Field monitoring and hydraulic modelling quantify untreated wastewater as dominant source of AMR in a small river running through a big city

Vikas Sonkar[a,*], Arun Kashyap[a], Rebeca Pallarés-Vega[b], Sai Sugitha Sasidharan[a], Ankit Modi[c], Cansu Uluseker[d], Sangeetha Chandrakalabai Jambu[a], Pranab Kumar Mohapatra[c], Joshua Larsen[e], David Graham[b], Shashidhar Thatikonda[a], Jan-Ulrich Kreft[d,*], on behalf of the AMRflows consortium

[a]Environmental Microbiology Laboratory, Department of Civil Engineering (Environmental Engineering), Indian Institute of Technology Hyderabad, Kandi, Sangareddy, 502284, Telangana, India

[b]School of Engineering, Newcastle University, Newcastle upon Tyne NE1 7RU, UK

[c]Department of Civil Engineering, Indian Institute of Technology Gandhinagar, Gandhinagar, Gujarat 382355, India

[d]Institute of Microbiology and Infection & School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

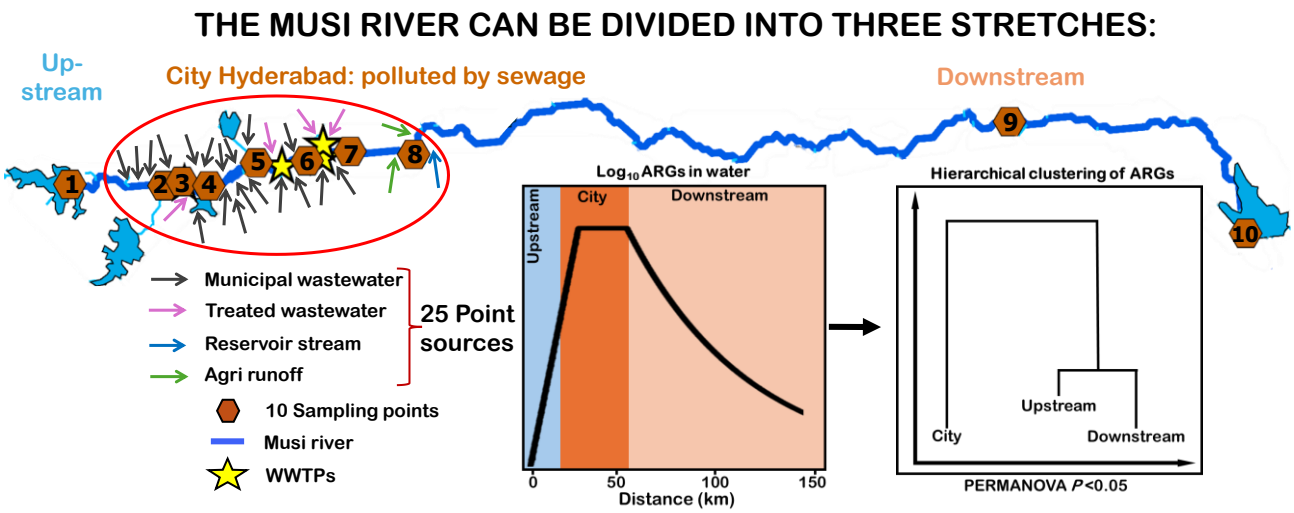
[e]School of Geography, Earth and Environmental Sciences, University of Birmingham, Birmingham, B15 2TT, UK

*Corresponding authors

E-mail addresses:

vikassonkar629@gmail.com (V. Sonkar)

j.kreft@bham.ac.uk (J.-U. Kreft)



Highlights

- **Within the city, the concentration of ARGs and ARBs increases in the dry season**
- **Clustering and ordination of ARGs separates upstream, city and downstream stretches**
- **PERMANOVA shows stretches and seasons are significantly different**
- **LDA can discriminate stretches and seasons based on water quality parameters**
- **Point source load calculations indicate 60-80% of river water is untreated wastewater**

Abstract

Antimicrobial resistance (AMR) is a silent pandemic, which is transmitted and spread through the environment. Few of many studies on waterborne AMR spread have characterised the prevalence, sources, and transport of AMR in adequate detail to inform mathematical modelling and exposure risk assessments. Here, we quantified taxonomic and resistance genes (ARGs), sensitive and resistant bacteria (ARBs), and environmental conditions in water and sediment samples at ten locations along the Musi River through Hyderabad, a city renowned for antimicrobial manufacturing, to develop a hydraulic model for AMR spread in the catchment. In the dry season, absolute water column ARG and ARB abundances increased rapidly upon entering the city, then declined downstream. In the wet season, the increase and decline of AMR levels was more gradual. Clustering and ordination of ARGs separated the river into three stretches: upstream, city, and downstream. Most ARGs and water quality indicators differed significantly between stretches and seasons ($p<0.05$; PERMANOVA). Linear Discriminant Analysis showed that various water quality parameters, such as lower dissolved oxygen and elevated total nitrogen, can distinguish between AMR-polluted and less-polluted sites and might be good proxies to detect AMR hotspots. Modelling showed that 60%-80% of river water in the city was untreated wastewater, suggesting municipal wastewater dominated AMR in the river, not manufacturing sources. This will be a typical situation where wastewater treatment is insufficient and dilution in receiving water bodies limited, highlighting the urgent need for improved wastewater management to make rivers like the Musi safer for use, such as irrigation, reducing AMR exposures across wider environments.

Keywords: Antibacterial resistance; Contagion; Emerging contaminants; Mass balances; Pollution; Sewage

1. Introduction

Antimicrobial resistance (AMR) is among the greatest health threats of the 21st century, impacting humans, animals, crops, and the environment around the world (UNEP 2023). Murray et al. (2022) estimated that 1.27 million additional deaths were attributable to bacterial resistance in 2019 and O 'Neill (2014) predicted that AMR could lead to up to 10 million deaths per year and $100 trillion total GDP loss annually by 2050. AMR is an ancient and natural phenomenon (D'Costa et al. 2011), but human activities have greatly increased its prevalence and spread across healthcare systems and the wider environment. Although the primary health risk is increasing AMR pathogens, resistance in pathogens is primarily conferred by AMR genes (ARGs) that can be acquired through horizontal gene transfer (HGT) from commensal and other resistant bacteria (ARBs) on mobile genetic elements (MGEs) (Martínez 2008). ARGs are often used as surrogates of AMR in environmental monitoring.

AMR prevalence is especially increasing in Low- and Middle-Income Countries (LMICs) (UNEP 2023). This is largely due to inadequate waste management and less regulated antimicrobial use in medicine and agriculture, resulting in AMR-laden wastes released to the environment, fuelling AMR transmission and spread via polluted water across the human, animal, crop, and environmental sectors (Klein et al. 2018; Kashyap et al. 2023; Sonkar et al. 2024). In parallel, many LMICs are experiencing accelerated urbanisation, which amplifies AMR transmission and spread due to local overpopulation. As an example, 34% of India's population lived in urban areas in 2021, which is increasing at 2.4% per year (The World Bank 2022), pressuring a civil infrastructure that is already overloaded. This is especially true of wastewater management systems and treatment plants (WWTPs), which reduce the release of AMR bacteria and genes into local water courses (Yee et al. 2021; Yang et al. 2022; Beltr et al. 2023). Such exposed water bodies become hubs for waterborne AMR spread, impacting the emergence and transmission of pan-resistant strains through mechanisms such as mutation and HGT (Graham et al. 2014; Farkas and Butiuc-keul 2016; Beltr et al. 2023). As such, characterising the spatiotemporal distribution of ARBs, ARGs, and MGEs in urban LMIC watersheds, which receive complex mixtures of wastes, has wide significance.

The dynamics of inadequate waste management and AMR spread is exemplified by conditions in the Musi River in southern India (**Figure 1**). The Musi has significant economic and societal importance (Diwan et al. 2018), but also has become a major waste disposal site for municipal and industrial wastewater from the growing city of Hyderabad, Telangana. Hyderabad has inadequate WWTP capacity and, as such, the Musi has become highly polluted due to diverse

wastewater inputs, including municipal and industrial wastes (Larsson et al. 2007; Gothwal and Shashidhar 2017; Konda et al. 2022, 2024). However, the quantitative consequences of such releases of AMR in the Musi, including source attribution, has not been determined, which is key to prioritising remedial actions to reduce AMR exposures across the watershed.

The shortage of useful data for decision-makers on AMR in Indian aquatic environments was recently reviewed (Rathinavelu et al. 2024). The Musi, which is our case study, appears to a good model for assessing AMR fate in LMIC river catchments (Caucci et al. 2016; Herrig et al. 2020; Kumar et al. 2020a, b; Yang et al. 2022; Beltr et al. 2023), and is relevant to understanding other Indian rivers, such as Sabarmati (Ram and Kumar 2020), Mutha (Marathe et al. 2017), Kshipra (Hanna et al. 2020), Brahmaputra (Hanna et al. 2020), Ganga and Yamuna (Gupta et al. 2022, 2023). However, past Indian river studies have not examined spatiotemporal variations of AMR levels in detail, including how water quality and AMR are related and impact AMR exposure risks (Kumar et al. 2020b; Xu et al. 2023). Surface water bodies can change seasonally, including temperature fluctuations and differences in rainfall intensity and frequency, and antimicrobial usage can vary between seasons due to different microbial infections in human and animal populations (Knapp et al. 2012; Caucci et al. 2016; Ott et al. 2021). Variations in rainfall also alter agricultural practices, and impact agricultural/urban runoff and wastewater discharges (Di Cesare et al. 2017; Arrieta et al. 2022). These factors collectively contribute to seasonal variations in the abundance and diversity of ARBs, ARGs, and MGEs that associate with AMR development and transmission (Rathinavelu et al. 2024).

Here we investigated and quantified seasonal variations in ARB, ARG, and MGE abundances along the Musi River in Telangana State, a place that experiences distinct dry and wet seasons, and includes antimicrobial manufacturing in its catchment. Water column and sediment samples were collected for both seasons from different parts of the catchment to assess how seasonal and spatial factors impact *in situ* AMR. Basic water and sediment quality data were collected across seasons as well as viable ARB colony counts, which were compared with ARG and MGE data in the river, and other Indian studies (Kumar et al. 2019; Ram and Kumar 2020; Gupta et al. 2022, 2023). Overall, the objectives were: (1) to characterise the impact of season on the abundances of ARGs and ARBs in the water and sediments as the Musi flows through Hyderabad, which is needed mathematical modelling and risk analysis, (2) to assess correlations between ARGs, MGEs, and water quality conditions in the watershed, (3) to attribute major drivers of AMR found in the river, and (4) to identify possible water quality proxies for AMR

to guide preventative action and prioritise sites for more detailed AMR monitoring and analyses.

2. Materials and methods

This study adhered to EMBRACE-WATERS reporting guidelines (Hassoun-Kheir et al. 2021) wherever possible, indicating any deviations.

2.1. Study area and sample collection

Sampling was performed at 10 locations in the dry and wet seasons along the Musi River through Hyderabad in Telangana State, Southern India **(Fig. 1)**. The Musi is a major tributary of the Krishna River, ~260 km in length and has an average annual rainfall in its catchment of ~800 mm, most of it falling in the monsoon or wet season (June to September). Two reservoirs, Osman Sagar and Himayat Sagar, exist where the Musi and Musa Rivers (a tributary of the Musi 60 km upstream of Hyderabad city) are blocked by dams, respectively. The Musi riverbed is generally rocky, consisting primarily of pink granite (Narshimha et al. 2020). The two dams are closed most of the year except for spillway overflows during the wetter months. Therefore, flows in the Musi are often very low upstream of the city (only leaks from the dams), and most of the flow within the city is from wastewater discharges, about 1.2 million m^3/d of partially treated municipal wastewater, mixed with industrial wastes from across the city. The wastewater discharged into the Musi is either untreated or released from municipal WWTPs (**Tracer.xlsx**). Two Common Effluent Treatment Plants for pharmaceutical and industrial waste releases also discharge into the Musi catchment, including: the Patancheru Enviro Tech Ltd. (PETL) and Jeedimetla Effluent Treatment Plant (JETL), release 1600–2000 and 1000-1200 m^3/day, respectively. Effluents from the PETL and JETL flow through an 18 km pipeline to the Amberpet WWTP (339 MLD), where the discharges mix with treated wastewater and flow to the Musi itself **(Fig. 1)** (CPCB 2005, 2010; Lübbert et al. 2017). In terms of land use, the Musi catchment is both rural and urban, including many residential communities, hospitals and factories. Downstream of the city, land use includes agriculture and aquaculture, with less human impact than the city stretch. Sampling sites were chosen to contrast upstream water conditions with sites in the city and downstream. Since the Musi flows from a reservoir upstream of the city, only one upstream sampling point was possible. Within the city, many sampling points were sampled, reflecting different inputs along the river.

Sampling campaigns were conducted in both dry and wet seasons to characterise general water quality conditions, the abundance of ARB and susceptible bacteria, and ARGs and other gene levels in water and sediment samples. Grab samples from 10 river water and sediment sites (**Fig. 1,** detailed description of sites given in **Tracer.xlsx**) were collected on two occasions, 18-

19 August 2022 (wet season) and 11-12 March 2023 (dry season). All samples were collected on days when no rain had occurred for five days (even wet season samples). At most sites, duplicate water samples were collected mid-stream from bridges along the river using a bucket suspended on a rope and transferred to pre-sterilised 2-L HDPE bottles for each site.

Sub-samples were preserved immediately using H_2SO_4 for chemical oxygen demand (COD) laboratory analysis, whereas water temperature, pH, dissolved oxygen (DO), TDS and conductivity were measured on-site with an Orion Star A329 portable multimeter with smart probes (ThermoFisher Scientific, USA). Sediment samples were collected in duplicate from 2 to 3 metres from the riverbank using a grab dredger, targeting the top 0-15 cm depth. Each sample was sieved through a 2 mm sterile net and transferred into pre-sterilised polypropylene bags (approx. 1 kg). All samples were stored in the dark on ice in a cooler and were transported back to the lab for further processing and analysis.

2.2. Water and sediment quality analysis

Water quality and sediment physicochemical parameters were measured using standard protocols (**Table S1** and **S2**). When required for analysis (*e.g.*, organic carbon, total nitrogen (TN), ammonia, nitrate, and nitrite), water samples were filtered using 0.45 µm polyvinylidene difluoride (PVDF) membrane filters (Merck Life Sciences, Merck, India) before analysis. To normalise the culture-based and genetic data from the sediment samples, dry matter (DM) and organic matter (OM) fractions were quantified using standard methods (**Table S2**).

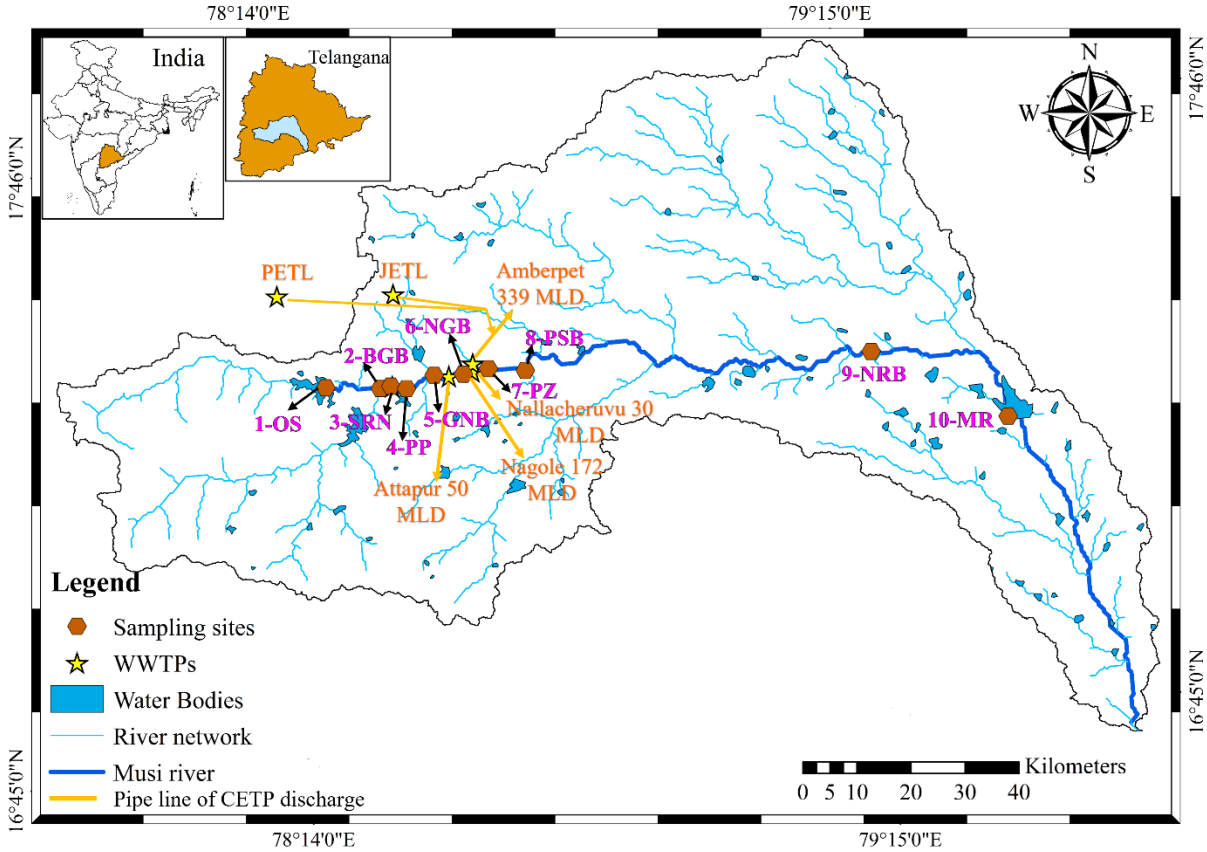


Fig. 1. The basin of the Musi River in Telangana, Southern India with the locations of sampling sites (numbered 1-10) and WWTP effluent discharges. Larger WWTPs use the Upflow Anaerobic Sludge Blanket (UASB) process apart from Attapur using the Sequencing Batch Reactor (SBR) process. A total of 25 point sources of untreated or treated sewage and other sources have been identified within Hyderabad, between sampling points 1 and 8, see **SI spreadsheet 1** for details.

2.3. Enumeration of *E. coli,* heterotrophic bacteria and antibiotic-resistant bacteria (ARBs)

Viable counts were performed with water and sediment samples using less and more selective media after serial dilution. To avoid substrate-accelerated death of environmental bacteria, serial dilutions were done with sterile MilliQ water (Bartscht et al. 1999). Before dilution, 1.0 g of each sediment sample was suspended in 10 mL of sterile MilliQ water, gently vortexed and allowed to settle at room temperature for 10-15 minutes before use (Bartscht et al. 1999; Tanaka et al. 2014). Then, 0.1 mL of well-mixed water or sediment samples were plated.

To quantify 'total culturable' bacterial numbers, a modified version of the OECD 2001 synthetic sewage medium (OSS) was used because it is quite similar to polluted rivers (OECD 2001;

188 O'Flaherty and Gray 2013; Tanaka et al. 2014), supplemented 100 mg/L SDS (sodium dodecyl
189 sulfate) to inhibit Gram-positive bacteria. *E. coli* was enumerated using the more selective
190 Tryptone Bile X-β-D-glucuronide agar (TBX) (HiMedia, India) as blue colonies (Baker et al.
191 2022). To enumerate viable Extended Spectrum β-lactamase (ESBL)-producing bacteria in
192 samples, the OSS medium was amended with the HiCrome™ ESBL supplement (HiMedia,
193 India) (*i.e.*, 3 mg/L ceftazidime, 3 mg/L cefotaxime, 2 mg/L ceftriazone, 2 mg/L aztreonam, and
194 10 mg/L fluconazole), creating OSS-ESBL media. Carbapenemase (CARB)-producing bacteria
195 were quantified using OSS media amended with 0.5 mg/L meropenem (TCI, Japan), 200 mg/L
196 cloxacillin (TCI, Japan), and 70 mg/L $ZnSO_4$, creating OSS-CARB media. The TBX, OSS-
197 ESBL and OSS-CARB plates were incubated at both 44°C (ISO 16649-3:2015 2015) and 30°C
198 to select for gut-relevant bacteria and mirror Musi water temperatures, respectively. All water
199 and sediment samples were processed within 24 hours. Triplicate plate counts were performed
200 after 24 hours.

201 **2.4. DNA extraction**

202 Water column sub-samples were aseptically vacuum filtered through sterile 0.2 µm
203 polyethersulfone (PES) membrane 47 mm disc filters (Pall Corporation, NY, USA) to collect
204 biomass. To account for differences in biomass across samples, different sample volumes were
205 filtered depending on the sample and total volumes were recorded. Filters were stored at -20°C
206 before extraction. DNA from both water and sediment samples was extracted in duplicate within
207 24 hours of sampling using the DNeasy® PowerWater® Kit (Qiagen, Germany) and DNeasy®
208 PowerSoil® Pro Kit (Qiagen, Germany) respectively, following the manufacturer's instructions.
209 After extraction, the duplicate extracts were pooled in a sterile centrifuge tube. DNA purity
210 (A_{260}/A_{280} ratio of 1.8–2.1) was determined by NanoDrop™ 2000/2000c (Thermofisher, US),
211 and DNA yields were measured using a Qubit™ 4 Fluorometer (Thermofisher, US).

212 **2.5. Measurement of ARGs by quantitative polymerase chain reaction (qPCR)**

213 ARG abundances were quantified by qPCR from the extracted DNA using primer sets for 10
214 selected genes (**Table S3**). The 16S rDNA gene was quantified as a proxy for total prokaryotes
215 and to compare with culturing data. ARG targets were chosen according to Berendonk et al.
216 (2015) and to reflect resistance to antibiotics used and produced in Hyderabad (**Fig. S1)**. Genes
217 included: fluoroquinolones (*qnrS*), aminoglycosides (*aph(3")-Ib*), sulfonamides (*sul2*),
218 carbapenems and third-generation cephalosporins (bla_{NDM} and $bla_{CTX\text{-}M}$), macrolides (*ermF*),
219 and tetracyclines (*tetW*). Two more genes were included: *uidA* to quantify *E. coli* numbers

220 (Frahm and Obst 2003; Silkie et al. 2008) and *intI1* to quantify class I integrons (Gillings et al.
221 2015).

222 All qPCR reactions were conducted with 10 µL volumes, including TB SYBR Green® Premix
223 Ex Taq^{TM} II (Takara, Japan) and BSA 4 mg/mL (m/v) (Sigma Aldrich, USA). Concentrations
224 of forward and reverse primers, amplicon size of target genes and annealing temperatures are
225 summarised in **Table S3**. After initial testing, DNA was diluted 10-fold to reduce inhibition and
226 2 µL of DNA template was added to each reaction. The reaction volume was made up to 10 µL
227 with DNase/RNase-free water (Honeywell, USA). All reactions were performed in a CFX96
228 Touch® Thermocycler from BioRad (USA), according to Pallares-Vega et al. (2019), but using
229 different annealing temperatures provided in **Table S3**. Synthetic DNA fragments (IDT,
230 Singapore) containing suites of target genes were used as positive controls and to create
231 standard curves, for which ten-fold serial dilutions of gene fragments were performed in
232 DNase/RNase-free water (Honeywell, USA). Every sample was analysed in technical
233 duplicates. Standard curves were included in each PCR plate with at least seven serial dilution
234 points and technical duplicates. A joint standard curve based on a standard curve from every
235 run was created for every gene set. Concentrations of genes in the samples were then calculated
236 using this joint standard curve.

237 **2.6. Estimating possible point-source pollutant sources along the Musi River**

238 While ARGs and ARBs were quantified here in Musi River water and sediment samples, it was
239 important to identify possible pollutant point sources along the river for modelling purposes.
240 As such, 25 possible point-sources of untreated and treated municipal wastewater, agricultural
241 runoff, and mixed waste sources in the catchment were identified, and attributions from each
242 source were numerically estimated (**Tracer.xlsx**).

243 For each wastewater source, wastewater production rates were estimated based on local
244 population size and typical municipal wastewater production rates (detailed methods given in
245 **SI text 1.1.)**. Further, flow records of WWTP and reservoir discharges were obtained.
246 Catchment sizes and locations were based on official records and digitised elevation maps. For
247 corroboration, an alternative approach to estimate population sizes was performed based on
248 census data combined with satellite images to identify dwellings and localise the data from the
249 High Resolution Population Density Maps + Demographic Estimates.

250 Results from the two approaches were reasonably consistent, comparing estimates using a
251 hypothetical tracer that reflected wastewater inputs from point sources. Our "tracer" was meant

to reflect the typical composition of wastewater and was set to measured values of COD in untreated (normalised to 100%) and treated wastewater (16%). Values for VS (12%), 16S (19%) and total ARGs (22%) were similar (see **Tracer.xlsx)**. Mass balance and load calculations of the tracer were performed using upstream river flow and concentration data and estimated point-source discharges to determine the relative impact of each point source on river water quality (**Tracer.xlsx**). For simplicity, the tracer was assumed to be inert, which is reasonable given short residence times.

2.7. Data processing and statistical analysis

Statistical analyses were performed with R 4.3.0 (R Core Team 2018), mainly packages ade4, vegan and MASS using the RStudio 2023.03.0 desktop (https://www.rstudio.com/) and fully documented in an R notebook (Supplementary File "Musi_analysis.Rmd"). The methods and approach, including Hierarchical clustering, Principal Component Analysis (PCA), Linear Discriminant Analysis (LDA) and PERMANOVA, were guided by (Borcard et al. 2011), but we are responsible for the choices made. However, two clustering methods and two dissimilarity measures were used to ensure that results are robust and not dependent on specific choices. We only report results supported by all method combinations. Ricotta and Podani (2017) was used for guidance in the choice of dissimilarity measures. Whether data should be scaled or standardised was decided after exploring and analysing the data. It was not possible to perform sample size (statistical power) calculations *a priori* due to a lack of preliminary data.

3. Results and discussion

Assessments of sources and distribution of AMR bacteria and genes in Indian rivers are rare, especially in catchments that include antimicrobial manufacturing and processing, like the Musi River. Here, we quantified ARGs, ARBs, and water quality and sediment conditions at 10 locations along the Musi in two seasons to identify primary drivers of elevated resistance in the river. The work provides essential new information to support One Health solutions to AMR to assist National Action Plans (NAPs), especially data on the environmental spread of AMR in Indian rivers. For this purpose, AMR and metadata from both seasons were analysed by clustering and ordination, and statistical correlations were performed between parameters, including ARGs, ARBs, and water quality conditions. Hydraulic modelling, mass balance and load calculations show that municipal wastewater pollution dominates *in situ* AMR signatures in the river, but simple water quality measures can be used as proxies for elevated AMR to guide deeper investigations within NAP development.

3.1. Patterns of genes and bacteria along the river in the dry and wet seasons

3.1.1. Resistance and other genes

The concentration of seven ARGs, *intI1, uidA*, and the 16S rDNA gene were quantified in river water (**Fig. 2**) and sediment samples (**Fig. S4**) from the wet and dry seasons. The most abundant genes in the water samples for both seasons were 16S rDNA, followed by *intI1*, *sul2*, *aph(3")-Ib, ermF*, *tetW* and *qnr*S. Conversely, $bla_{\text{CTX-M}}$, bla_{NDM}, and *uidA* had the lowest concentrations in both seasons. The abundances of all ten genes followed consistent spatial patterns across samples, with the lowest levels found upstream, highest within the city, and lower downstream, albeit similar to or higher than upstream of the city (**Fig. 2**). During the wet season, water column gene levels increased more gradually as water passed through the city; also, gene levels declined more gradually downstream.

No consistent seasonal patterns in gene levels were evident in the sediment samples, although concentrations were generally higher, albeit more variable, within the city (**Fig. S4**). Overall, the analysed genes in water and sediment columns indicated that Musi in Hyderabad is highly polluted with ARGs and ARBs in both seasons, although spatial patterns of detected genes differed between sediment and water column samples. On a positive note, ARGs and ARBs declined downstream of the city, suggesting natural recovery is possible when pollutant sources diminish.

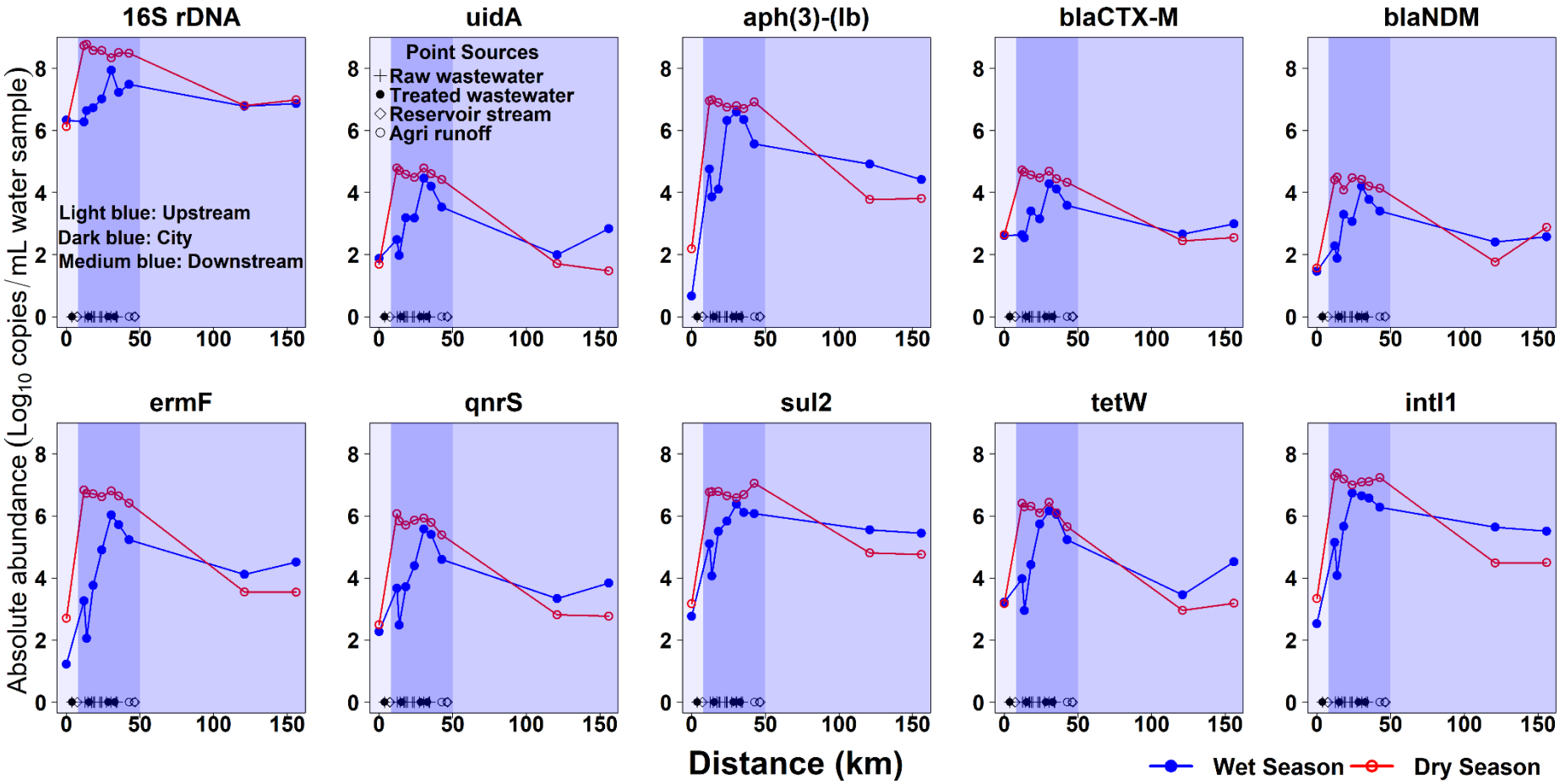


Fig. 2. Absolute abundance of genes along the Musi River in dry (red) and wet season (blue) water samples. All panels have the same scale showing log transformed absolute abundances of 16S rDNA, *uidA*, intI1 and seven ARGs. The river can be divided into three stretches: (a) upstream (light blue), (b) within the city (dark blue) and (c) downstream of the city (medium blue). Point sources of raw wastewater, treated wastewater, a mixture of both, reservoir flows, and agricultural runoff are indicated by symbols. See **Fig. S3** for equivalent sediment sample data.

3.1.2. Bacterial plate counts and their correlations with genes

'Total culturable' bacteria, *E. coli*, heterotrophic bacteria (OSS), and ESBL- and CARB-resistant bacteria were quantified in the water column and sediment samples. In general, using 30°C and 44°C as incubation temperatures did not alter observed patterns of culturable total bacteria or *E. coli* in the water samples (**Fig. S5**; see **SI 2.1.** for details). That is, viable bacteria concentrations were lower upstream, much higher in the city, and lower again downstream in the dry season. In contrast, abundances were elevated everywhere in the wet season, similar to those in dry season city samples.

The pattern for ESBL-resistant bacteria was similar with <3 CFU/mL (*i.e.*, below detection limit (BDL)) upstream, higher in the city, and returned to BDL downstream in the dry season. In the wet season, levels were similar to those found within the city during the dry season. Conversely, CARB-resistant bacteria were BDL in the city in the dry season (and elsewhere), while the wet season pattern reflected ESBL. The consistently high ESBL and CARB counts in the wet season (**Fig. S6**) suggest elevated flows reduce sedimentation of water column bacteria and/or increase scouring of organisms previously deposited in sediments, which can occur in rivers in monsoonal climates (Knapp et al. 2012).

Plate count data for sediment samples differed from their parallel water samples (**Fig. S5 B**). Bacterial abundances were generally higher in the wet season than in the dry season on all media. Still, there was no clear distinction between upstream, in-city, and downstream samples in the dry season, and in the wet season, abundances were slightly higher in the city and downstream samples compared with upstream samples. However, differences were less dramatic than in the water samples. ESBL- and CARB-resistant plate counts were all BDL in sediments in the dry season with one exception, while they were all at similarly high levels in the wet season.

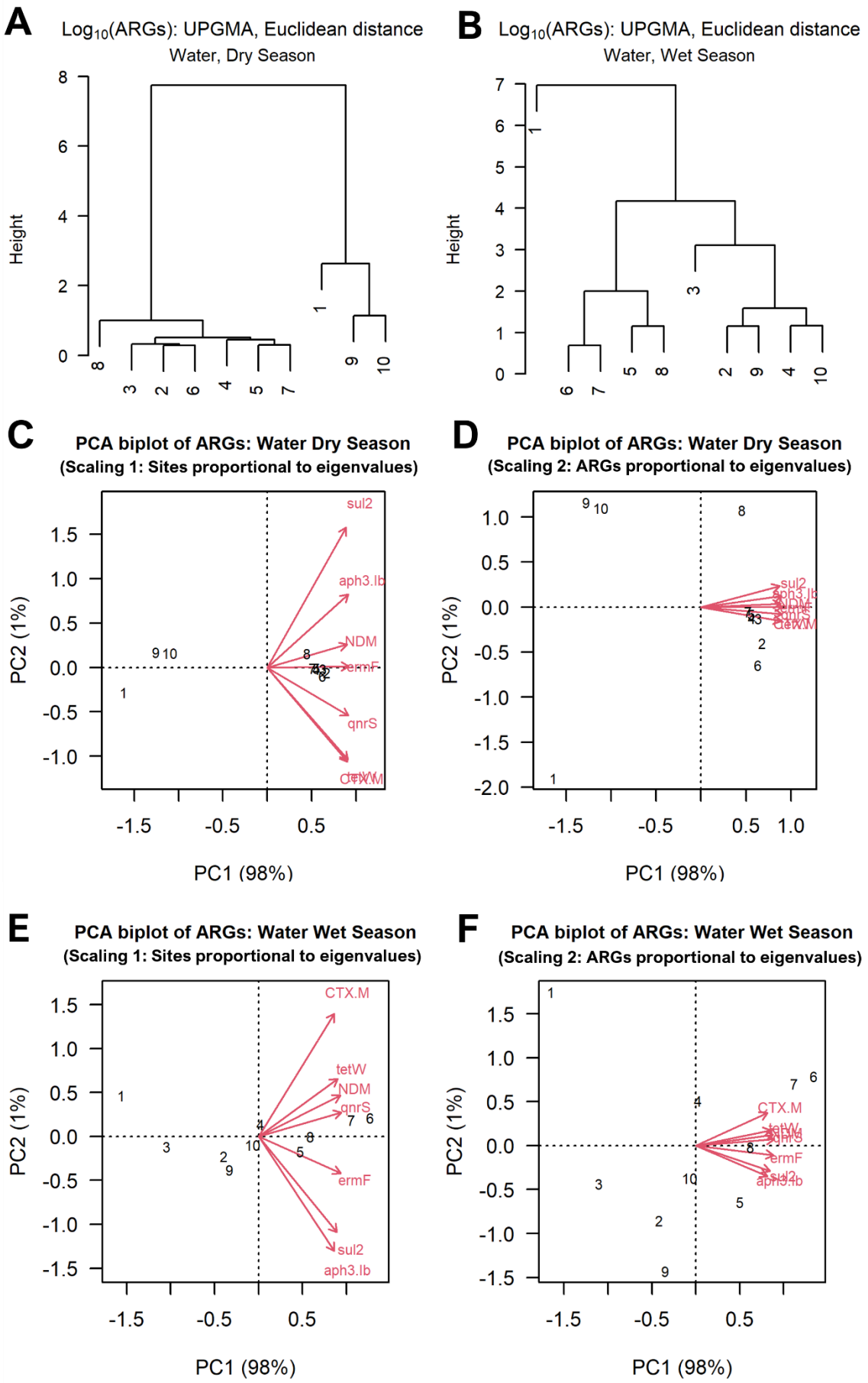
Different hypotheses regarding relationships between viable counts versus different conditions and viable counts versus genes quantified by qPCR quantification. These include: (i) colony counts at higher temperatures would be less than counts at the lower temperature on the same medium; (ii) counts on CARB media would be less than ESBL media; (iii) counts on ESBL media would correlate with *E. coli* counts because they relate to levels of municipal wastewater pollution; (iv) counts on OSS media would be less those estimated from 16S rDNA gene data; and (v) counts of *E. coli* would correlate with *uidA* gene concentrations. All five hypotheses were broadly confirmed using log-transformed data, although no absolute conclusions could be

made because of the highly scattered viable count data (**Fig. S8, S9** and **Table S4**). The gene data was clearly more consistent across samples, which will be used in most subsequent numerical analysis.

3.2. Analysis of gene patterns with clustering and ordination

3.2.1. Gene distribution based on water column samples

Preliminary analysis indicated that the Musi can be divided into three stretches: upstream, city, and downstream. In terms of water column data, hierarchical clustering and ordination were employed to objectively verify AMR and water quality patterns before testing for statistical significance. In the dry season, city locations formed a distinct cluster, whereas upstream and downstream locations clustered together (**Fig. 3A**). Conversely, in the wet season, city samples split with late-city sites remaining a distinct cluster, but early-city sites clustered with downstream samples - the upstream site remained distinct (**Fig. 3B**). This in-city split suggests the city contains two sub-stretches in the wet season, which differs from the dry season. PCA results confirmed these patterns, showing a tight cluster of in-city sites in the dry season and more spread and mixed patterns in the wet season (**Fig. 3 C-F**). All seven ARG vectors pointed in similar directions. It should be noted that **Fig. 3** was based on UPGMA clustering with Euclidean distance. Ward's method and Bray-Curtis dissimilarity analysis gave similar results, confirming the robustness of the results (see Supplementary File "**Musi_analysis.Rmd**").


A
Log10(ARGs): UPGMA, Euclidean distance
Water, Dry Season
Height
B
Log10(ARGs): UPGMA, Euclidean distance
Water, Wet Season
Height
C
PCA biplot of ARGs: Water Dry Season
(Scaling 1: Sites proportional to eigenvalues)
sul2
aph3.Ib
NDM
ermF
qnrS
tetW
CTX.M
PC2 (1%)
PC1 (98%)
D
PCA biplot of ARGs: Water Dry Season
(Scaling 2: ARGs proportional to eigenvalues)
PC2 (1%)
PC1 (98%)
E
PCA biplot of ARGs: Water Wet Season
(Scaling 1: Sites proportional to eigenvalues)
CTX.M
tetW
NDM
qnrS
ermF
sul2
aph3.Ib
PC2 (1%)
PC1 (98%)
F
PCA biplot of ARGs: Water Wet Season
(Scaling 2: ARGs proportional to eigenvalues)
CTX.M
ermF
PC2 (1%)
PC1 (98%)

357

Fig. 3. Hierarchical clustering and ordination of water samples comparing dry and wet seasons based on Log_{10} transformed absolute abundances of seven ARGs. Sampling locations are numbered along the river: upstream (1), city (2-8) and downstream (9-10). **(A-B)** Hierarchical clustering (UPGMA) of Euclidean distances shows the separation of the city from up- and downstream samples in the dry season, whereas only downstream city sites cluster in the wet season. **(C-F)** PCA biplots for dry **(C-D)** versus wet **(E-F)** seasons.

3.2.2. Relationship between water and sediment samples

Before making comparisons, the water and sediment ARG concentration data were scaled to reduce differences due to different denominator units (see R notebook for scaling details): *i.e.*, gene copies per mL were used for water samples and gene copies per mg of organic matter (OM) was used for sediments. OM was employed instead of dry mass because the fraction of inorganic particles such as sand, which differs across locations, contributes to dry mass but is inaccessible to bacteria and their genes (Czekalski et al. 2014).

Scaled data show distinct clusters of genes in sediment and water samples in the wet season but not in the dry season (**Fig. 4**). This suggests that higher river flows in the wet season disrupt water-sediment connections, delocalising AMR in sediments under high flow conditions. In the dry season, genes in city water samples remain distinct, whereas up- and downstream water samples cluster with sediment gene levels, and inner-city sediment samples represent a separate group. Gene levels in inner-city sediment samples formed distinct clusters in both seasons. For water column samples, inner-city genes clustered only in the wet season, whereas samples from across the city clustered in the dry season. This pattern indicates persistent AMR pollution year-round in central Hyderabad.

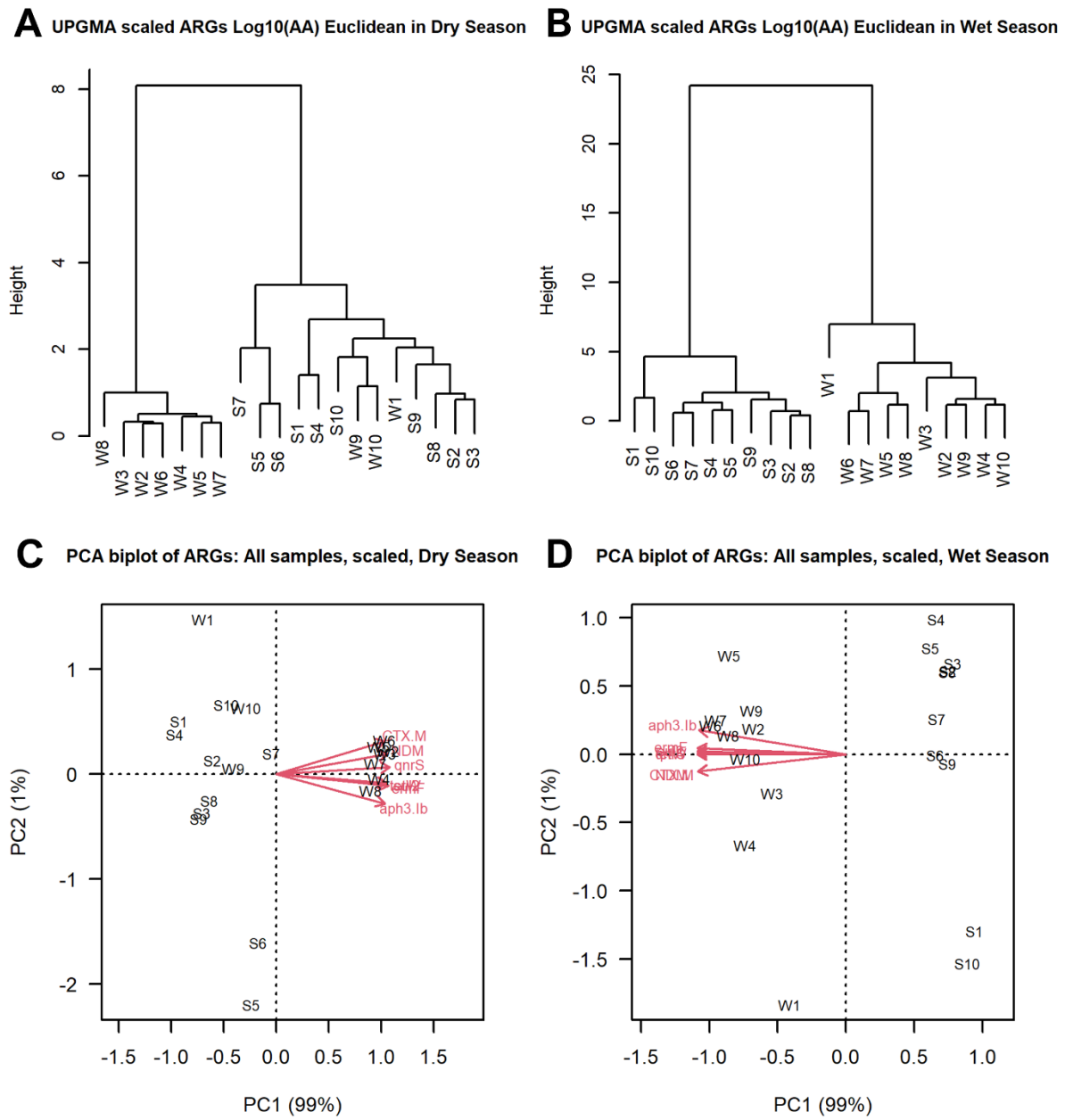


Fig. 4. Clustering of sediment and water samples along the Musi in the wet and dry seasons. Sampling locations are numbered along the river: upstream (1), city (2-8) and downstream (9-10), while W represents water and S represents sediment samples. This joint analysis of water and sediment samples is based on Log_{10}-transformed absolute abundances of ARGs. Since water and sediment ARG concentrations have a different basis, data were scaled to make them comparable. (**A-B**) Joint hierarchical clustering of the Euclidean distances between ARGs, either in the dry (**A**) or wet season (**B**). (**C-D**) PCA biplots, either in the dry (**C**) or wet (**D**) season. These biplots used Scaling 2, which correctly depicts genes (vectors).

3.3. Stretches and seasons were significantly different

Gene, plate count, and water quality (WQ) data imply that Musi has three stretches: upstream, city, and downstream. Clustering and ordination identified patterns but did not demonstrate statistical significance. Since the sampling design was not balanced (one upstream, seven city, and two downstream sites), and variables were neither independent nor evenly distributed, PERMANOVA (permutational MANOVA using adonis2 from the vegan package) was used to statistically compare differences between "season" and "stretches" for all measured parameters.

Stretch and season were significantly different (p-values <0.05) for most variables in the water samples, but comparisons among sediment samples were less evident (**Table S5**). For water samples, the stretch was significant for all genes, six of seven WQ parameters (not nitrite), and all plate counts, *i.e.*, 19 of 20 parameters (counts on "resistance" plates had too many NAs (not available) to analyse). The season was significant for nine out of 10 genes (not *tetW*), five of seven WQ parameters (not DO nor nitrite) and all plate counts, *i.e.*, 17 of 20 variables. For the sediment samples, stretch only was significant for five of the 10 genes (*intI1*, *aph(3")-Ib, ermF*, *qnrS*, *tetW*), the sum of ARGs, two of seven of the WQ parameters (TN and Conductivity) and two of the three plate counts, *i.e.*, nine of 20 parameters. Season only was significant for sediment samples for two of the 10 genes (*uidA*, $bla_{\text{CTX-M}}$), six of seven WQ parameters, and all plate counts. Further interpretations of these results and connections to the LDA are provided in Section 3.4 (see **SI text 2.3.**). Statistics are provided in **Table S5**.

3.4. Water quality parameters as proxies for microbial distributions and AMR

Water quality parameters were measured in all water column samples, and some parameters were measured in sediment samples (see **SI text 2.3**., **Figs. S10-S12** for details). In brief, indicators of wastewater pollution, such as lower DO and higher nitrogen, are less evident upstream, very evident in the city and less evident downstream, especially in the dry season. This pattern is similar to the 16S and other gene data, implying wastewater inputs reduce water quality in the city and are strongly associated with elevated bacterial abundances and AMR. Specifically, wastewater inputs are decomposed by river microbes, removing oxygen and releasing ammonia. However, lower DO levels in the river in the city and subsequent downstream reoxygenation drive nitrogen cycling, which makes individual nitrogen species less reliable indicators of wastewater pollution than total nitrogen (TN), which statistical analysis confirms (see **SI text 2.3.** and **Table S5** for details).

To compare water quality, microbial distributions in the river, and AMR prevalence, Linear Discriminant Analysis (LDA) was performed after data standardisation (see SI for details). Ott et al. (2021) had suggested DO and ammonia-nitrogen are potentially good proxies for wastewater pollution and, in turn, AMR in rivers, and we wanted to assess whether this was the case in the Musi and refine the analysis using LDA. Our LDA, which included seven standardised water quality parameters (WQ7), clearly discriminated between stretches and seasons (**Fig. 5A**). However, to develop the simplest model, parameters were removed from the full model one parameter at a time (since there were 127 possible combinations of the seven parameters).

Nitrate, ammonium, and nitrite were found to be collinear due to their connection in the nitrogen cycle (although ammonium did distinguish between seasons); therefore, TN was retained to reflect nitrogen conditions. This yielded a model with four variables (WQ4: pH, DO, COD, TN). This simpler model had reduced accuracy (**Fig. 5B**), but it separated the stretches well and is logical in terms of river water chemistry and microbiology. In a second stage of simplification, parameters were then removed from the WQ4 model, ultimately finding that DO alone was a sensible proxy for water conditions, although PERMANOVA showed that DO alone could not significantly distinguish between seasons (only stretches). As such, WQ4 parameters were added back to the DO model and it was found that DO with TN could discriminate stretches better than the other options, albeit not as good as the WQ4 model (**Fig. 5C**).

To test the DO+TN model, we re-examined the results from Ott et al. (2021) on the River Skudai in Malaysia, which suggested DO and ammonia as good proxies for AMR pollution. Their DO+ammonia model provided similar results to our DO+TN model, which proved better with the Musi data (**Fig. S13**). Overall, LDA shows that a simple model based on water quality data can discriminate between wastewater polluted sites or not, and thus, ARG prevalence, at least in the Musi. In reality, Ott et al. (2021) and our study agrees that ambient DO is a good proxy for AMR. However, their versus our recommendation for a nitrogen proxy differ. Ott et al. (2021) chose ammonia as the better proxy for AMR, but they did not analyse TN, whereas work suggests that TN is a better and more general proxy for AMR, which is supported by Musi data.

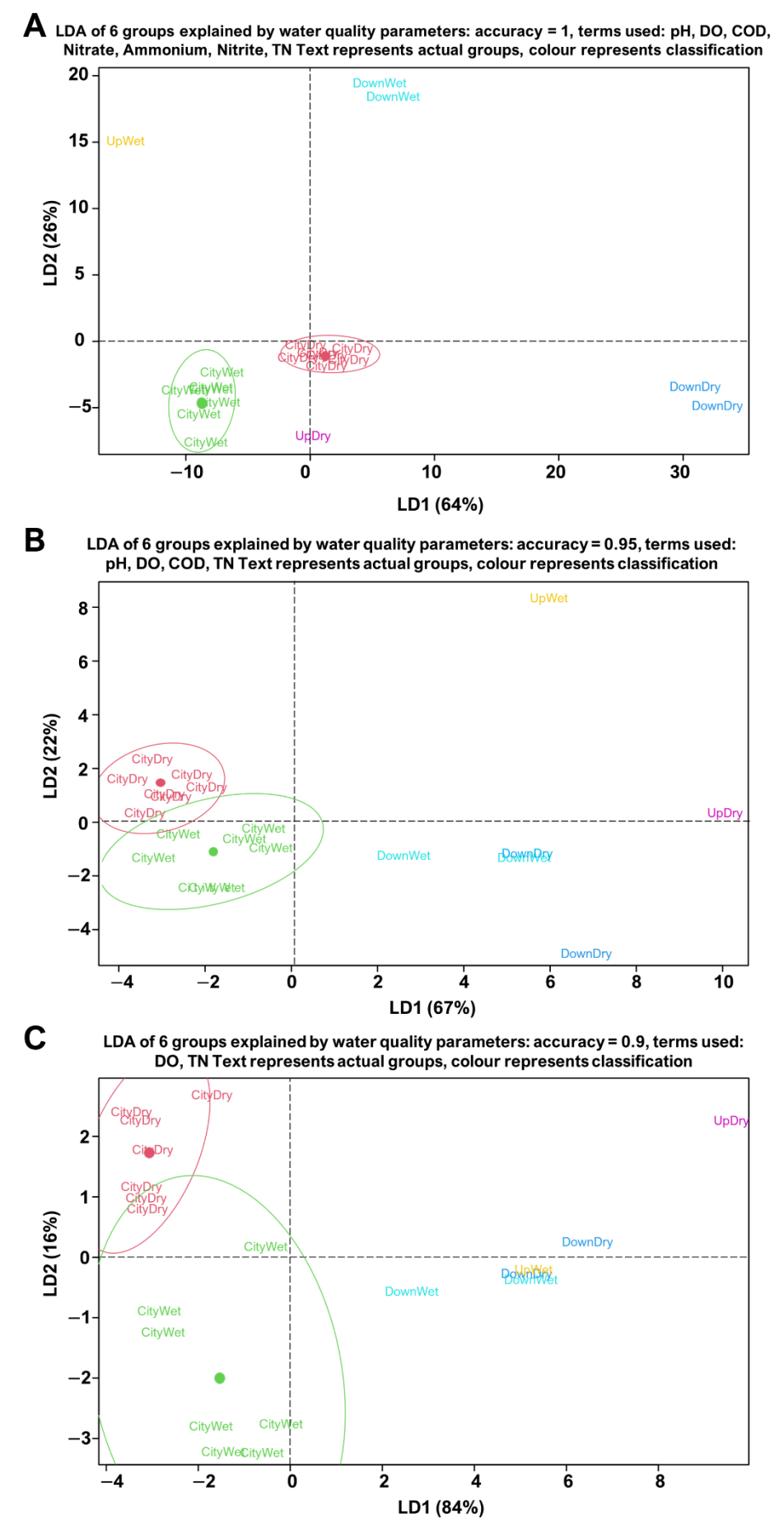

A
LDA of 6 groups explained by water quality parameters: accuracy = 1, terms used: pH, DO, COD, Nitrate, Ammonium, Nitrite, TN Text represents actual groups, colour represents classification
LD2 (26%)
LD1 (64%)
DownWet
UpWet
CityDry
CityWet
DownDry
UpDry
B
LDA of 6 groups explained by water quality parameters: accuracy = 0.95, terms used: pH, DO, COD, TN Text represents actual groups, colour represents classification
LD2 (22%)
LD1 (67%)
UpWet
CityDry
CityWet
UpDry
DownWet
DownDry
C
LDA of 6 groups explained by water quality parameters: accuracy = 0.9, terms used: DO, TN Text represents actual groups, colour represents classification
LD2 (16%)
LD1 (84%)
CityDry
UpDry
CityWet
DownDry
DownWet

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Fig. 5. Linear discriminant analysis (LDA) showing that the river stretches identified by clustering and ordination of ARGs can be discriminated by environmental characteristics in both seasons. Text labels combine actual stretch (Up, City, Down) and season (Dry, Wet) while colours indicate the LDA classifications of the sites, which are mostly consistent (accuracy of classification 90%-100%). Ellipses show 95% confidence intervals. **(A)** Discrimination using the full set of seven independent water quality parameters (WQ7, seven more were measured but were colinear). **(B)** Discrimination without the N-cycling ammonium, nitrite and nitrate (WQ4). **(C)** Discrimination with only DO and TN as water quality parameters (DO plus TN).

3.5. Explaining why TN is a good proxy for AMR in rivers like the Musi River

Water quality in the Musi is relatively good upstream of Hyderabad, but wastewater inputs from the city drive the river DO down, and TN rises before quality conditions improve downstream. This pattern is pronounced in the dry season and becomes less evident in the wet season because higher flows of re-entrainment sediments that blur the local impact of wastewater inputs, similar to Knapp et al. (2012) in the Almendares River in Cuba. In general, wastewater releases into a river increase organic carbon, nitrogen, and bacterial loadings, impacting water quality, bacterial abundances, and in turn, AMR. However, this does not explain why TN is a better proxy than ammonia for wastewater-related AMR for flagging “hot spots” in environmental monitoring.

Organic carbon and nitrogen in releases into a river deplete oxygen levels and release ammonia in wastewater. However, as DO drops, denitrification degrades residual nitrite and nitrate when DO is low, but as DO increases downstream due to reduced inputs, denitrification stops and nitrification occurs, causing transient increases in nitrite and nitrate farther downstream (*e.g.*, site 9). As such, ammonia, nitrite, and nitrate levels rise and fall along the river based on changes in DO, making TN a better general proxy for N-related pollution in a river and, in turn, AMR spread, as suggested by the LDA. In Ott et al. (2021), the river’s DO was consistently high, reducing N-cycling and making wastewater ammonia a decent proxy. However, when DO varies like in the Musi, TN is a better general proxy for wastewater pollution and AMR in more rivers.

3.6. Hydraulic modelling, mass balances, and mass loadings of wastewater pollution in the Musi River

Clustering and ordination analysis divided the Musi into three stretches based on water quality AMR conditions. PERMANOVA analysis confirmed that these patterns were significantly different between the stretches, and then LDA showed that DO and TN levels distinguish between stretches and AMR prevalence in the river. To model AMR conditions for predictive purposes, 25 point-sources of raw wastewater, treated wastewater, and other inputs to the river, as well as mass balances, were performed. Point-source discharges were estimated for modelling based on "sewershed" population sizes and per capita wastewater production rates in Hyderabad. A hypothetical, inert tracer model was then used to represent untreated (100% tracer) and treated wastewater (16%), respectively, and loadings were calculated using mass balances and discharge estimates. The tracer level in treated wastewater was set to 16% because water quality data indicated that treated wastewater content, on average, about 16% of raw wastewater COD content, which was consistent with VS (12%), 16S rDNA (19%), and total ARG (22%) data. Details of point sources, sampling sites, discharge, tracer, and load calculations can be found in Supplementary File **Tracer.xlsx**. Measurements of WWTP influent and effluent are in Supplementary File **WWTP_influent_effluent_data.xlsx**.

Flow, tracer concentrations, and load calculations are provided in **Figure 6**, which indicates that about 60 to 80% of flowing water in the Musi in the city functionally is raw wastewater (mostly municipal). This input is also evident when one compares the upstream and first city sampling points, after which untreated wastewater dominates conditions in the river, implicitly including AMR. It should be noted that WWTP points, *i.e.*, with pollutant inputs reduced to 16%, result in local drops in pollutant levels, indicating that WWTPs broadly improve local water quality. This is in stark contrast to countries where the bulk of the wastewater is treated before entering rivers, so WWTPs pollute the rivers rather than making them cleaner as with the Musi (Brown et al. 2019; Lee et al. 2021; Kirschner et al. 2024).

Comparing the tracer predictions with the untransformed absolute abundances of the 16S rDNA gene (**Figure 6A**) indicates that bacterial levels (which mirror ARGs and other variables) spiked when the tracer concentration also spiked, but then the 16S broadly declined downstream of inputs. This decline is likely due to microbial die-off, which was not included in the tracer mass balance calculations, as die-off rates are not known and differ due to various factors. Die-off will be included in future models. Regardless, untreated municipal wastewater releases dominate conditions in the river, including bacterial abundance and AMR levels. Interestingly,

however, if one considers the river catchment at a system level, the data imply that the Musi River itself acts as a "pseudo-WWTP" for site wastewater, reducing carbon levels by about 59% of the city's wastewater (see Supplementary File **Tracer.xlsx**). A similar phenomenon was seen in community sewer lines in a European study on the fate of AMR at urban catchment scales (Li et al. 2021).

These mass balance calculations show the importance of considering mass loading levels (*i.e.*, mass transferred per time) into and along a river rather than just concentrations, which is key to developing practical solutions to problems like AMR spread in rivers. Current "WHO Guidance on waste and wastewater management in pharmaceutical manufacturing with emphasis on antibiotic production" uses point-source concentrations in the guidance, assuming a fixed dilution factor of 10 after entry into a river. Such an approach does not consider the impact of volumetric flow rates and mass load inputs in absolute terms and is much less effective from an environmental protection perspective. The current approach allows polluters to reduce point source concentrations through dilution without reducing the actual mass load of pollutants into a receiving river. Such a loophole can be addressed by also stipulating load limits in environmental standards, especially when considering AMR genes and bacteria released into the environment.

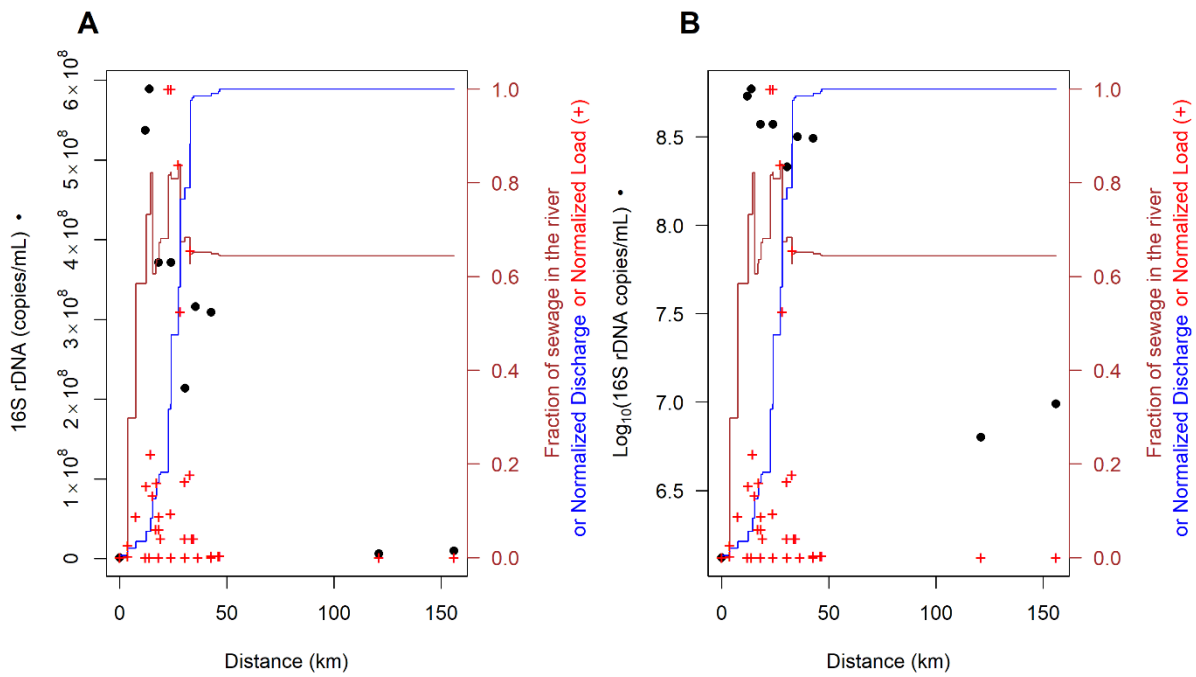

Fig. 6. Direct comparison of the absolute abundance of the 16S gene in the river with the estimated wastewater fraction. The tracer concentration in raw wastewater (brown stairs) was set to 1 to track wastewater loads. It was assumed to be inert in contrast to 16S rDNA levels (•), which declined along the river. **(A)** Comparing the actual concentration of the 16S gene (not log-transformed) with the wastewater fraction being the assumed main source of the 16S gene-carrying bacteria. **(B)** Comparing Log_{10} transformed 16S concentrations with the tracer concentrations as most figures are showing log-transformed data, visualising the relationship between untransformed and transformed data.

3.7. How important are antimicrobial manufacturing wastewater releases to AMR in the Musi River?

India has over 400 rivers and approximately 2.4 million other freshwater bodies (Rathinavelu et al. 2024), and the questions are 1) how typical is the Musi of Indian rivers and 2) does manufacturing waste inputs alter AMR in the river? In terms of the first question, rapid urbanisation in India has greatly outpaced the development of new WWTPs and supporting sewerage infrastructure. Specifically, the "National Inventory of Sewage Treatment Plants" of India (CPCB 2021) indicated that only 28% of municipal wastewater is treated nationwide and only 17% in compliant WWTPs. In Hyderabad, about 41% of wastewater is treated (see Supplementary File **Tracer.xlsx**), which makes Hyderabad better than average in terms of

wastewater treatment, although almost 60% of municipal waste releases are untreated, making the Musi fairly typical of Indian rivers.

Relative to the second question, a unique aspect of the Hyderabad sewershed is that it has extensive antimicrobial manufacturing and processing operations. which discharge wastewater into the local sewer drains. Hyderabad has been historically infamous for pollution from manufacturing since a report (Larsson et al. 2007) found ciprofloxacin concentrations in surface waters near the city up to 31 mg/L. Cipro concentrations were subsequently found as high as 5.5 mg/L within the city during the dry season (Gothwal and Shashidhar 2017). However, these reported concentrations were recorded before the construction of two new wastewater treatment plants for the antimicrobial manufacturing cluster, PETL and JETL, which appear to have consequentially reduced the manufacturing contribution to AMR in the catchment.

Treated effluents from the PETL and JETL discharge into a sewer line that feeds into the Amberpet STP, the largest municipal WWTP in Telangana State, and the industrial effluents are further diluted before reaching the Musi River. Recent work has shown much lower concentrations of antimicrobials in surface waters in the region (Konda et al. (2024, 2022), compiled in **Table S6**). For example, detected cipro levels were much lower than previous observations, *i.e.*, only 19.3 µg/L in local sewer drains and 7.28 µg/L in local industrial effluents and much lower in the river. Our modelling supports the positive impact of the PETL and JETL WWTPs, indicating only ~4% of original volumes from the facilities actually reach the river, and these calculations did not consider treatment and other losses along the sewer flow path (see Supplementary File **Tracer.xlsx**).

Therefore, the manufacturing waste inputs and impacts on the Musi River appear to be small relative to municipal inputs at a catchment scale. This does not mean such facilities have zero influence on AMR in Indian rivers. Marathe et al. (2017), Kumar et al. (2020b), and Lamba et al. (2020) detected ARGs, such as those coding resistance to aminoglycoside (*aph(3”)-lb*), macrolide (*ermF*), fluoroquinolone (*qnrS*), and sulfonamide (*sul2*) antibiotics, which were found in the Musi River, possibly related to local manufacturing near Hyderabad (Javvadi and Mohan 2023). However, untreated municipal wastewater from the city containing antimicrobials (**Fig. S1**) dominates AMR in the Musi River.

3.8. Study limitations

Although EMBRACE-WATERS guidelines for studying AMR in wastewater and aquatic environments were used (Hassoun-Kheir et al. 2021), several limitations existed in this study,

including sampling and analytical challenges. For example, although most field sampling was conducted from bridges, which were preferable during the wet season because heavy rainfall made bankside sampling hazardous, some sampling was actually performed at the riverbank on consecutive days due to the distance between the sites. Resource constraints also limited sampling to a single instance during both the dry and wet seasons, precluding a more extensive temporal analysis.

The study faced three analytical challenges. First, discrepancies between water and sediment samples arose due to different denominators for developing equivalent concentrations. To mitigate this, ARG concentrations were scaled in water samples to align with sediment concentrations, although some discrepancies persisted. Second, variability in sediment compositions, ranging from mineral-rich (sandy) to organic-rich samples, complicated comparisons between samples. We addressed this by normalising concentrations to the organic fraction (organic matter) of the sediments. This approach, while not ideal, was deemed the best available option. Further research across diverse sediment types is necessary to refine this method for future updates of the EMBRACE-WATERS guidelines.

Finally, correlations between viable bacterial counts and gene copy numbers from qPCR were poor. The discrepancy was likely due to differences in the sample preparation methods: larger water volumes (~200 mL) for qPCR versus diluted 100 µL samples for viable counts, potentially affected by inconsistent bacterial detachment from particles during sample homogenisation. This highlighted the need for methodological improvements and standardisation to enable reliable data aggregation across studies.

4. Conclusions and future directions

Here, we showed that AMR bacteria and genes in the Musi River are primarily sourced from untreated municipal wastewater released into the river through Hyderabad. By combining ARG abundance, plate count, water quality data, hydraulic modelling, and waste loading and mass balance calculations, the relative contributions of different waste sources to AMR levels were quantified in the river. Loading and mass balance calculations showed that up to 60 to 80% of the total Musi River flow in the city was associated with untreated municipal wastewater inputs. The percentage decreases downstream due to natural cleansing processes once the wastewater inputs into the river decline. Importantly, the influence of pharmaceutical manufacturing and processing waste inputs within the sewer catchment had minimal impact on *in situ* AMR, including both ARGs and ARBs. Given that Hyderabad has greater than average WWTP coverage than other cities in India, it is probable that sources and conditions observed in the Musi are common in India and other places with insufficient sewerage infrastructure and wastewater treatment relative to population sizes for their municipal wastes.

On a technical level, our LDA showed that selected water quality parameters could serve as useful proxies for wastewater pollution and, in turn, ARBs, ARGs, and probably pathogens in the river. Specifically, lower DO and higher TN levels are closely associated with elevated *in situ* AMR. DO plus TN serves as a good proxy model to predict locations where elevated ARGs and ARBs are likely prevalent. They are especially valuable as proxies because they can be analysed along a river to identify locations with a greater chance of AMR-related pollution, reducing the cost of surveillance, *i.e.*, they can triage AMR potential in rivers. However, work is needed in other rivers to verify the predictive capability of these parameters, especially coupled field-based methods, such as DO test strips, to empower citizens to monitor and identify local AMR "hot spots" in their own catchment.

Finally, this work highlights the value of simple hydrological and hydraulic modelling in predicting pollution loads, dilution factors, and concentrations in receiving rivers based on publicly available data. This approach helps identify areas of greatest AMR exposure risk as well as locations of greatest risk of spread, using mass loading data rather than concentration data in field assessments. Our methodology provides a framework for structuring and parameterising exposure pathways in future risk assessments, which are critical to better understanding, preventing, and prioritising mitigation action against AMR in places like the Musi River.

The AMRflows consortium includes

Panagiota Adamou, Shubham Anurag, Ewelina Bien, Sangeetha Chandrakalabai Jambu, David W Graham, Colin Harwood, Siu Fung Stanley Ho, Rupert Hough, Chaitanya Janivara Chandregowda, Kelly Jobling, Arun Kashyap, Jan-Ulrich Kreft, Soumendra Nath Kuiry, Joshua Larsen, Thara Methale Velakkath Paramba, Ankit Modi, Pranab Kumar Mohapatra, Anantha Barathi Muthukrishnan, Indumathi Manivannan Nambi, Julián Osvaldo Ovis Sánchez, Rebeca Pallarés Vega, Ravikrishna Raghunathan, Sasikaladevi Rathinavelu, Aravind Kumar Rengan, Wahidullah Hakim Safi, Shubhangi Sanjeev, Sai Sugitha Sasidharan, Vandit Kumar Shah, Vikas Sonkar, Rupali Srivastava, Shashidhar Thatikonda, Mads Troldborg, Cansu Uluşeker, Rama Vaidyanathan, Willem van Schaik, Anjali Vasudevan, Kiruthika Eswari Velmaiel, Arathy Viswanathan.

CRediT authorship contribution statement

Vikas Sonkar: Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Validation, Visualization, Writing – original draft, review & editing. **Arun Kashyap:** Conceptualization, Methodology, Data curation, Investigation. **Rebeca Pallarés-Vega:** Conceptualization, Methodology, Investigation, Formal analysis, Validation, Writing – review & editing. **Sai Sugitha Sasidharan:** Methodology, Data curation, Investigation. **Ankit Modi:** Methodology, Investigation, Data curation, Formal analysis. **Cansu Uluseker:** Methodology, Investigation, Data curation. **Sangeetha Chandrakalabai Jambu:** Conceptualization, Writing – review & editing, Project administration. **Pranab Kumar Mohapatra:** Conceptualization, Formal analysis, Writing – review & editing, Supervision, Funding acquisition, Project administration. **Joshua Larsen:** Conceptualization, Formal analysis, Writing – review & editing, Supervision, Funding acquisition. **David Graham:** Conceptualization, Formal analysis, Writing – original draft, review & editing, Funding acquisition. **Shashidhar Thatikonda:** Conceptualization, Investigation, Methodology, Writing – review & editing, Supervision, Funding acquisition, Project administration. **Jan-Ulrich Kreft:** Conceptualization, Formal Analysis, Writing – original draft, review & editing, Supervision, Funding acquisition, Project administration.

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.

Data availability

All data and their statistical analysis are made available as part of supplementary materials and files.

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