., 2019a; Zhang et al., 2020; Chen et al., 2019b). Although several reports are present on unravelling the resistome aspects, there remains a significant lacuna in the comprehensive assessment of the microbial diversity of the peri-urban rivers and the influence of urban waters on the connected peri-urban stretches.

Pune city, in Pune district of Western Maharashtra, is the ninth-largest city in India with a population of around 5.9 million and is also among the fastest-growing cities observing rapid industrialisation and urbanisation (Marathe et al., 2017). The riverine system flowing in the Pune district can be differentiated based on their overall path of flow. The Rivers Mula, Ramnadi, Pawana, Mutha, and Mula-Mutha traverses through the heavily populated and industrialised urban settlements of Pune while the river Bhima and its tributary Indrayani mostly flowing through the peri-urban and agricultural dominant region join Mula-Mutha River in the Pune city outskirts. Several reports on these rivers (Water Quality Database; Yewale et al., 2019; Nawani et al., 2016) have shown the deteriorated condition of these rivers. Previous studies (Marathe et al., 2017, Yewale et al., 2019, Razavi et al., 2017) gave an account of ARGs and the pathogens prevalent in the city stretch some of these rivers. Marathe et al., in 2017, elaborately profiled ARGs in the upstream and city part of the Mutha River and discussed the other essentials that could impact public health. On the other hand, a recent study by Yewale et al. (2019) employed a PCR-based strategy to characterise Mutha River's resistome. Nevertheless, the studies exploring microbial diversity and delineating the community differences in the urban and peri-urban waters is still scarce.

The previous studies on some of these rivers were well within the city limits and did not cover the connected peri-urban and rural stretch. A detailed investigation of the overall microbial ecology and virulence trait of the riverine system is still lacking. Here, we hypothesized that the microbial communities of the riverine system differ in the connected urban and peri-urban riverine stretch. The prime focus of studying this riverine system is to i). assess the influence of urban water on the connected peri-urban stretch by deciphering the dissimilarity in the microbial communities between the two stretches and ii). to understand the overall virulence potentials of the riverine system. We used a rapid, real-time long read MinION-based sediment shotgun sequencing to address these existing lacunas. The present study is one of the most comprehensive shotgun studies investigating the differences in the city stretch's microbial communities and the connected peri-urban stretch to the best of our knowledge.

2. Materials and methods

2.1. Description of the study sites

The major rivers flowing through Pune city includes Mula, Mutha, Mula-Mutha (formed after Mula and Mutha meet in the centre of the Pune city), Pawana, and Ramnadi. The River Pawana and Ramnadi are the major tributaries of the Mula river meeting at the left and right banks, respectively. These rivers traverse through urban settlements is immensely affected by wastewaters from industries, domestic sources, and hospitals. The Mula River dammed at Mulshi lake travels around 52 km, and Mutha river, dammed twice at Panshet dam and Khadakwasla dam, flows about 21 km to join Mula river. On the course of its flow, Mula merges with its tributaries, i.e., Pawana River on the left bank and Ramnadi on the right bank before its confluence with Mutha, and after that it flows downstream as Mula-Mutha River. The River Mula-Mutha is one of the major tributaries of the Bhima River. The Bhima River originates from the Western ghats and meets Indrayani river on the right bank at Tulapur. It travels more than 20 km and joins Mula-Mutha River in the outskirts of Pune city near the village Ranjangaon Sandas in the peri-urban region. After this point, the river flows downstream as Bhima River and joins to Krishna River, which empties itself in the Bay of Bengal. Altogether, depending on the geographical locations, population, urban-influenced water, and population census (Census India 2011; Dhawde et al., 2018; Hui and Wescoat Jr, 2019), we divided the riverine water into two groups, namely urban waters (sites in Pune city and downstream) ($n = 10$) and peri-urban waters ($n = 14$) ('n' denotes the number of sites or samples) (Fig. 1).

2.2. Sample collection details

The water-saturated sediments often harbour the maximum biomass responsible for the metabolic cycles (Gibbons et al., 2014). The wet sediment samples ($n = 24$, from top 5 to 10 cm) were collected during December 2018 in gamma-irradiated sterile containers and stored at 4 °C until further processing. The elaborated details of the sample sites and their coordinates are stated in (Supplementary Tables 1 and 2). The urban water includes R2, RM2, RM3, PM1, PM2, MM1, MM2, MM3 MM4, MM5, BMM1, BMM2, BMM3, MT1 sites, whereas the peri-urban water samples include M1, M2, R1, MT3, I1, B1, B2, B3, B4, and BMM4 sites.

2.3. Library preparation and sequencing

The total DNA was extracted from 400 mg of sediment samples using DNeasy PowerSoil Pro Kit (Qiagen) following the manufacturer's instructions. The DNA quality and concentrations were checked by Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific) followed by its qubit analysis on Qubit fluorometer (Thermo Fisher Scientific) using Qubit Broad range (BR) assay kit and Qubit High sensitivity (HS) assay kit (Thermo Fisher Scientific, Q32853 and Q32851 respectively). The DNA was stored at −80 °C until further processing. Only those DNA that had a purity of 1.8 (OD 260/280) were further processed. The library preparation was performed using 1D Ligation sequencing kit SQK-LSK109, and the 24 samples were barcoded using Native barcoding EXP-NBD104 and NBD114 as per the manufacturer's

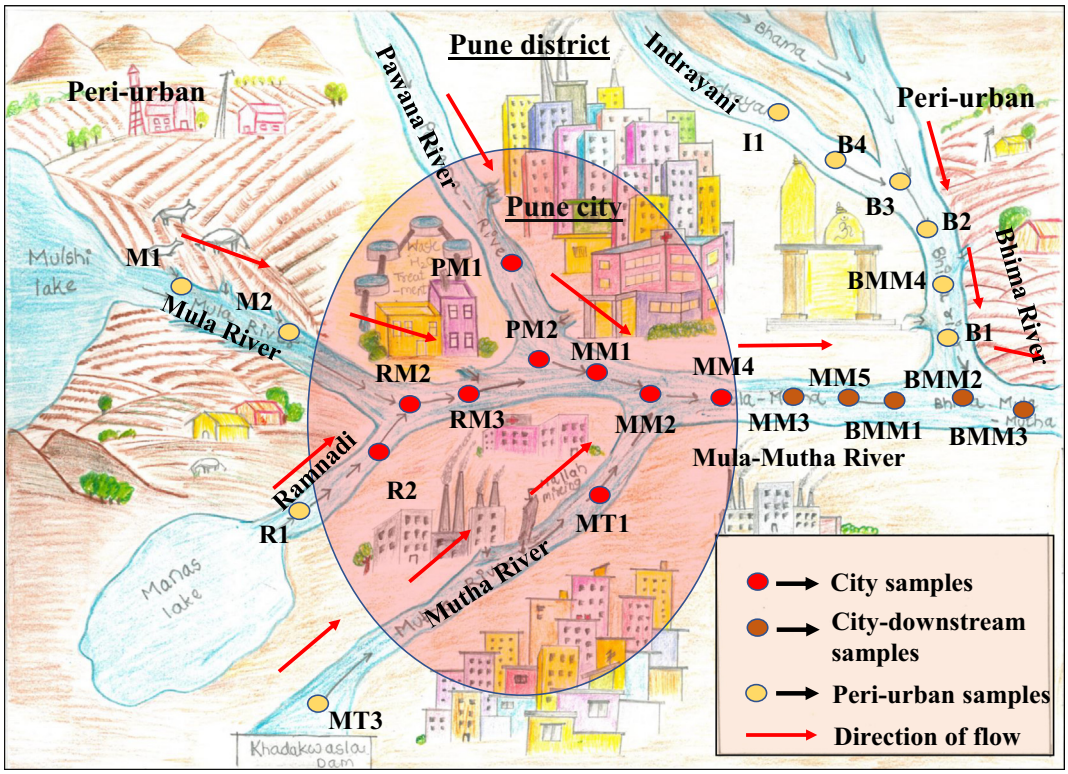

Fig. 1. Schematic illustration of the riverine system. The figure displays the sampling sites. The riverine system is broadly divided into urban (R2, RM2, RM3, PM1, PM2, MM1, MT1, MM2, MM4, MM3, MM5, BMM1, BMM3) and peri-urban stretch (M1, M2, R1, MT3, I1, B4, B3, B2, BMM4, B1) depending on the geographical location and population density. From MM2 site, River Mula flows as Mula-Mutha River and from point BMM2, the River Mula-Mutha flows as the Bhima River.

instructions. The resultant library was quantified by Qubit High sensitivity (HS) assay kit, and around 500 ng of the library was loaded on FLO-MIN 106D R9 Version.

2.4. Shotgun metagenomic data processing and analysis

The raw reads were base-called using Albacore (v2.3.4). The resultant fastq reads were demultiplexed using qcat (v1.0.1), followed by the adapter trimming from the end and the middle of the reads using Porechop (v0.2.4). Data rarefactions were carried out using single_rarefaction.py and rarefy even depth function in QIIME (Caporaso et al., 2010) and R, respectively. The taxonomic classification was performed using Kaiju (version 3) (Menzel et al., 2016) with default parameters. Kaiju carries out a six-frame translation of each metagenomic reads and classifies them using an annotated NCBI BLAST nr protein database (Menzel et al., 2016). The alpha diversity indices Chao1 and Shannon function was calculated using R package Phyloseq on the unfiltered data (Lahti and Shetty, 2017). We used the core protein dataset of the Virulence factors database (VFDB) (Chen et al., 2005) to map virulence genes and the pathogens. Here, we used DIAMOND (Buchfink et al., 2015) for the alignment with parameters –min-score 60, –id 60 and –more-sensitive mode. These parameters were carefully chosen based on a previous study by Pearson (2013). Furthermore, for genome mapping and coverage estimation, we used GraphMap (Sović et al., 2016). We used R packages such as the phyloseq package (McMurdie and Holmes, 2013), microbiome (Lahti and Shetty, 2017), MicrobiomeAnalyst (Dhariwal et al., 2017) for preparing the illustrations.

2.5. Statistical analysis

To overcome the variable sequencing depth, we normalised the data using DESeq2 package. The Non-metric multidimensional scaling (NMDS) was constructed for beta diversity estimation using a counts-based Bray-Curtis dissimilarity measure. The dissimilarity significance was assessed by ANOSIM and PERMANOVA (Adonis test, p-value threshold of 0.05). Betadisper test (p-value threshold of 0.05) was done to assess compositional homogeneity among the groups. The data distribution was checked using the Shapiro-Wilk normality test (p-value threshold of 0.05), followed by the construction of the Q-Q plot. STAMP (Statistical Analysis of Metagenomic Profiles) (Parks et al., 2014) and METAGENassist (Arndt et al., 2012) were used for analysing the differentially abundant significant features between city and non-city samples. Here, we used Welch's *t*-test (p-value threshold of 0.05) and Wilcoxon rank-sum test along with Benjamini-Hochberg FDR for multiple test corrections. Fold-change analysis was carried out in METAGENaaist.

3. Data availability

The datasets supporting the conclusions of this article are publicly available in the MG-RAST with a project name of "PU_River_metagenomics_2019" and NCBI accession number PRJNA666546.

4. Results

The MinION sequencing generated 3.6 million reads yielding around 19.5 Gbases with an average read length of ~5 Kb. The Kaiju analysis assigned 2.58×10^6 reads to bacteria and 7.23×10^4 reads to archaebacteria. The average read count was 1.08×10^5 and 3.02×10^3 for bacteria and archaebacteria, respectively. The rarefaction plot (Supplementary Fig. 1A and B) showed sufficient sequencing depth for all the samples.

4.1. Microbial community composition

4.1.1. Bacterial community composition

Altogether, the taxonomic classification revealed 149 bacterial phyla and 457 families in the riverine system. *Proteobacteria* (mean

relative abundance %) (35.04%), followed by *Actinobacteria* (12.51%), *Bacteroidetes* (8.43%), *Chloroflexi* (7.74%), and *Acidobacteria* (6.29%) were among the most dominant bacterial groups in the riverine system (Fig. 2A). Although not significant (Welsch's *t*-test, Benjamini-Hochberg FDR test), the members of *Firmicutes* (4.9% vs 2.8%) and *Bacteroidetes* (9.6 vs 5.9%) were more enriched in the urban waters as compared to the peri-urban waters. *Planctomycetes*, *Verrucomicrobia*, *Cyanobacteria*, *Nitrospirae*, and *Gemmatimonadetes* were the other considerably prevalent bacterial phyla in the riverine system (≥Total abundance of 1%) (Fig. 2B). Furthermore, the fold-change analysis revealed a significantly higher abundance of *Spirochaetes*, *Synergistetes*, and *Tenericutes* in the urban waters of the riverine system (Supplementary Fig. 2A, B, C). Overall, *Actinobacteria*, *β-Proteobacteria*, *δ-Proteobacteria*, *α-Proteobacteria*, *γ-Proteobacteria*, *Anaerolineae*, *Bacteroidia*, *Clostridia*, *Planctomycetia*, *Bacilli*, *Flavobacteria*, and *Cytophagia* were the most abundant classes (1%) in the riverine system (Supplementary Fig. 3). Among these prominent bacterial classes, *γ-Proteobacteria*, *Bacteroidia*, and *Clostridia* were significantly enriched in the urban sites (Wilcoxon rank-sum test <0.05 with FDR correction).

Furthermore, at the lower classification hierarchy (Fig. 2B), *Streptomycetaceae*, *Comamonadaceae*, and *Planctomycetaceae* were among the most supremely (≥1%) abundant bacterial groups. Notably, among the dominant families (≥0.5%), we observed significant enrichment (Supplementary Fig. 2D) of *Methylococcaceae* in the urban part, especially at the BMM2 site (a confluence site of River Bhima and Mula-Mutha). The fold change analysis also revealed the differential abundance of 37 bacterial families (Supplementary Table 3) in the urban sites compared to four (*Conexibacteraceae*, *Gemmatimonadaceae*, *Myxococcaceae*, and *Solirubrobacteraceae*) in the peri-urban waters.

4.1.2. Archaebacterial community composition

In total, we observed 18 archaebacterial phyla and 45 families in the riverine system. We observed higher enrichment of *Euryarchaeota* in the urban waters while *Thaumarchaeota* in the peri-urban waters (Fig. 2C and D). Notably, *Thaumarchaeota* members were relatively higher in some urban sites (MM4, MM3, MM5, BMM1, and BMM3). The examination at the class level revealed a significant increase in *Methanobacteria* and *Methanomicrobia* (especially at the BMM2 site) while a significant decrease in *Nanohaloarchaea*, *Halobacteria*, *Archaeoglobi*, and *Thermococci* in the urban-influenced waters (Student's *t*-test p-value <0.05 with FDR correction) (Supplementary Fig. 4). The archaeal family *Methanosetaceae* was significantly enriched (fold-change analysis) in the urban-influenced waters, while *Nitrososphaeraceae* was higher in most peri-urban sites. Similar to the bacterial composition pattern, the confluence site BMM2 showed major variations being highly populated with Thaumarchaeotal group *Nitrososphaeraceae* compared to its pre-confluence sites (BMM1 and B1) and post-confluence site (BMM3) (Fig. 2C and D).

5. Microbial community diversity

We used the Chao1 richness estimator and Shannon index to understand the overall microbial richness and diversity of a riverine system. Although not significant, we observed higher bacterial richness and diversity in urban waters (Fig. 3A). On the contrary, peri-urban sites showed significantly higher archaeal richness (Welsch's t-test, $p < 0.05$) (Fig. 3B) than the urban waters. Beta diversity for understanding the overall community differences was estimated using the Bray-Curtis dissimilarity matrix. NMDS plot showed significant dissimilarity in the bacterial (ANOSIM, $R = 0.17$, p-value <0.05, Stress value = 0.1) as well as archaebacterial (ANOSIM, $R = 0.18$, p-value<0.05, Stress value = 0.1) composition of the urban and peri-urban waters (Fig. 3C and D).

6. Virulence factors in the riverine system

Here, we used the VFDB database to characterise the virulence potential of the riverine system. Our analysis observed 295 virulence genes mapping to 38 different pathogenic bacterial genera in the entire riverine system. The virulence genes such as *flrA*, *fleQ*, *fliP*, *flhA*, *cheV3*, *tlpA*, *tlpC*, *tufA*, *fliN*, *pilB*, and *pilT* were among the most prevalent (≥2%) in the riverine system. Altogether, 17% of virulence gene hits were mapped to *Aeromonas* followed by (≥2%) *Pseudomonas*, *Helicobacter*, *Vibrio*, *Legionella*, *Burkholderia*, *Francisella*, *Yersinia*, *Klebsiella*, *Listeria*, and *Neisseria*. The genome coverage for the global priority pathogens in the riverine system was estimated by mapping the

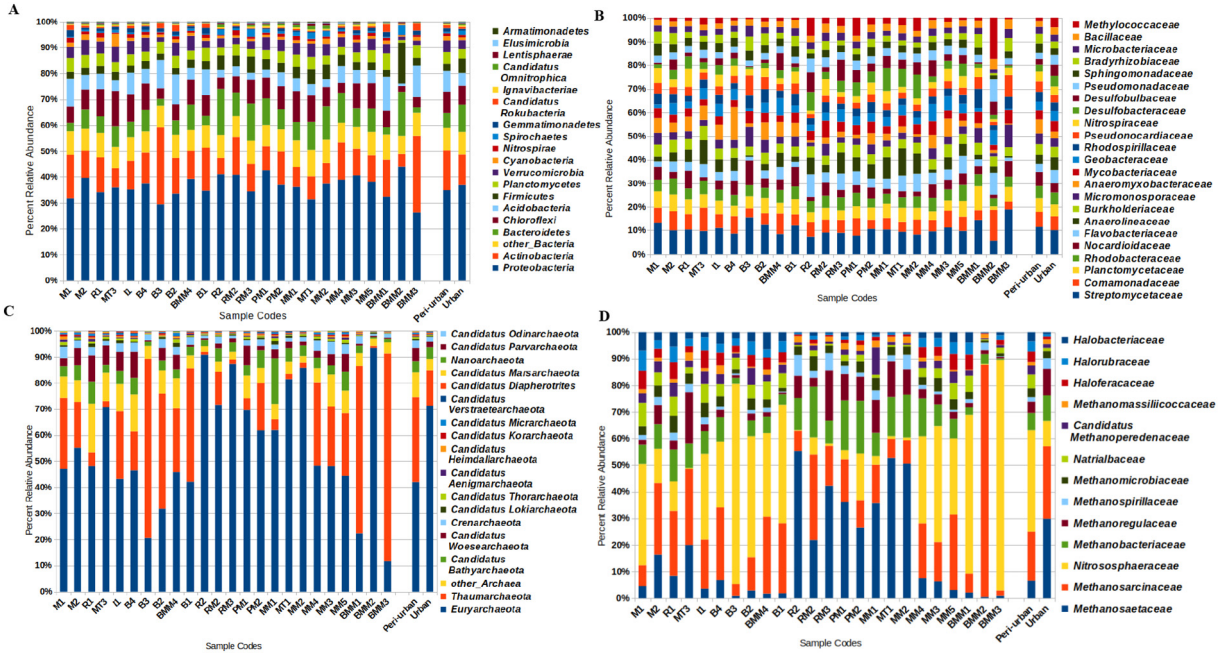

Fig. 2. Microbial community pattern of the riverine system. The stacked bar plot indicate the percent relative abundance of bacterial composition at phylum and family level (A and B, respectively), and archaebacterial composition at phylum and family level (C and D, respectively) in the riverine system.

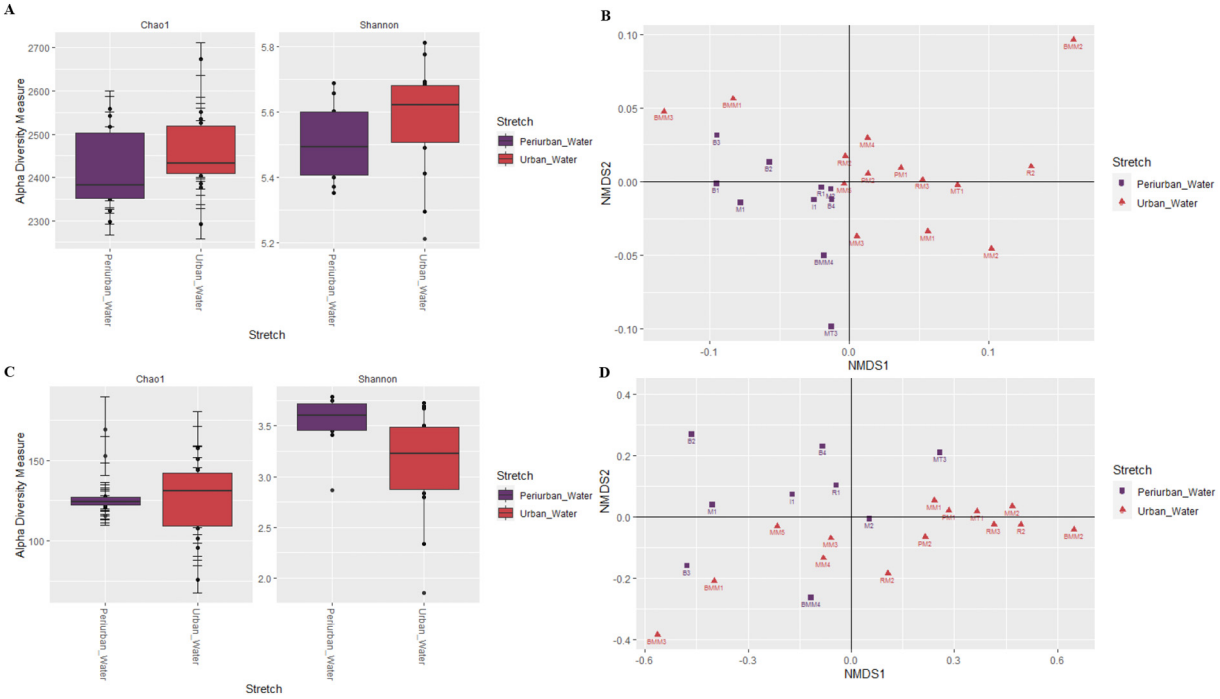

Fig. 3. Microbial diversity of the riverine system. A and B. Bacterial alpha diversity (Welch Two Sample *t*-test, p-value >0.05) and beta diversity (ANOSIM, p-value<0.05, R = 0.17), respectively with NMDS stress value = 0.1. C and D. Archaebacterial alpha diversity (Welch Two Sample t-test, p-value < 0.05) and beta diversity (ANOSIM, p-value<0.05, R = 0.18), respectively.

metagenomic data with reference genomes of priority pathogens. We obtained more than 60% coverage (Table 1) with most of the reference genome of pathogenic strains except for the *H. pylori* (49%) with higher coverage depth.

7. Discussion

Over the last decade, the rivers in Pune have been subjected to immense anthropogenic activities with unfavourable environmental impacts (Water Quality Database; Yewale et al., 2019). Wastewaters from different sources frequently contaminate the urban surface waters resulting in a higher risk of antimicrobial resistance and pathogens. This comprehensive shotgun study was conducted to comprehend the overall microbial diversity and virulence traits of the riverine system in Pune. We also deciphered the influence of urban-traversing river water on the connected peri-urban riverine stretch's microbial diversity.

The taxonomic analysis revealed that the river harbours a diverse microbial population (Fig. 2A, B, C, and D). Bacterial diversity discerned were mostly consistent with the previous other studies (Marathe et al., 2017; Zhang et al., 2019; Samson et al., 2019; Jani et al., 2018; Lin et al., 2019; Ibekwe et al., 2016), which reported the microbial composition for the rivers that traverse through the urban settlements. A significant increase of bacterial phyla such as *Bacteriodetes*, *Firmicutes*, *Spirochaetes*, *Synergistetes*, *Tenericutes* and classes viz., *γ-Proteobacteria*, *Bacteroidia*, and *Clostridia* in the urban-influenced stretch implicates the influence of faecal pollution in the riverine system (Paruch et al., 2019; Lin et al., 2019). The urban riverine system is infused continuously with recalcitrant pollutants from treated and untreated wastewaters of various industries. We observed a higher abundance of bacterial groups such as *Sphingomonadaceae*, *Xanthomonadaceae*, *Pseudomonadaceae*, *Mycobacteriaceae*, and *Burkholderiaceae* utilising such pollutants as their nutrient source (Ghosal et al., 2016). Further, significant enrichment of microbial populations in the urban waters compared to peri-

Table 1
Estimation of the genomic coverage. GraphMap was used for estimation of genome coverage of the pathogens (whose virulence genes were predicted) from the metagenomic sequences.

Pathogens	Number of bases covered	Reference genome length	Coverage for reference genome	Mean coverage depth (approximate × coverage)
Pseudomonas aeruginosa PAO1	~6.26×10^6	6.26×10^6	99.9%	700×
Aeromonas hydrophila subsp. *hydrophila* ATCC 7966	4.69×10^6	4.74×10^6	98.9%	356×
Legionella pneumophila subsp. *pneumophila* str. Philadelphia 1	2.14×10^6	3.4×10^6	62.9%	28×
Burkholderia pseudomallei K96243	7.12×10^6	7.25×10^6	98.2%	575×
Yersinia enterocolitica subsp. *Enterocolitica* 8081	2.79×10^6	4.61×10^6	60.4%	92×
Neisseria meningitidis MC58	2.09×10^6	2.27×10^6	91.9%	89×
Klebsiella pneumoniae subsp. *pneumoniae* NTUH-K2044	5.12×10^6	5.33×10^6	95.9%	385×
Haemophilus influenzae Rd KW20	1.58×10^6	1.83×10^6	86.1%	22×
Listeria monocytogenes EGD-e	2.11×10^6	2.94×10^6	71.5%	13×
Bordetella pertussis Tohama I	4.08×10^6	4.09×10^6	99.9%	914×
Mycobacterium tuberculosis H37Rv	~4.41×10^6	~4.41×10^6	99.9%	534×
Helicobacter pylori 26695	8.21×10^5	1.67×10^6	49.2%	46×
Acinetobacter baumannii ACICU	3.40×10^6	4.33×10^6	78.5%	25×
Shigella dysenteriae Sd197	3.33×10^6	4.37×10^6	76.2%	55×
Staphylococcus aureus subsp. *aureus* MW2	2.73×10^6	2.82×10^6	96.9%	40×

urban stretch indicates anthropogenic activities on the river microbial diversity. The urban water showed significantly higher enrichment of methanogenic *Euryarchaeota* such as *Methanobacteria* and *Methanomicrobia* compared to peri-urban waters. Such anoxic methanogenic archaebacterial *groups* in polluted river water are consistent with previous studies (Zhang et al., 2019; Wang et al., 2018). While the urban-influenced waters were rich in *Euryarchaeotal* group, the peri-urban stretch showed a significant predominance of *Thaumarchaeota* members. The agricultural runoffs in the peri-urban stretch are quite luxurious with ammonium due to the use of biosolids and fertilisers, thus supporting ammonium oxidising archaea (AOA) group such as *Nitrososphaeraceae* that carry out ammonium oxidation in nitrogen cycling (Pratscher et al., 2011). Nevertheless, we also observed a considerable abundance of AOA in the urban water sites (MM4, MM3, MM5, BMM1, and BMM3), suggesting that they play a critical role in ammonia oxidation in the polluted urban rivers (Tu et al., 2019). The BMM2 site showed significant variations in the overall microbial diversity. The site is a confluence site of two rivers, i.e. Mula-Mutha and Bhima Rivers, which are differently impacted by anthropogenic activities. The Mula-Mutha River travels mostly through urban settlements and relatively is subjected to more anthropogenic pressure. We observed an upsurge in the abundance of *Bacteriodete*s and *Firmicutes* phyla and the corresponding increase of *Flavobacteriaceae*, *Desulfobulbaceae*, and *Pseudomonadace*ae bacterial families at BMM2 site. Moreover, the site also showed a significant increase in methanogenic microbial populations. The increase in the methanogenic microbes can also be attributed to the excessive water hyacinth growth seen at BMM2 during the sampling (Avila et al., 2019). Samson et al. (2019) also observed such dynamic alterations in the microbial community at the confluence of two differently anthropogenically impacted rivers. Overall, these observations at the BMM2 confluence site probably indicated the influence of urban surface waters on the microbial diversity of the interconnected peri-urban rivers.

The wastewaters from cities containing various chemicals, oils, hospital wastes, detergents, and human secretions enter the surface waters through well-planned drainage systems, while the peri-urban stretch mainly receives agricultural runoffs. The higher bacterial diversity and a significantly lower archaebacterial diversity ($p < 0.05$) in the urban riverine sites relative to the peri-urban sites probably indicate the influence of different wastewater received by both the riverine stretch. Similarly, the beta diversity analysis showed significant microbial dissimilarity between the riverine stretches; however, we observed lower ANOSIM R-values (0.17 and 0.18 for bacteria and archaebacteria), indicating significant differences with overlapping similarities. These similarities can be reasoned: Firstly, the rivers examined in this study are located in similar climatic conditions (with rivers as the variable factor we obtained PERMANOVA, $p > 0.05$), and secondly, the riverine sites MM3, MM5, BMM1, BMM2, and BMM3 grouped into the urban waters geographically are at the outskirts of the Pune city. However, unlike the other peri-urban sites in this study, MM3, MM5, BMM1, BMM2, and BMM3, being the downstream city sites, receive all the urban waters and thus are "urban-influenced". Upon excluding these samples from the urban water groups, the analysis revealed a significant dissimilarity in the microbial community between the riverine system's urban and peri-urban sites with a higher ANOSIM R-value of 0.55 (Supplementary Fig. 6). Based on the diversity analysis, it can be concluded that the urbanisation and increased anthropogenic activities in the cities have not only a crucial impact on the microbial diversity of the urban surface water but also the downstream stretch of an interconnected riverine system.

The World Health Organization (WHO) published a global priority pathogens list and categorized them to include Critical, High, and Medium groups based on ten parameters such as mortality, antibiotic resistance, and their treatability (Asokan et al., 2019). Widespread occurrences of the virulence genes encoding toxins, inflammatory mediators, adhesins, resistance have been reported in the environment with the PCR and culture-based approach (Søborg et al., 2013; Hamelin et al., 2007). Here, the analysis predicted wide ranges of virulence genes mapping to different bacterial pathogenic genera, including all the global priority and ESKAPE pathogens (Asokan et al., 2019) in the riverine system. Most of the dominant virulence genes detected in the rivers facilitate adhesion and assist in the motility functions (Supplementary Fig. 5). The *fleQ* gene is an essential regulator of flagellar biogenesis in *Pseudomonas aeruginosa* (Dasgupta et al., 2003). The critical group pathogen *P. aeruginosa* is an opportunistic pathogen and is involved in a range of life-threatening infections such as cystic fibrosis (Savoia, 2014). The *flr* and *laf* gene products are the virulence factors of *Aeromonas*, providing them with the motility functions (Chen et al., 2005; Tomás, 2012; Rabaan et al., 2001). The Environmental Protection Agency (EPA) has put this ubiquitous waterborne bacterium *Aeromonas* on the Contaminant Candidate List due to its ability to cause human infections (Seshadri et al., 2006). The *pil* gene is pili forming genes that facilitate attachment to the host cells, helping in twitching motility and biofilm formation (Comolli et al., 1999). The *clpC* and *clpE* encode stress proteins, basically an ATPase, of *Listeria* pathogens that provide them multiple virulence potentials such as high-temperature tolerability and escaping phagocytosis activity (Chen et al., 2005). Further, the higher coverage for the genome of priority pathogens in our metagenome validates their higher prevalence in this riverine system.

8. Conclusion

This study comprehensively characterised the differences in the microbial community between the urban and peri-urban waters and also identified the pathogenic potentials of the riverine system using high-throughput shotgun sequencing. Significant enrichment of faecal indicator bacterial groups in the urban waters indicated the release of untreated sewage of faecal origin. Further, a higher prevalence of pollutant degrading bacteria in urban waters probably indicated their adaptive potentials towards the recalcitrant pollutants. The peri-urban waters were seemed to be most affected by agricultural runoffs as they were highly enriched with ammonium oxidising microbes. The analysis of the peri-urban, urban, and the directly influenced city outskirts sites indicated significant alterations in overall microbial diversity. Besides, it also shows the influence of urban-influenced waters on the connected downstream rivers, thereby affecting the overall lotic ecosystems. Higher coverage of the priority pathogens and their virulence genes further implicates the ill-effects of increasing anthropogenic stress on the riverine system. Overall, this detailed study revealed a clear distinction of the microbial communities on the based pollution sources and the level of urbanisation. It also signifies the need to properly treat the wastewaters to reduce the environmental stress on the urban rivers and the connected peri-urban rivers.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Rakeshkumar Yadav: Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Vinay Rajput:** Formal analysis, Methodology. **Mahesh Dharne:** Conceptualization, Supervision, Writing – review & editing, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors are grateful to the Director, CSIR-NCL, for providing facilities, infrastructure and support. RKY acknowledges the University Grants Commission (UGC), New Delhi for fellowship. We acknowledge technical assistance of Mr. Digeshwar Rane during sample collection.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.146960.

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Ecotoxicology and Environmental Safety 202 (2020) 110938

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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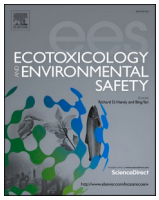

Comprehensive metagenomic insights into a unique mass gathering and bathing event reveals transient influence on a riverine ecosystem

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ARTICLE INFO

Keywords:
Mass bathing
Virulence genes
Antibiotic resistance
Priority pathogens
MinION sequencing

ABSTRACT

The religious mass gathering and bathing can pose a multitude of significant public health challenges and lead to severe alterations in the river microbial ecology. The Pandharpur Wari is an annual pilgrimage of Maharashtra, India, where millions of devotees carry the footprints of the saint-poets and pay their obeisance to Lord Vitthal on the 11th day of moon's waxing phase (Ashadi Ekadashi). As a part of the ritual, the engrossed devotees, walk over 250 km, take a first holy dip in a sacred river Indrayani at Alandi and secondly in Bhima River at Pandharpur. The MinION-based shotgun metagenomic approach was employed to examine the impact of spiritual mass bathing on environmental changes (concerning the river microbial community structure and functions); and public health aspects (in terms of changes in the pathogenic potential and antibiotic resistance). The analysis of bathing and post-bathing samples of both the rivers revealed alterations in the alpha and beta diversity, indicating significant spatiotemporal variations in the overall microbial structure and function. Furthermore, the analysis revealed up to 80% of differences in the abundance of virulence genes between the bathing and post bathing samples. We observed parallel increase of priority skin and enteric pathogens (ranging from 11% to 80%) such as Acinetobacter baumannii, *Staphylococcus aureus*, Streptococcus pyogenes, *Mycobacterium tuberculosis*, and *Pseudomonas aeruginosa* during the bathing event. Moreover, we observed a significant increase in the antibiotic resistance in the bathing samples of Bhima and Indrayani rivers respectively. Altogether, this is the first comprehensive metagenomic study unravelling the influence of religious mass-bathing on the riverine ecosystem.

1. Introduction

Increased magnitude of anthropogenic activities has resulted in significant deterioration of river water quality (Labbate et al., 2016; Wang et al., 2018). Consequently, most of the rivers across the world are facing global threats such as elevated antimicrobial resistance and waterborne diseases (Yewale et al., 2019; Marathe et al., 2017; Wang et al., 2020; Hamner et al., 2019). The ritual mass bathing in rivers where millions of people plunge into rivers increases the anthropogenic stress on the river ecosystem to several-fold (Tyagi et al., 2013). Kumbh-Mela in India is the world's largest religious mass bathing event, where people take a dip in holy water with a belief to wash away their sins (Jani et al., 2018a, 2018bbib_Jani_et_al_2018abib_Jani_et_al_2018b). Similar to Kumbh Mela, the Pandharpur Wari or simply known as "Wari", is also one of the most important pilgrimages in India that are undertaken by millions of Warkaris (pilgrims) for more than 800 years (Mathesul et al., 2016).

Pandharpur, regarded as "Heaven on Earth" by the devotees, is a small town in the Solapur district of Maharashtra, India (Baad, 2016). As per the Hindu tradition, the 11th day in the moon's waxing phase (Ashadi Ekadashi) is considered to be auspicious to undertake Wari. Overall, there are mainly four Waris that is conducted in Hindu months of Ashadh (June), Kartik (November), Magh (February), and Chaitra (April). Out of these, the Ashadi Wari attracts the most number of devotees. Briefly, the devotees form a small group ('dindis') that carries the paduka (footwear) of a saint-poets in a palkhi (palanquin) (Baad, 2016; Koiso, 2017). They walk a route (over 250 Km) in western Maharashtra, from Dehu and Alandi to Pandharpur ultimately offering their respects to Lord Vitthal in Pandharpur on the holy occasion of

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https://doi.org/10.1016/j.ecoenv.2020.110938
Received 24 May 2020; Received in revised form 21 June 2020; Accepted 23 June 2020
Available online 11 July 2020

Ashadi Ekadashi (Koiso et al., 2016). River Indrayani and River Bhima (also known as Chandrabhaga because it resembles a half-moon shape in Pandharpur) are revered holy rivers. Bhima River flows through south-east of India for 860 km before it enters in Krishna River, while Indrayani River originates in Sahyadri mountains flows towards east and meet Bhima. During the Wari event, millions of devotees engrossed in the hymn, offer their obeisance to Lord Vitthal and take a holy bath in Indrayani River at Alandi and Bhima River at Pandharpur.

It is very important to understand the source of organic pollution of river water and develop knowledge about the implications of mass bathing (Purohit et al., 2020). Due to communal eating and bathing, these mass gathering events possess a potential risk of waterborne illness (Vortmann et al., 2015). Also, many recent studies explained the severe consequences of mass gathering and bathing on public health and the river ecosystem (Tyagi et al., 2013; Jani et al., 2018a; Blyth et al., 2010; Shafi et al., 2008; Memish et al., 2012). Recently, a targeted amplicon sequencing study by Jani et al., in 2018a, gave an account of the alterations in microbial diversity in Godavari River during Kumbh Mela. Furthermore, the influence of mass gatherings on public health is undoubtedly inevitable and requires utmost monitoring and precautions (Shafi et al., 2008). Collectively, these events not only possess the risk of infections and diseases but also lead to major alterations in the microbial ecology of the rivers, therefore affecting the overall indigenous functioning and quality of the aquatic ecosystem. Comprehensive shotgun metagenomics has the potential to provide a detailed understanding of the broader changes that occur during such mass gathering events. As per our best knowledge, to date, in-depth studies addressing the impact of mass-bathing events on the river ecosystem is still scarce. The paucity of information regarding the overall ecological changes during such large anthropogenic stress calls for a comprehensive investigation.

Here, we aimed to investigate the changes in the overall microbial ecology, their functionality and potential health impacts associated with religious mass bathing events. Moreover, particular emphasis was given to assess the changes in the antibiotic resistance and pathogenicity during mass bathing in Wari using the latest shotgun-based MinION nanopore sequencing technology. As per our knowledge and literature survey, this is the first comprehensive shotgun-based metagenomic study that unravels the impact of mass bathing on the river ecosystem and public health.

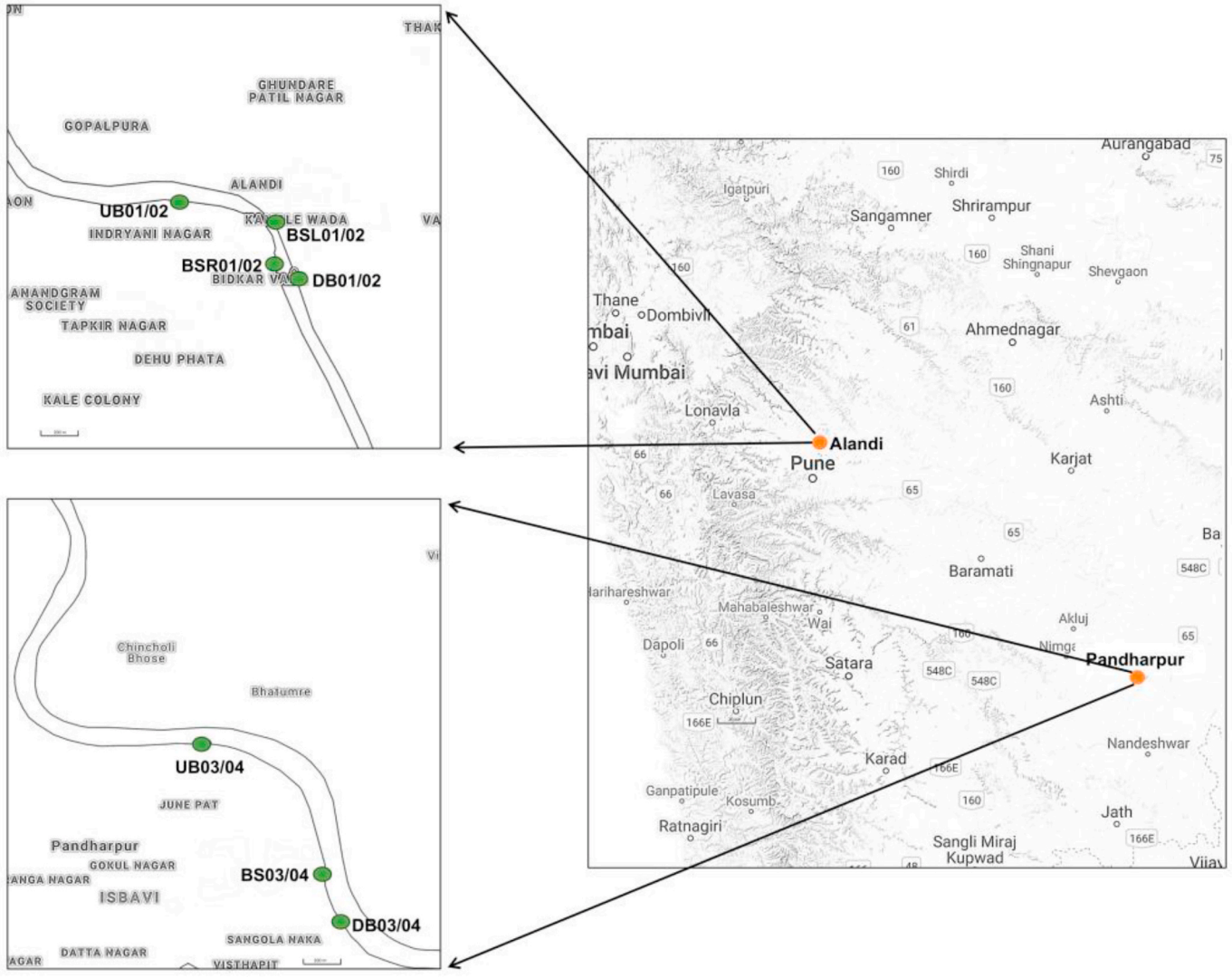

Fig. 1. Map illustrating the sampling locations. The water samples were collected from the Indrayani River at Alandi (Bathing samples: UB01, BSL01, BSR01, and DB01; post bathing samples: UB02, BSL02, BSR02, and DB02) and from Bhima River at Pandharpur (Bathing samples: UB03, BS03, and DB03; Post bathing samples: UB04, BS04, and DB04).

2. Materials and methods

2.1. Sample collection

We carried out sampling during the bathing events at two locations, i.e. at Alandi of Indrayani River and Pandharpur of Bhima River and also collected the post-bathing samples from the same sites to evaluate the influence of mass bathing. Water samples (bathing and post-bathing during Wari, 2019) were collected in gamma-irradiated sterile containers from two locations i). Alandi, on the bank of River Indrayani (n = 8, n is the number of samples) and from ii). Pandharpur, on the bank River Bhima (the sampling site is also known as Chandrabhaga) (n = 6). The sample codes and their description are as follows: River Indrayani at Alandi (Bathing sample codes: UB01, BSL01, BSR01, and DB01; Post-bathing sample codes: UB02, BSL02, BSR02, and DB02). River Bhima at Pandharpur (Bathing sample codes: UB03, BS03, DB03; Post-bathing sample codes: UB04, BS04, DB04) (Fig. 1).

2.2. DNA extraction from water samples

The water was subjected to following successive filtration process in absolute sterile conditions using a sterile water-filter assembly. Firstly, coarse filtration of 500 ml of water was carried out using sterile 11 μm pore-sized Whatman Grade 1 Qualitative Filter paper. The filtrate collected was further subjected to filtration using a sterile 0.4 μm PES membrane filter (Millipore Express). The resultant filtrate was re-filtered using 0.22 μm PES membrane filter (Millipore Express). The filter paper was stored in −80 °C in a sterile Petri-plate until DNA extraction. The DNA extraction was carried out using DNeasy PowerWater Kit, Qiagen as per the protocol mentioned by the manufacturer. The DNA quality and concentrations were checked by Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific) followed by its qubit analysis on Qubit fluorometer (Thermo Fisher Scientific) using Qubit High sensitivity (HS) assay kit (Thermo Fisher Scientific Q32851 respectively). The DNA was further stored in −80 °C until further processing.

2.3. Library preparation and metagenomic sequencing

DNA with the purity of 1.7–1.9 (260/280) was processed for library preparation. We started library preparation with a DNA concentration of 1.1 μg/48 μl using 1D Ligation sequencing kit SQK-LSK109. Briefly, the following protocol was followed:

a. End-repair was performed using 3.5 μl of each NEBNext FFPE repair buffer, Ultra II End-prep buffer, 2 μl of FFPE repair mix and 3 μl of Ultra II End-prep enzyme mix. The reaction was thermally cycled at 20 °C for 10 min and 65 °C for 10 min.
b. 2.5 μl of NBD104 and 114, 25 μl of Blunt/TA Ligase Master Mix was mixed with 600 ng (Qubit estimated) of repaired DNA for barcoding (incubated at 28 °C for 10 min).
c. Lastly, all the barcoded samples were pooled (each 100 ng) and were mixed with 8 μl of Adapter mix II (AMII) and 60 μl Blunt/TA ligase (incubated at 28 °C for 10 min). The library was eluted by elution buffer (EB) at 37 °C for 10 min.

Each step mentioned above were followed by DNA purification using 60 μl of AMPure beads and 70% of ethanol on Magnetic Separation Rack (New England BioLabs, #S1509S).

2.4. MinION data processing and analysis

The base-calling for raw data was carried out using Albacore (v2.3.4). The generated fastq reads were demultiplexed using qcat (v1.0.1). The adapter trimming from the end and the middle of the reads were carried out using Porechop (v0.2.4). Kaiju (Menzel, 2016) was used for the taxonomic classification. NanoARG (Arango-Argoty et al., 2019) was used for the prediction of Antibiotic resistance genes (ARGs) and Mobile genetic elements (MGE). NanoARG uses DIAMOND for the alignment of reads and bedtools to cluster the local best hits into regions (Arango-Argoty et al., 2020). We used Virulence factors database (VFDB) (Chen et al., 2005) for mapping of virulence genes and pathogens. Briefly, we used DIAMOND (Buchfink et al., 2015) for the alignment with a parameter of (evalue of 0.00001, –more-sensitive, –identity of 60%). MG-RAST (Glass et al., 2010) with E-value cut-off = $1 \times e{-}5$ and sequence identity of 60% using the KEGG database was used for functional annotation. Furthermore, R packages such as phyloseq (McMurdie and Homes, 2013), microbiome (Lahti and Shetty, 2017), and MicrobiomeAnalyst (Dhariwal et al., 2017) was used for generating alpha diversity, beta-diversity plots and other statistical analysis. Percent difference was calculated by dividing the differences of the two values with the mean of two values followed by multiplication with hundred.

2.5. Isolation of bacteriophages

Five MDR strains each of *Klebsiella pneumoniae*, Acinetobacter baumannii, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and some common waterborne pathogenic bacteria viz. Escherichia spp. Enterobacter spp. Shigella spp., Salmonella spp., and Vibrio spp. were used. Fresh culture of each of the 20 MDR strains was prepared by inoculating the culture of bacteria in sterile 30–40 ml Tryptone soy broth, which were incubated for 18–24 h at 37 °C. Briefly, the water samples were filtered through a laboratory-grade sterile filter for coarse filtration. Further filtration was carried out by using a 0.22 μm pore-sized nitrocellulose water filter. The filtered environmental sample (1 ml) was added with 1 ml of the overnight grown host culture and was incubated for 18–24 h at 37 °C for the enrichment of phage (Crothers-Stomps et al., 2010). The enriched solution was centrifuged at 3783×*g* at 4 °C for 10 min. 1 ml of supernatant lysate was pre-incubated with 1 ml fresh host culture for 15 min at 37 °C for the interaction of host and phage followed by their plating on a Tryptone Soya Agar with 0.05% 2,3,5-Triphenyl Tetrazolium Chloride (TTC). The plates were incubated for 24 h for observing (Khairnar et al., 2014).

2.6. Statistical analysis

Data rarefaction was carried out in QIIME (Caporaso et al., 2010). The distribution of data was checked using the Shapiro-Wilk normality test (p-value threshold of 0.05) followed by the confirmation using a Q-Q plot. Beta-diversity estimation was carried out using the Bray-Curtis dissimilarity measure, and statistical significance was checked using Adonis and Permanova test (p-value threshold of 0.05). STAMP (Statistical Analysis of Metagenomic Profiles) (Parks et al., 2014) was used for analyzing the differentially abundant significant features between bathing and post-bathing samples. Here, we used Welch's *t*-test (p-value threshold of 0.05) for normally distributed data and White's non-parameter test (WP test) (p-value threshold of 0.05) for the data which was not normally distributed. Furthermore, the statistical significance of the alpha diversity data between bathing and post-bathing samples was checked using the Kruskal-Wallis test (p-value threshold of 0.05) and *t*-test (p-value threshold of 0.05).

3. Results

The MinION sequencing generated around 18 Gb of base-called data with average quality score and length of 8.75 and 4 Kb, respectively. The Kaiju analysis classified around 70% of total reads, among which ~93% was assigned to bacteria, and 0.8% was assigned to archaebacteria. The overall read statistics are listed in Supplementary Table 1.

3.1. Influence of mass bathing on the microbial community

The members of Actinobacteria (Welsch's two-sided *t*-test, p-value <0.05) (mean%±Standard deviation%) (39.93±5.29) were highly abundant followed by Proteobacteria (21.60±1.85), Planctomycetes (10.66±1.87), Bacteroidetes (5.2±0.62) in the bathing samples of Bhima River (Fig. 2A). In comparison to the Bhima River, the bathing samples of Indrayani possessed significantly higher prevalence of Proteobacteria (72.21±16.07) (WP test, p-value <0.05), however lower (48.11%) at the upstream site (UB01), followed by Bacteriodetes (6.43±5.86), Firmicutes (4.45±0.50) (WP test p-value<0.05), and Actinobacteria (4.81±4.39) (Fig. 3A). We observed a significant increase (WP test, p-value<0.05) in the population of Actinobacteria (17.76±1.33), Verrucomicrobia (9.99±1.55) and Planctomycetes (2.21±0.19) in the post-bathing samples. At the family level, we observed higher enrichment of Rhodocyclaceae (33.61±6.96) at the BSL01, BSR01 and DB01 while a decrease in its prevalence (0.47±0.06) in post-bathing samples of the Indrayani River (Fig. 3B). The bacterial family such as Commamonadaceae was relatively higher in the post-bathing samples of both the rivers. The Mycobacteriaceae (21.14±3.85) was the most dominant group in the bathing sample,

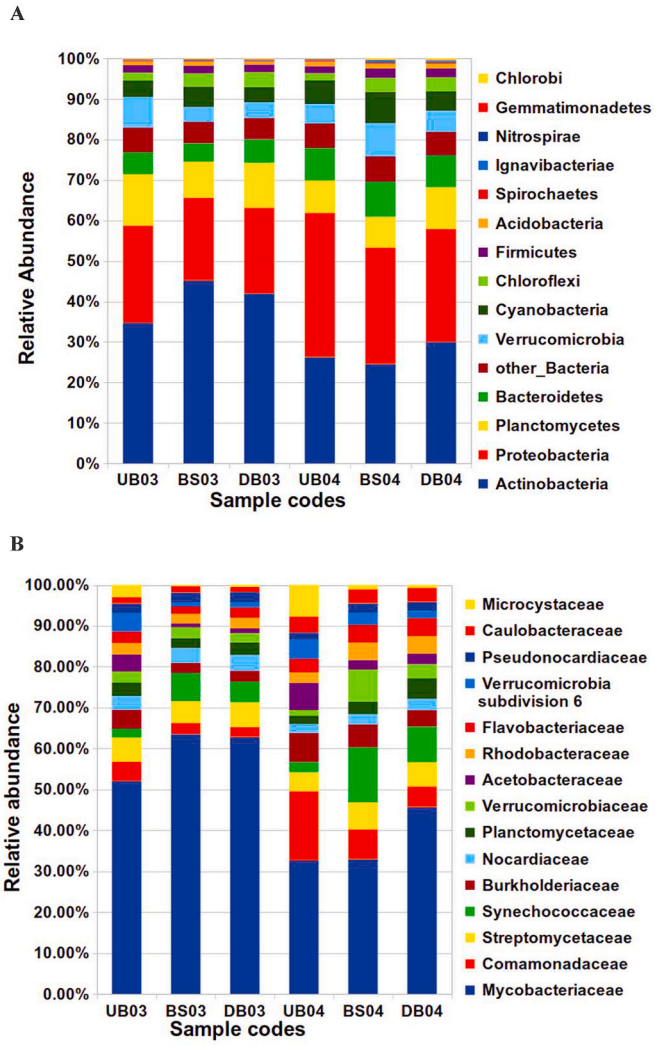

Fig. 2. Bacterial community pattern of Bhima River during Wari. A. Bacterial Phyla in Bhima River. B. Bacterial family in Bhima River. (UB01, DB01, BSL01, BSR01, UB03, BS03, DB03 are Bathing samples of respective rivers whereas UB02, DB02, BSL02, BSR02, UB04, BS04, and DB04 are Post-bathing samples of respective rivers).

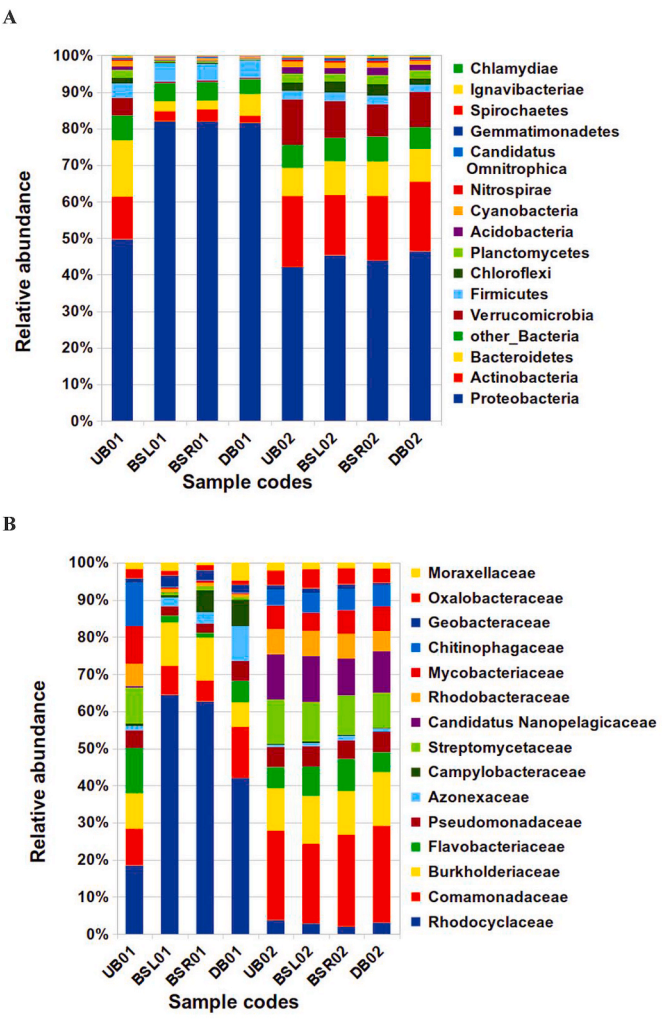

Fig. 3. Bacterial community pattern of Indrayani River during Wari. A. Bacterial phyla in Indrayani River. B. Bacterial family in Indrayani River. (UB01, DB01, BSL01, BSR01, UB03, BS03, DB03 are Bathing samples of respective rivers whereas UB02, DB02, BSL02, BSR02, UB04, BS04, and DB04 are Post-bathing samples of respective rivers).

whereas its prevalence slightly decreased (10.54±1.24) in the post-bathing samples of Bhima River (Fig. 2B). The members of Euryarchaeota were the most abundant (55.98±3.93, 65.60±4.77 and 65.93±2.14, 64.59±4.90) archaeal phylum during bathing as well as the post-bathing event in Bhima and Indrayani River, respectively (Supplementary Fig. 1A and 1B). The Candidatus Bathyarcheaota and Thaumarcheota were other considerable archaeal phyla prevalent during bathing and post-bathing in both the rivers.

3.2. Impact of mass bathing on microbial community structure and function

Overall, we observed 18.45% and ~6.78% (Shannon indices) differences in the bacterial diversity between the bathing and post-bathing samples of Indrayani and Bhima Rivers, respectively. In the case of bacteria, the assessment showed significantly lower (*t*-test, p-value<0.05) (Fig. 4A) while the archaebacteria displayed higher diversity during the time of bathing in the Bhima River (Supplementary Fig. 2A and 2B). Similarly, the diversity indices revealed lower bacterial diversity (Fig. 4C) and higher archaebacterial diversity during the bathing event in Indrayani River (Supplementary Fig. 2B). Notably, in comparison with the other samples during the eve of bathing, the upstream of the bathing site (UB01, UB03) had relatively higher bacterial diversity in

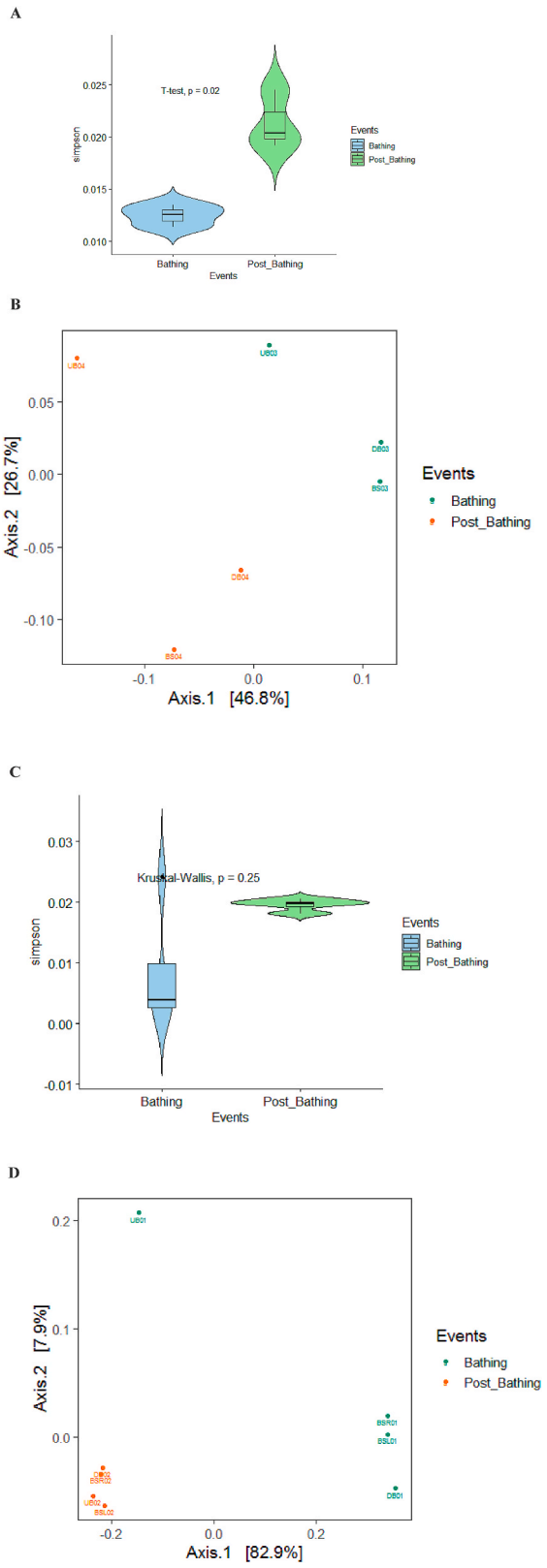

Fig. 4. Spatiotemporal variations of Bacterial Community. A and C..Violin plot depicting alpha diversity between bathing and post-bathing samples of Bhima River (*t*-test p-value <0.05) and Indrayani River (Kruskal-Wallis test, p-value>0.05) respectively. B and D. Beta diversity estimation (PCoA plot using Bray-Curtis dissimilarity matrix) of River Bhima (Adonis test and Permanova p > 0.05) and Indrayani (Adonis test and Permanova p < 0.05) respectively during Wari.

both the rivers. Furthermore, we observed significant differences in the microbial communities (Fig. 4B and D; Supplementary Fig. 2B and 2D) (significant for bacteria and archaebacteria from Indrayani River, Permanova (Adonis test) p-value<0.05, betadisper p-value>0.05). Further, we performed in-silico functional gene analysis using MG-RAST tool. The ordination analysis (Supplementary Fig. 3A and 3B) indicated functional dissimilarity for the bathing and post-bathing samples of Indrayani (Permanova (Adonis test) p-value<0.05, R^2 = 0.45 and betadisper p-value>0.05) and Bhima (Permanova, p > 0.05).

3.3. Higher prevalence of virulence genes

Here, we used the Virulence Factor Database (VFDB) to get insights into the alterations in the pathogenic potentials during such mass bathing events. We observed a higher difference in the prevalence of virulence genes between the bathing and post-bathing samples (82.60% in Indrayani and 54.78% in Bhima River). Also, the permanova analysis suggested the massive impact of mass bathing as significant changes ($p < 0.05$, $R^2 = 0.19$) were observed in the virulence genes (VG) of Indrayani River. Further, our analysis showed enrichment of VG mapping (ranging from 11% to 80% higher prevalence in bathing samples) (Fig. 5) to pathogens such as Acinetobacter baumannii, *Staphylococcus aureus*, Streptococcus pyogenes, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* and the members of the Enterobacteriaceae group during the bathing event.

3.4. Increase of antimicrobial resistance during mass bathing

In total, we observed resistance against 29 categories of antibiotics (Fig. 6A) (142 and 333 antibiotic-resistant genes (ARGs) sub-types in Bhima and Indrayani Rivers respectively), with the predominance of ARGs that provides resistance to the multiple drugs. The rpoB2 (14.81±5.92 and 10.19±2.31), ugd (24.14±0.86 and 0.86±0.45), bacA (20.63±11.23 and 4.38±1.66), penA (21.42±12.75 and 3.59±1.43), mdtC (23.13±14.5 and 1.87±0.34), and ompR (18.45±9.93 and 6.55±1.0) were among the top abundant antibiotic resistance genes predicted during bathing and post-bathing events in Indrayani River respectively. Likewise, rpoB2 (23.08±4.65 and 10.25±5.02), efpA (27.36±8.53 and 5.97±3.66), tetA (24.03±4.95 and 9.3±5.73), tap (27.10±9.70 and 6.22±3.53), and ompR (23.31±3.98 and 10.02±6.35) were among the dominant ARGs in the Bhima River during the bathing and post-bathing events in Bhima River, respectively. The bathing and post-bathing samples of Bhima and Indrayani river accounted for ~110% difference in the abundance of the ARGs (Fig. 6B). Moreover, the significant (Supplementary Fig. 3C and 3D) variations of ARGs during the bathing and post-bathing in the Bhima and Indrayani River (Permanova, Adonis p < 0.05) indicate the allochthonous inputs of ARGs during such events. We also observed higher differences in MGEs

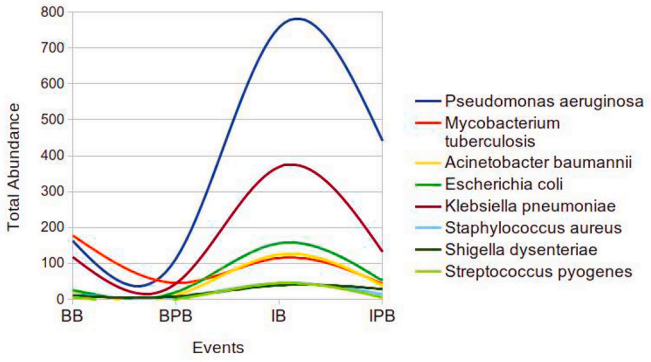

Fig. 5. Influence of mass bathing on the priority pathogens. BB- Bhima bathing samples; BPB- Bhima post-bathing samples; IB- Indrayani bathing samples; and IPB- Indrayani post-bathing samples.

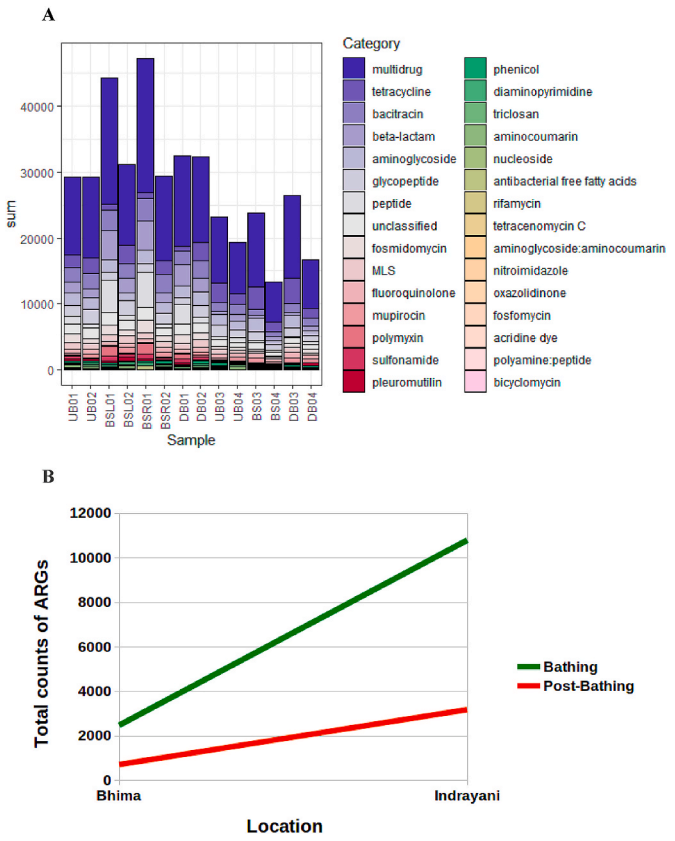

Fig. 6. Impact of mass bathing on Antibiotic resistance. A. The abundance of ARGs during bathing and post bathing in both the rivers. B. Level of antibiotic resistance during a bathing event of both the rivers. (UB01, DB01, BSL01, BSR01, UB03, BS03, DB03 are Bathing samples of respective rivers whereas UB02, DB02, BSL02, BSR02, UB04, BS04, and DB04 are Post-bathing samples of respective rivers).

(Supplementary Fig. 4) such as transposases, recombinase and integrases (~34% and ~85%) between the bathing and post-bathing samples of Bhima and Indrayani River, respectively. The scarcity in the development of newer antibiotics for MDR pathogens displays a very problematic scenario (Ventola, 2015). To tackle the observed multi-drug resistant (MDR) pathogenic bacteria in this study, we subjected the water samples against 20 MDR and some common waterborne pathogens to isolate phages. We successfully isolated phages against strains of *Klebsiella pneumoniae*, Acinetobacter baumannii and *Staphylococcus aureus* (Table 1).

Table 1
Isolation of Bacteriophages. Bacteriophages isolated against multi-drug resistant and waterborne pathogens using water samples collected during Wari.

Sr. No.	Host	Water samples from Bhima River	Water samples from Indrayani
1	*Klebsiella pneumoniae*	+	–
2	*Acinetobacter baumannii*	+	+
3	*Pseudomonas aeruginosa*	–	–
4	*Staphylococcus aureus*	+	+
5	*Escherichia*	–	–
6	*Enterobacter*	–	–
7	*Shigella*	–	–
8	*Salmonella*	–	–
9	*Vibrio*	–	–

4. Discussion

In the current study, we observed major alterations in the overall microbial ecological structure and functions of the rivers during the religious mass gathering and bathing event during Pandharpur Wari in Maharashtra, India. We observed considerable differences in the drug resistance and the virulence potential amid the surge in their prevalence during mass bathing.

The anthropogenic activities and the resulting pollutants profoundly impact riverine systems affecting as well as governing their overall microbial ecology (Jani et al., 2018b; Li et al., 2018). Our microbial community analysis revealed significant variability in the abundance of dominant bacterial populations such as *Proteobacteria, Firmicutes, Actinobacteria* and others, between the bathing and post-bathing samples of both the river. Moreover, a higher predominance of skin, as well as gut-associated bacterial phyla such as *Actinobacteria, Proteobacteria, Firmicutes*, and *Bacteriodetes*, was recorded in the bathing samples. This implies the influence of bathing as well as open defaecation observed during Wari (Jani et al., 2018b; Grice and Segre, 2011; Thursby and Juge, 2017). The effect of mass bathing becomes even more evident at the lower level of the taxonomy. The sites under this study are of religious importance and mostly observe gatherings throughout the years and therefore are more susceptible to anthropogenic activities. However, during the Wari event, the numbers of pilgrims increase hugely, and consequently, the ecological perturbations become more severe. Thus, the increase in the members of Rhodocyclaceae and decrease in the common freshwater Commamonadaceae could probably be attributed to such anthropogenic disturbances which result in increase carbon loading (Moon et al., 2018; Balmonte et al., 2016). Additionally, the increase in Mycobateriaceae, a group containing many known human pathogens (Malhotra et al., 2017) further implicates the ill-effects of such mass bathing events.

Anthropogenic stress has a wide range of consequences on the microbial structural pattern and their functionality, especially when it comes to the aquatic ecosystems, which is more dynamic and vulnerable (Nogales et al., 2011; Won et al., 2017). A previous study by Jani et al. (2018a), has reported a decrease in the bacterial richness and diversity during such mass bathing event. Similarly, here the analysis for alpha diversity revealed notable alterations in the microbial diversity during the bathing and the post-bathing events. Evidently, the bacterial diversity decreased significantly in the bathing samples of both the rivers. Moreover, we observed higher differences in the overall microbial richness and diversity of the rivers between the bathing and post-bathing samples. Additionally, higher diversity in the upstream samples (UB03, UB01) could probably be attributed to lesser usage of these sites during mass bathing. Further, the beta diversity analysis unveiled the critical impact of mass bathing. The higher R^2 value (0.58 and 0.48 for bacteria and archaebacteria, respectively) strongly suggested the influence of mass bathing which resulted in significant alterations in the overall microbial community structure. The alterations in the microbial community also lead to changes in the functional potentials of the riverine system. We observed substantial differences in the functionality of the river ecosystem during the mass bathing. Overall, the observed spatiotemporal alterations in the overall microbial community and functionality can be associated with the input of allochthonous microbes in these rivers during mass bathing.

The type of microbial community is one of the crucial factors that determine the water quality and thus, pathogen-containing water will have very grave consequences on human health (Azzam et al., 2017). Previously, influenza outbreaks were reported during the mass gatherings of World Youth Day 2018 (Blyth et al., 2010). During such mass bathing events, millions of people interact, gargle and are in direct contact with the water, and thus such events provide multiple chances to the infectious and multi-drug resistant pathogens to propagate. Here, we observed higher differences in the prevalence of virulence genes. Moreover, the significant dissimilarity in the virulence gene structure

between the bathing and post bathing samples indicate the addition of pathogens during the mass bathing. Since mass bathing involves the influx of a large number of pilgrims; we gave special emphasis to the skin and intestinal disease-related pathogens. These virulence genes predicted were mapped to various global priority and ESKAPE pathogens such as Acinetobacter baumannii, *Staphylococcus aureus*, Streptococcus pyogenes, *Mycobacterium tuberculosis* and others, possessing the potential to cause multiple intestinal and skin-related infections (Asokan et al., 2019; Findley and Grice, 2014; Mulani et al., 2019). Altogether, the increase in the prevalence of priority pathogens and their virulence genes undoubtedly confirms the increase for the potential of water-borne infections and also display the dismal conditions of the rivers during the mass bathing.

Antimicrobial resistance (AMR) is currently the biggest threat to the entire global health and is progressing with an unmatched pace (https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance). Our analysis detected ARGs against all the clinically important classes of antibiotics. The inflated levels of ARGs in the bathing samples further substantiated the impact of mass bathing in these rivers. Probably, the river ecosystem will further act as a suitable niche for the interaction of these MDR pathogens, and as a result, it will facilitate the emergence and propagation of newer antibiotic resistance. When considering the AMR problem, it is also essential to consider the mobilome of the pathogens that amplify this antibiotic resistance via horizontal transfer of such ARGs (Stokes and Gillings, 2011). This notable increase of MGEs such as transposases, recombinase and integrases in bathing samples further exacerbates the antibiotic resistance problems during such bathing event. Collectively, the mass bathing and the associated anthropogenic activities resulted in significant alterations in the overall microbial ecology and their functionality.

5. Conclusions

To conclude, Wari is a unique religious occasion which is a symbol of unity that brings millions of devotees belonging to different caste together. The overall aim of this comprehensive study was to unravel the microbial ecological changes in the river ecosystem and estimate the potential danger during this mass bathing event. This information would help the government to plan the upcoming Wari event in a more meticulously manner to minimize river pollution due to open defecation and reduce the chance of infections. As to our best knowledge, this comprehensive shotgun-based study is the first report addressing the implication of mass gathering and bathing during the event of Wari. Overall, we observed inflated levels of pathogens, virulence genes, and ARGs during the bathing event of Wari which resulted in higher differences in their prevalence between the bathing and post-bathing samples. Higher prevalence of priority pathogens and their virulence genes during bathing further provides a potential opportunity for the pathogens to rapidly spread. In addition to a holy bath, the devotees also drink a handful of water as a part of the ritual, which results in the enormous intake of disease-causing pathogenic organisms. Moreover, the spatiotemporal variations in the overall microbial ecology collectively illustrated the impact of mass bathing. The comparison with the post-bathing samples helped us to conclude about the ecological alterations occurring in the river during bathing. As we observed that post-bathing samples relatively recovered (lesser prevalence of ARGs, virulence and increase in the bacterial diversity as compared to bathing samples) from the sudden impact of the mass bathing, however, the uncertainty remains. Therefore, our future studies would undertake the long-term monitoring of these rivers post the Wari event to delineate the nature of its impact on the riverine ecosystem. Altogether, our results emphasize a need for proper surveillance, planning and infrastructure developments to tackle the challenges of water pollution and risks associated with public health.

Research data

The data-sets supporting the conclusions of this article are available in the MG-RAST in the project entitled as “Wari_2019”. Following are the MG-RAST Ids for the metagenome samples (Sample ID-MGRAST ID): DB01- mgm4869419.3, UB02-mgm4869420.3, BSL02-mgm4869421.3, DB03- mgm4869422.3, BS04- mgm4869423.3, DB04- mgm4869424.3, BSR02-mgm4869425.3, BSR01-mgm4869426.3, BSL01-mgm4869427.3, UB01- mgm4869428.3, BS03- mgm4869429.3, UB03-mgm4869430.3, UB04- mgm4869431.3, DB02- mgm4869432.3.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions

Rakeshkumar: Data curation, Investigation, Methodology, Writing original draft; Vinay: Investigation, Reviewing and Editing, Formal analysis, Krishna: Investigation, KG: Investigation, Reviewing and Editing; Mahesh: Supervision, Conceptualization, Resources

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Authors are grateful to the Directors of CSIR-NCL and CSIR-NEERI for providing facilities, infrastructure and support. RKY would also acknowledge University Grants Commission (UGC), New Delhi, India for fellowship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.110938.

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