Environmental Microbiome

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Antimicrobial resistance transmission in the environmental settings through traditional and UV-enabled advanced wastewater treatment plants: a metagenomic insight

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Abstract

Background Municipal wastewater treatment plants (WWTPs) are pivotal reservoirs for antibiotic-resistance genes (ARGs) and antibiotic-resistant bacteria (ARB). Selective pressures from antibiotic residues, co-selection by heavy metals, and conducive environments sustain ARGs, fostering the emergence of ARB. While advancements in WWTP technology have enhanced the removal of inorganic and organic pollutants, assessing ARG and ARB content in treated water remains a gap. This metagenomic study meticulously examines the filtration efficiency of two distinct WWTPs-conventional (WWTPC) and advanced (WWTPA), operating on the same influent characteristics and located at Aligarh, India.

Results The dominance of Proteobacteria or Pseudomonadota, characterized the samples from both WWTPs and carried most ARGs. *Acinetobacter johnsonii*, a prevailing species, exhibited a diminishing trend with wastewater treatment, yet its persistence and association with antibiotic resistance underscore its adaptive resilience. The total ARG count was reduced in effluents, from 58 ARGs, representing 14 distinct classes of antibiotics in the influent to 46 and 21 in the effluents of WWTPC and WWTPA respectively. However, an overall surge in abundance, particularly influenced by genes such as *qacL*, $bla_{OXA-900}$, and *rsmA* was observed. Numerous clinically significant ARGs, including those against aminoglycosides (*AAC(6')-Ib9*, *APH(3")-Ib*, *APH(6)-Id*), macrolides (*EreD*, *mphE*, *mphF*, *mphG*, *mphN*, *msrE*), lincosamide (*lnuG*), sulfonamides (*sul1*, *sul2*), and beta-lactamases (bla_{NDM-1}), persisted across both conventional and advanced treatment processes. The prevalence of mobile genetic elements and virulence factors in the effluents possess a high risk for ARG dissemination.

Conclusions Advanced technologies are essential for effective ARG and ARB removal. A multidisciplinary approach focused on investigating the intricate association between ARGs, microbiome dynamics, MGEs, and VFs is required to identify robust indicators for filtration efficacy, contributing to optimized WWTP operations and combating ARG proliferation across sectors.

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Keywords Antibiotic resistance, Municipal wastewater treatment plants, Antibiotic resistance genes, Bacteria, UV disinfection

Background

In recent years, antimicrobials, particularly antibiotics, have attracted significant attention due to their large-scale increase in consumption resulting in a severe impact on the environment [1]. Antimicrobial resistance (AMR) is growing progressively worse, with the predicted annual death toll of approximately 10 million people by the year 2050 [2]. It is reported that AMR has caused 1.27 million deaths in the world in the year 2019 alone, compared to a model scenario where all infections were susceptible to treatment [3]. Understanding the epidemiology underlying the emergence of AMR at global and local levels, its selection and transmission, and individual ARGs (Antibiotic Resistance genes) are essential for developing sustainable strategies to combat this global threat.

One of the major sources or reservoirs for ARGs and antibiotic resistant bacteria (ARB) are the municipal wastewater treatment plants (WWTPs). These are becoming hotspots for the emergence of ARGs and ARB [4]. WWTPs are potential reservoirs where the initial mobilization of resistance genes occurs [5]. The injudicious consumption of antibiotics is common, but there is a lacuna in understanding the final fate of these antibiotics. The human body digests antibiotics partially, and approximately 30-90% of residues are excreted, depending on the class of antibiotics consumed [6]. Animals absorb approximately 25% of antibiotics; the remaining 75% are excreted [7]. Now, the question arises: where do these remnants go, and how do they contaminate the environment? These antibiotic residues ultimately reach WWTPs and exert selective pressure, facilitating ARG proliferation. WWTPs foster optimal conditions, including temperature, pH, nutrients, and, consequently, a conducive environment for the growth and proliferation of bacteria [7–9]. WWTPs are an important interlink for AMR dissemination between the human population and the environment. For surveillance, WWTPs are a cost-effective matrix to survey entire cities for the fluctuating ARGs [11].

WWTPs have advanced technologically but do not warrant the absolute removal of bacteria and ARGs [12, 13]. The prevalence of mobile genetic elements such as Class I integron facilitates the horizontal gene transfer and indicates the abundance of multi-drug resistant (MDR) strains [12]. The frequently reported ARGs which persist in samples at all stages of WWTPs are sulfonamide resistance genes (*sul1*, *sul2*), tetracycline resistance genes (*tetA*, *tetC*, *tetM*, *tetO*, *tetQ*, *tetX*, *tetW*, *tetG*), *qacEdelta1*, aminoglycosidases (*aadA* and *strB*), beta-lactamases (*blaOXA*, *blaTEM*), and MLSB (macrolide-lincosamide-streptogramin B) resistance genes (*ermB*, *ermF*) owing to their extensive administration, capability to survive in variable environments and efficient resistance mechanisms [12–15]. Above mentioned studies have reported increased ARGs against ciprofloxacin, ampicillin, cefoperazone, sulfamethoxazole, and tetracycline in downstream samples. Horizontal gene transmission by MGEs, such as transposase (*tnpA*), integrase (*intI1*), and insertion sequences (*ISAba3* and *ISPps*) prevalent in microorganisms at each sampling sites in WWTPs worsen the calamity [13].

In this study, the primary objective is to establish a comparative assessment of the ARG and ARB removal efficiency at various stages of two distinct types of WWTPs. The aim was to assess the alterations in microbiome diversity, their co-association with ARGs, abundance of virulence factors, and the role of mobile genetic elements in AMR dissemination at different stages of treatment processes used in this study. These WWTPs cater to approximately 0.05 million population including hospital wastewater. The first one is a Conventional Wastewater Treatment Plant (WWTPC), and the other one is an Advanced Wastewater Treatment Plant (WWTPA), both located at the same place in Aligarh, India. WWTPs are the reservoir for ARG and ARB dissemination. With inadequate standard measures, microorganisms carried at each sampling sites in WWTPs can possibly disseminate drug-resistant bacterial infections to the exposed environment. WWTPs primarily focus on removing inorganic pollutants not on ARG removal. This study aims at understanding the impact of technological advancement in treatment technologies such as UV, UASB and solar-powered oxidation, on filtering ARG and ARB.

Methods

Sample site selection

For this study, two WWTPs situated within the campus of the Aligarh Muslim University, Aligarh, India (27°55'18.5"N 78°03'38.7"E) were selected. Both the WWTPs are receiving the same influent quality from the institution which has a residential population of about 55,000. However, the approaches to degrade the organic matter and removal of pollutants of these treatment processes are different. One of them is based on a conventional process that is trickling filter technology whereas other is an integration of anaerobic digestion followed by a nature-based solution 'wetlands technology'. The conventional WWTP uses attached media system where a

biofilm grows as wastewater passes through in a vertical movement once sprinkled from the top that operates at an HRT (Hydraulic Retention Time) of 8.5 h on an average flow. The later one consists of an anaerobic digester, i.e., Upflow Anaerobic Sludge Blanket (UASB) with HRT of 10.5 h as a primary treatment followed by vertical and horizontal subsurface flow constructed wetlands system commonly known and falls under the category of 'nature-based solution (NbS)', and finally, disinfection units that are based on UV and solar-driven anodic oxidation (AO) processes. Both the wastewater treatment plants are operated on a continuous flow, under steady state condition, and thus maintains an average HRTs as per their respective design. The sampling of influent and effluent from these wastewater treatment plants was done when these were on a continuous mode of operations. The physicochemical parameters of wastewater were noted.

Sample collection, DNA extraction, library preparation, and sequencing

A total of seven samples were collected, including influent ($n = 1$) and effluents at different stages of the WWTPC ($n = 3$) and WWTPA ($n = 3$) (Table S1). From the main sewage-water pipeline receiving influents from every other pipeline from the AMU campus, multiple samples were collected from adjacent points in sterile bottles and pooled into one sample of untreated sewage water. The same protocol was followed in collecting samples at each stage of treatment in the WWTPC and WWTPA. Samples were transported to the laboratory on ice for DNA extraction. The samples were homogenized using a vortex; 50 mL was collected, centrifuged at 7000 x g for ten minutes at 4 °C to sediment the cell pellet, and stored at -20 °C until DNA extraction.

DNA was extracted using the DNeasy PowerSoil Kit (Qiagen). The quality of the extracted DNA was checked using NanoDrop™ 2000 Spectrophotometer (Thermo-Fisher Scientific). Sample DNA concentrations were quantified using Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific) and Qubit 3.0 Fluorometer (Thermo Fisher Scientific). Starting with 100 nanograms of intact DNA, enzymatic fragmentation was done using Covaris to achieve fragment sizes ranging from 200 to 300 base pairs [17]. The ensuing end-repair process involved converting overhangs resulting from fragmentation into blunt ends. This entailed the removal of 3' overhangs through the 3' to 5' exonuclease activity, complemented by polymerase activity to fill in the 5' overhangs. The blunt-ended fragments underwent adenylation, adding a single 'A' nucleotide to the 3' ends. Adenylated fragments were ligated with adapters, and subsequent cleavage was carried out using the uracil-specific excision reagent (USER) enzyme. The DNA underwent further purification through the utilization of AMPure beads.

After enzymatic processes, the DNA underwent polymerase chain reaction (PCR) with six cycles, utilizing NEBNext Ultra II Q5 master mix, Illumina universal primer, and sample-specific octamer primers for amplification. Post-amplification, AMPure beads were used to clean the DNA. The final DNA library was eluted in 15 µL of 0.1X Tris EDTA buffer.

For quantification purposes, 1 µL of the library was subjected to analysis using the QUBIT 3 Fluorometer with dS DNA HS reagent. Fragment analysis was performed using an Agilent DNA 7500 chip on the Agilent 2100 Bioanalyzer, loading 1 µL of the library. The DNA was subsequently sequenced on the Illumina HiSeq 4000, employing a 2 × 150 bp paired-end run.

Metagenome assembly and genomic annotation

The demultiplexing of sequence reads by barcode was done with bcl2fastq v2.1.9. Assessment of sequence data quality involved the utilization of FastQC v0.11.9 and MultiQC v1.9, evaluating parameters such as base call quality distribution, the percentage of bases above Q20 and Q30, %GC content, and the presence of sequencing adapter contamination (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The raw reads were cleaned and filtered using fastp v0.23.2 [18].

For microbiome composition and contig-based ARG analyses, the clean reads underwent assembly using MEGAHIT v1.2.9 with specific parameters, namely –k-min 35, –k-max 141, and –k-step 28 [19]. Contigs shorter than 200 bp were excluded from subsequent analysis to enhance data reliability. The assembly quality was further validated using Bowtie2 v2.1.0 [20]. The contigs were annotated by PROKKA (Prokaryotic Genome Annotation) v1.12 [21].

Identification of microbiome abundance and diversity

The microbiome composition and taxonomic diversity in the filtered reads were classified by Kraken2 using the standard database of bacteria consisting of RefSeq complete bacterial genomes/proteins. The assembled reads were also classified to correlate ARGs and microbiome [22].

Identification of ARGs and calculation of ARG abundance

The ARGs were identified using Resistance Gene Identifier (RGI) v6.0.1 by aligning against the latest Comprehensive Antibiotic Resistance Database (CARD) [23]. The criteria for ARGs were ≥ 90% identity and ≥ 90% coverage against the reference. The abundance of ARGs was calculated in terms of RPKM (Reads Per Kilobase Million).

Identification of plasmid-mediated ARGs

The plasmid-mediated and chromosomally mediated ARGs were distinguished by PlasFlow v.1.1 and PlasClass,

which determined the plasmid origin of individual contigs. A cut-off of 0.7 was set for identifying a contig of plasmid origin. Abricate v1.0.1 identified the replicon types of plasmids against the PlasmidFinder database.

Mobile genetic elements (MGEs)

The mobileOG-db annotated and classified MGEs as plasmids, phages, integrative, transposable, and conjugative elements [24].

Virulence factors

The virulence factors were detected using Abricate v1.0.1 (https://github.com/tseemann/abricate) using the Virule nce Factor Database (VFDB), with the cut-off being ≥ 80% identity and ≥ 70% coverage.

ARG-microbiome co-association

The ARG-taxa correlation was identified by calculating Spearman's rank correlation at a cut-off value of 0.8 and further represented by Cytoscape v.3.9.1.

Metagenome-assembled genomes

To construct metagenomic assembled genomes (MAGs), we employed MEGAHIT v1.2.9 with a specified minimum contig length of 1000 [19]. Following assembly, Bowtie2 v2.5.1 was used for read mapping back to their respective assemblies, and SAMtools v1.16 facilitated the conversion of reads to BAM format [20, 25]. Metagenomic binning was executed through MetaBAT2 v1.7, incorporating specific parameters like --minContigLength 1500, generating 15 bins [26]. Quality assessments for Metagenome-Assembled Genomes (MAGs) were performed using CheckM v1.0.18, taking into account completion (> 90% for high-quality bins) and contamination (< 5% for high-quality bins), with similar criteria for medium and low-quality bins [27]. 76 MAGs failing to meet the specified criteria (contamination > 10%) were excluded from subsequent analysis. Taxonomic assignments for the MAGs were determined using gtdb-tk v.2.2.6 [28]. For the identification of known ARGs, MAGs underwent screening by aligning predicted open reading frames to CARD v. 3.0.7 using diamond blastp, with specific parameters such as -e value 1e-10 and --id 90 [23].

Statistical analyses

All the statistical analyses, i.e., alpha and beta diversity, were calculated in R Studio v. 2021.09.0.

Results

Sampling from WWTPs

The influent for both the WWTPs was common, receiving wastewater from the entire campus of Aligarh Muslim University (AMU, Aligarh, India), a residential campus with more than 30,000 students living in hostels, more than 1,500 teaching faculty members, and around 7000 non-teaching staff members living with their families in and around the campus. The university also has a tertiary care hospital catering to the needs of people living in the Aligarh district and adjoining areas. The campus has a state-of-the-art sewage-water pipeline network that takes all wastewater from the hostels, the hospital, and the residential quarters to the WWTPs. The traditional WWTPC processes the wastewater to release it into the nearby agricultural fields. AMU also has a WWTPA that treats this wastewater and makes it drinkable by the local human and animal populations. WWTP-1 is the influent treated with grit removal sluice gate. WWTP-2 corresponds to effluents of primary clarifier, WWTP-3 is the effluent of trickling filter, and WWTP-4 is the effluent of aeration, and secondary clarifier (Fig. S1).

At the first stage of wastewater treatment in traditional WWTPC, large and medium-sized solid waste is removed. Sequentially, the suspended solids and organic matter linked to the suspended solids are removed at the primary clarifier. The trickling filter, consists of a fixed-bed biological reactor working under aerobic conditions. In the trickling filter process, wastewater is evenly distributed over a bed of porous media made up of rocks, known as the trickling filter. This bed provides a surface for microorganisms to colonize. The microbial activity transforms the pollutants into simpler, less harmful substances. Throughout this process, the organic content of the water is significantly reduced. A secondary clarifier removes organic matter, such as nitrogen and phosphorus, from the water. WWTP-5, 6, and 7 were the influents from different stages of potable WWTP. WWTP-5 is the effluent of primary treatment by UASB, WWTP-6 is discharged after treatment from vertical flow constructed wetlands and horizontal flow constructed wetlands, and WWTP-7 is potable water used for irrigating the nearby fields after disinfection and removal of pathogens by solar-powered anodic oxidation and UV reactors [29] (Table 1, Fig. S1).

Pseudomonadota predominant at each stage of WWTPs

The sequenced raw reads ranged from 17 to 30 million, with bacteria comprising 75% of the total reads (Table S1). The microbiome composition and abundance at each taxa level were comparable for each sampling sites at WWTPC and WWTPA (Fig. 1 and Fig. S2 and S3). We didn't witness any remarkable variation at different treatment stages at WWTPC or WWTPA, except at WWTP-5, where certain abrupt fluctuations were observed. The alpha diversity was comparable across samples except WWTP-4 and 5 where a slight variation was observed (Table S1 and Fig. S4b). Compared to influent, no variation in alpha-diversity was observed in the effluent of WWTPC and WWTPA. The

Table 1 The average concentration for the physiochemical parameters of wastewater collected from: a) conventional wastewater treatment (WWTPC)

a) Conventional wastewater treatment (WWTPC)

Parameters ⇒ Location ⇓	**BOD_5 mg/l**	**COD mg/l**	**TSS mg/l**	**TN mg/l**	**TP mg/l**	**FC MPN/100ml**
Raw Sewage	170	350	300	32	12	10^7
Trickling Filters	20	70	50	18	7	10^4

b) Advanced wastewater treatment plant (WWTPA)

Parameters ⇒ Location ⇓	**BOD_5** mg/l	**COD** mg/l	**TSS** mg/l	**TN** mg/l	**TP** mg/l	**FC** MPN/100ml
Raw Sewage	170	350	300	32	12	10^7
UASB	100	190	150	28	10	10^6
Constructed Wetlands	1	8	Nil	Nil	Nil	10^4
Solar Driven Disinfection Units (AO&UV)	Nil	5	Nil	Nil	Nil	<50

top members comprising each level of taxonomy were common. Pseudomonadota, formerly known as Proteobacteria, decisively prevailed at each stage of WWTPs (Fig. 1). A substantial fluctuation was noted exclusively at WWTP-4, wherein Pseudomonadota decreased from 85.5% in the influent (WWTP-1) to 55.4% in the effluent (WWTP-4). In contrast to WWTPC, Pseudomonadota exhibited an elevation in the effluent of WWTPA relative to the influent. Both Bacteriodota and Bacillota increased from WWTP-1 to WWTP-4 in WWTPC. In WWTP-5, Bacillota and Bacteriodota content was higher than the influent, i.e., WWTP-1 but at further stages of treatment, i.e., in WWTP-6 and WWTP-7, their abundance reduced. The class Gammaproteobacteria predominated in all samples, but its relative abundance reduced from two-thirds to half in the effluent of

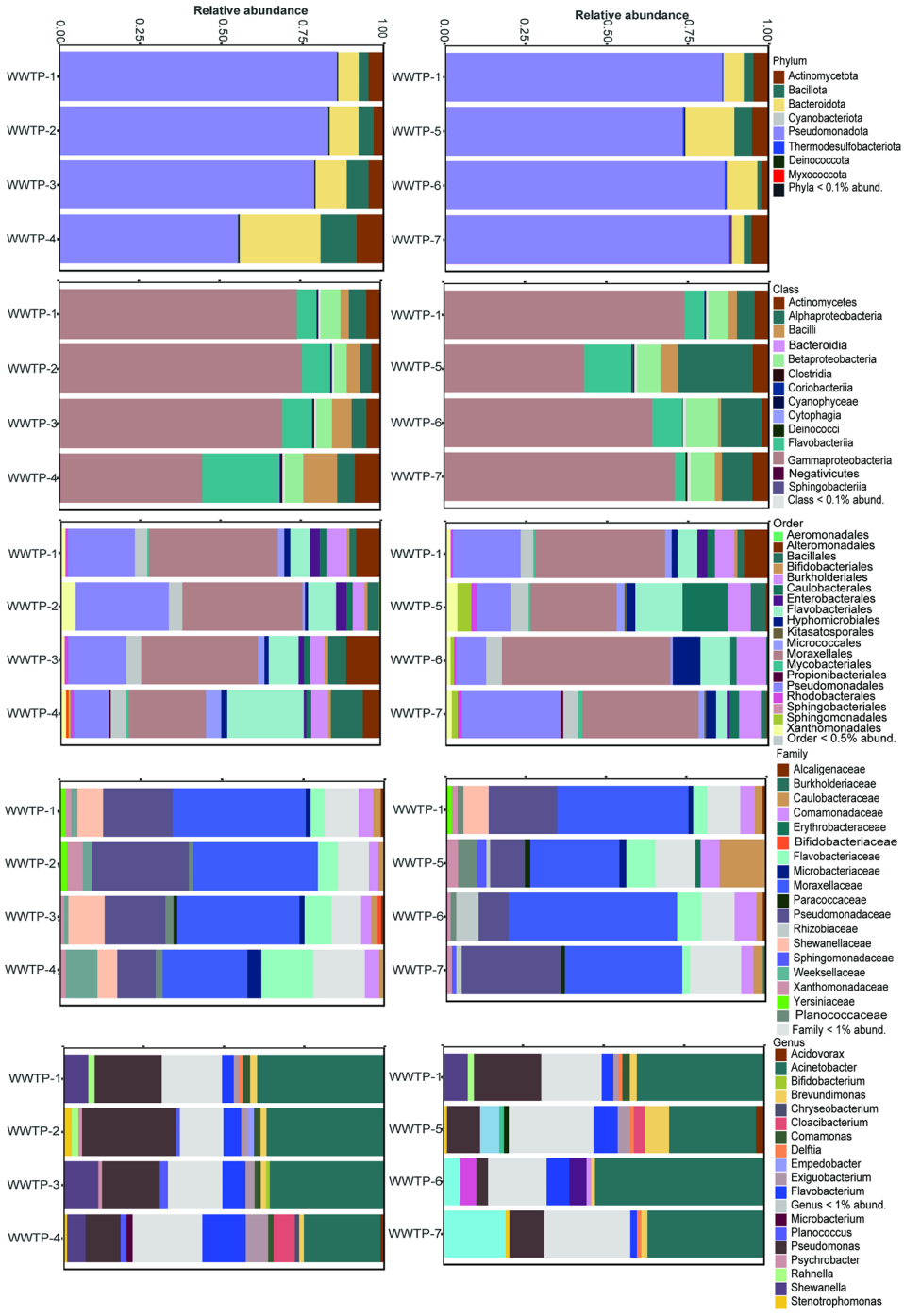

Fig. 1 Microbiome abundance in WWTPC and WWTPA. Relative abundance of microbiome at different taxonomic levels: Phylum, Class, Order, Family and Genus

WWTPC. Although it declined at WWTP-5, it subsequently increased, comprising two-thirds of the effluent (WWTP-7) bacterial composition. Flavobacteriia increased with successive treatment stages in WWTPC but, except for WWTP-5, decreased in the effluent from WWTPA. At WWTP-5, Alphaproteobacteria exhibited a sharp increase from 5 to 23%, later decreasing in the effluent but remaining double the amount detected in the influent. The order Moraxellales exhibited the highest abundance, followed by Pseudomonadales and Flavobacteriales, with Flavobacteriales showing higher abundance at WWTP-4 and WWTP-5. Predominant families, including Moraxellaceae, Shewanellaceae, and Flavobacteriaceae reduced in the effluent from both WWTPC and

WWTPA. Pseudomonadaceae decreased in the effluent of WWTPC but constituted a larger portion in the effluent of WWTPA. *Acinetobacter*, followed by *Pseudomonas*, *Shewanella*, and *Flavobacterium* dominated each stage. Among the most abundant genera in the collected samples, approximately all the genera except Flavobacterium reduced from influent to effluent in both WWTPs. Flavobacterium increased with treatment at WWTPC (Fig. 1). *Acinetobacter johnsonii* predominated at each stage of treatment but comprised half the composition in WWTP-7. The most abundant species across all samples, such as *A. johnosonii*, *Pseudomonas fragi* and *Acinetobacter lwoffii* decreased from influent to the final effluent at both WWTPC and WWTPA (Figure S2 and S3).

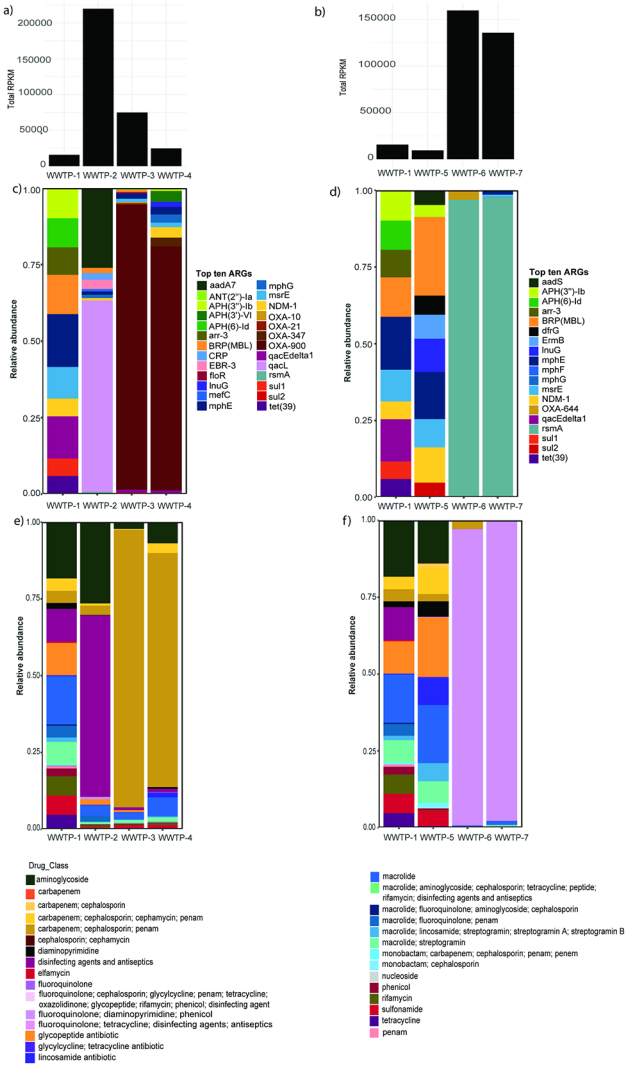

Fig. 2 ARGs abundance in WWTPC and WWTPA. (**a**) Total abundance of ARGs (RPKM) at different treatment stages of WWTPC. (**b**) Total abundance of ARGs (RPKM) at different treatment stages of WWTPA. (**c**) Top ten ARGs at different treatment stages of WWTPC. (**d**) Top ten ARGs at different treatment stages of WWTPA. (**e**) The total abundance of ARGs against the drug classes in WWTPC. (**f**) The total abundance of ARGs against the drug classes in WWTPA. The drug classes and their corresponding ARGs are represented by the colour gradient

Antimicrobial resistance in WWTP

In the comprehensive analysis of ARGs across various stages of wastewater treatment, a total of 115 ARGs were detected (Table S1). Among these, 58 ARGs, representing 14 distinct classes of antibiotics, were identified in the influent. Notably, this count exhibited a reduction in the effluents of WWTPC with 46 ARGs and further decreased to 21 in the effluents of WWTPA. Despite an overall decline in the total number of ARGs during successive treatment stages, a remarkable surge was observed after the treatment at the primary clarifier, specifically at WWTP-2. The total abundance of ARGs (RPKM) exhibited an exponential increase at WWTP-2 and WWTP-3 (Fig. 2a). At both WWTPC and WWTPA, the total abundance of ARGs in effluent was exceptionally high. Although there was a reduction at WWTP-5, a subsequent stage led to another surge (Fig. 2b). The manifold increment in total ARG abundance was attributed to specific ARGs such as *qacL* (disinfecting agents and antiseptics resistance) and *aadA7* (aminoglycosidase) constituting 84% of the total abundance at WWTP-2, $bla_{\mathrm{OXA-900}}$ (beta-lactamase) representing 90%, and 73% of total RPKM in WWTP-3 and WWTP-4 respectively, and *rsmA* (fluoroquinolone resistance) contributing to 97% of total ARG abundance in WWTP-6 and WWTP-7 (Fig. 2c and f). In summary, WWTPA demonstrated comparatively higher efficiency in filtering out ARGs. Notably, the alpha-diversity of ARGs also reduced considerably more in WWTPA (Fig. 3c). On the basis of ARG distribution (in terms of RPKM), the final stages of wastewater treatment shared higher similarities at both WWTPC (WWTP-3 and 4) and WWTPA (WWTP-5 and 6) (Figure 2a and b). The total abundance of ARGs did not follow a linear trend of increment or reduction, primarily due to the disproportionately high abundance of specific ARGs.

The core ARGs which weren't filtered at any stage of treatment included ARGs against aminoglycoside (*AAC(6')-Ib9*, *APH(3")-Ib*, *APH(6)-Id*), macrolide (*EreD*, *mphE*, *mphF*, *mphG*, *mphN*, *msrE*), lincosamide (*lnuG*), and sulfonamide (*sul1*, *sul2*) (Fig. 3a). $bla_{\mathrm{NDM-1}}$ was persistent at each stage except WWTP-6. Several ARGs such as beta-lactamases $bla_{\mathrm{NDM-43}}$, and bla_{OXA} variants ($bla_{\mathrm{OXA-114c}}$, $bla_{\mathrm{OXA-140}}$, $bla_{\mathrm{OXA-21}}$, $bla_{\mathrm{OXA-246}}$, $bla_{\mathrm{OXA-282}}$, $bla_{\mathrm{OXA-283}}$, $bla_{\mathrm{OXA-284}}$, $bla_{\mathrm{OXA-347}}$, $bla_{\mathrm{OXA-373}}$, $bla_{\mathrm{OXA-496}}$, $bla_{\mathrm{OXA-58}}$, $bla_{\mathrm{OXA-644}}$, $bla_{\mathrm{OXA-646}}$, $bla_{\mathrm{OXA-900}}$, and $bla_{\mathrm{OXA-915}}$) were absent in the influent but emerged at later stages of treatment (Fig. 3b). Among the top ten ARGs, aminoglycosidases, beta-lactamases, carbapenemases, macrolide, sulfonamide, and tetracycline

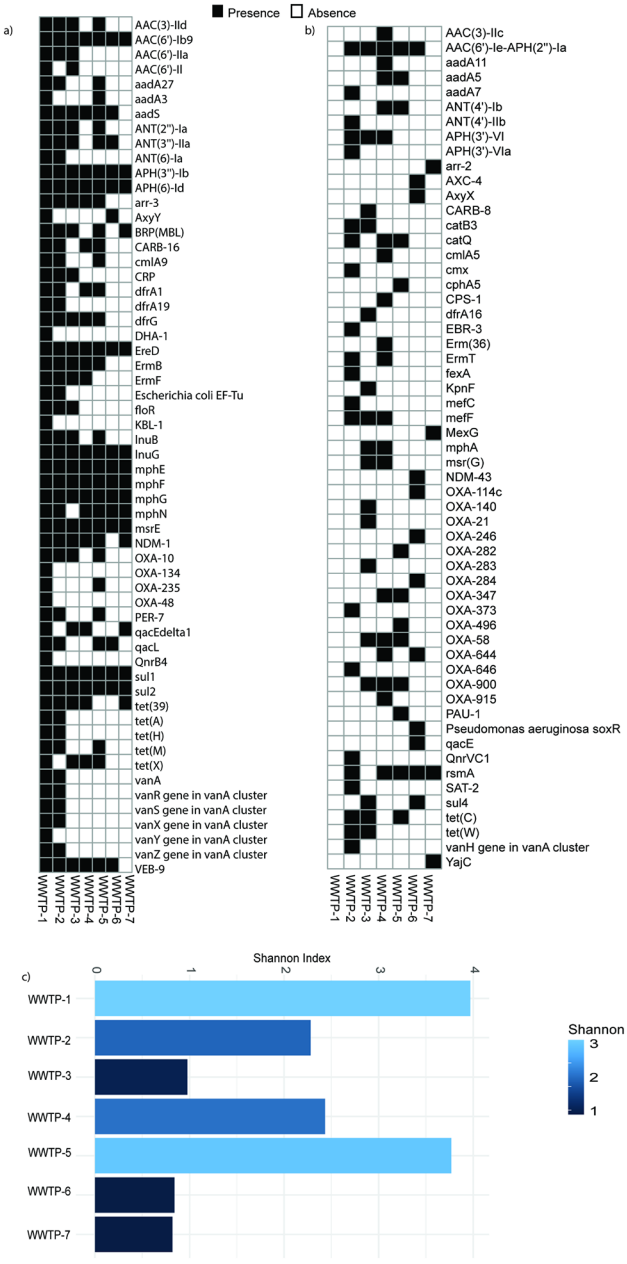

Fig. 3 Presence-absence heatmap and alpha-diversity of ARGs. (**a**) The fate of ARGs detected in the influent across the WWTPC and WWTPA treatment. (**b**) The ARGs absent in influent but emerging at later stages of treatment. (**c**) The alpha-diversity of ARGs represented by Shannon index

resistance genes were prevalent (Fig. 2). The principal mechanisms conferring antibiotic resistance were predominantly associated with antibiotic inactivation, succeeded by antibiotic target replacement and antibiotic efflux among most ARGs. These findings underscore the significance of these specific resistance mechanisms in contributing to the overall antibiotic resistance profile. On the basis of prevalence of ARGs against different drug classes, the final stages (WWTP-3 and WWTP-4, WWTP-6 and WWTP-7) showed remarkable similarities (Figs. 2 and 3 and S4a).

Plasmid-mediated ARGs and MGEs

ARGs were predominantly plasmid-mediated at all stages of WWTP. Approximately 3/4th of the ARGs were carried by plasmids. In compliance with the microbiome composition, Pseudomonadota carried most of the ARGs (Table S1). The plasmid replicons were identified as IncFIA(HI1), ColRNAI, IncP(6), IncQ1, IncQ2, repA_2_pKPC-2, Col(MG828), repUS12__rep(pUB110), rep22_1_repB(pUB110). The most prevalent IS elements were IS_Pl3_3, ISPpu12, ISplu7D_orfA, and ISl2 (Table S1). The overall count of MGEs, encompassing bacteriophage fragments, insertion sequences, integrative elements, and plasmids, demonstrated a more pronounced reduction in WWTPA than WWTPC. This observation suggests that WWTPA exhibits a higher efficacy in mitigating the presence of diverse MGEs than WWTPC.

Virulence factors

We have identified 103 virulence factors in the influent, mainly encoding flagellar proteins, twitching motility proteins, alginate biosynthetic proteins, and type III and VI secretion systems (Table S1). The majority of these virulence factors originated from *Pseudomonas aeruginosa.* In the subsequent stages of wastewater treatment, the count increased to 163 in the effluent at WWTP-2, decreased to 77 in sample WWTP-3, and further reduced to 43 in sample WWTP-4. Similarly, at WWTPA, there was an increment in the total number of virulence factors observed in the effluents at WWTP-5 (117), which was reduced at WWTP-6 (68) and WWTP-7 (57). Despite variations in the abundance of virulence factors, the constituents remain consistent.

ARG-microbiome correlation

The non-random co-occurrence of ARG and taxa is a plausible indicator for host information. We detected a significant Spearman's rank correlation (Spearman's $r = 0.8$ to 1 and $p > 0.05$) between the microbiome and ARG diversity (Fig. 4). The network analysis elucidated co-occurrence patterns between ARG subtypes and bacterial taxa. We detected 27 unique species as possible hosts for the top ten ARGs, of which 20 were Proteobacteria, 4 were Firmicutes, and 3 were Bacteroidetes. *Acinetobacter spp.* (*Acinetobacter baumannii*, *Acinetobacter calcoaceticus/baumannii complex*, *Acinetobacter indicus*, *Acinetobacter lwoffii*, *Acinetobacter radioresistens*, *Acinetobacter sp. NEB149* and *Acinetobacter variabilis*) were the most probable host for maximum ARGs in the top ten list.

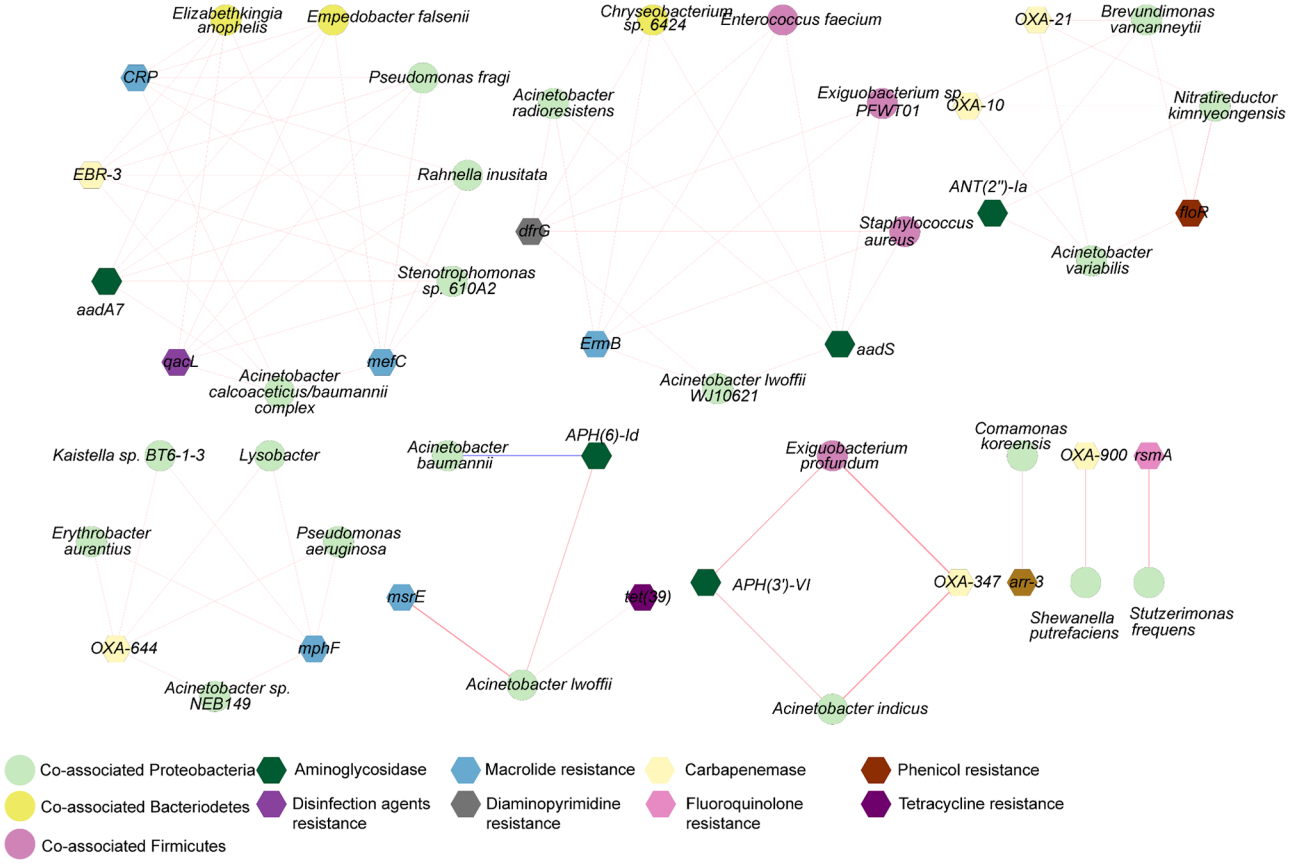

Fig. 4 ARG-taxa co-association. The co-association of ARGs, represented by different drug classes with the abundance of microbiome. The spheres represent microbiome, coloured by the phylum they belong to. Hexagon represents ARGs depicted by different colours according to their drug classes

Metagenome-assembled genomes

To identify the taxonomy of putative ARG hosts, metagenome-assembled genomes (MAGs) were constructed. A total of 115 MAGs (13 high quality, 26 medium-quality, and 76 low-quality) were recovered (Fig. 5a). Of these 115 MAGs, 57 had a sufficient signal for gtdb-tk to predict genus level taxonomic assignments. While acknowledging that including low quality bins is likely to incur errors, we used them to produce a more comprehensive list of potential hosts. The most commonly recovered genera were *Flavobacterium* (9/57), *Commamonas* (7/57), and *Leucobacter* (7/57). Of the 41 unique genera identified, 20 were unique to just 1 MAG. Next, we screened the MAGs for known ARGs. We identified a total of 30 unique ARGs in 28 MAGs (including low-quality MAGs) (Fig. 5b) conferring resistance to twelve different drug classes, including macrolide ($n = 12$), cephalosporin ($n = 4$) and aminoglycosides ($n = 3$). These included $bla_{\text{OXA}-1}$, an extended spectrum beta-lactamase (ESBL) putatively carried in a *Leucobacter sp.* (bin71, medium quality).

Discussion

Municipal WWTPs are important reservoirs for harboring ARGs and ARB. The selective pressure exerted by antibiotic residues, co-selection by heavy metals, and conducive environment sustains ARGs and facilitates the emergence of ARB. The influent of a WWTP exclusively receiving hospital wastewater exhibits a markedly reduced richness and abundance of ARGs compared to WWTPs involved in municipal wastewater treatment [30]. With advancement in WWTP treatment technology, the sewage treatment has improved tremendously in terms of inorganic wastes, biological oxygen demand (BOD) and chemical oxygen demand (COD) but still, there is lacunae in assessing the ARG and ARB content in filtered water. In this study, we quantified the ARG and ARB removal efficiency of two types of WWTPs, a conventional plant (WWTPC) and an advanced treatment plant (WWTPA), by evaluating their capacity to reduce ARG and ARB concentrations in the effluent. Our results showed that WWTPC reduced the total number of ARGs by 20.7%, whereas WWTPA demonstrated a significantly higher reduction efficiency of 63.8% (Table S1). Additionally, the predominant ARB in the influent belonged to the Pseudomonadota phylum, and their abundance was

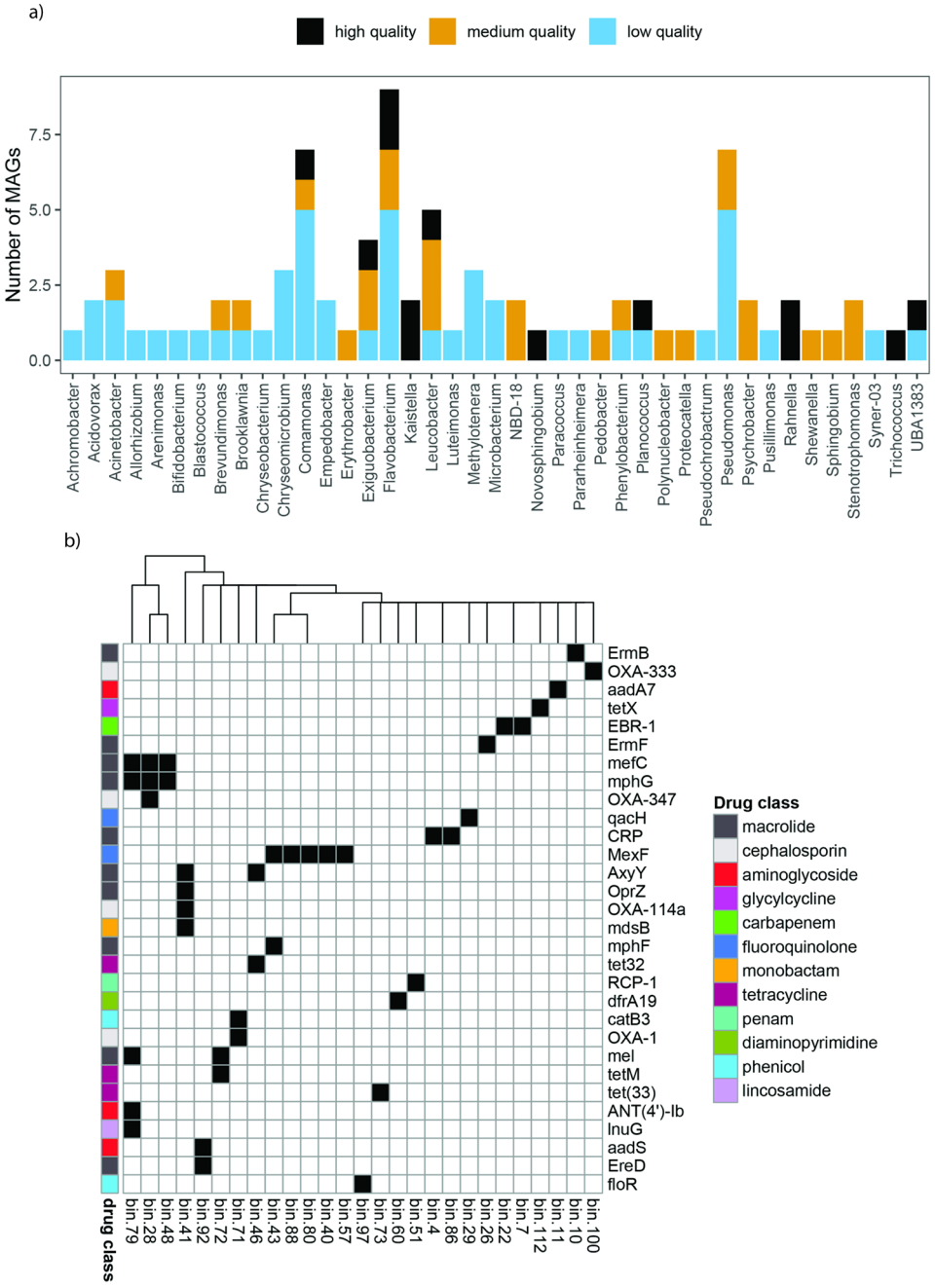

Fig. 5 Metagenome Assembled Genomes **a**) Number of MAGs identified in all the samples, black representing the high-quality ones. **b**) ARGs in MAG

notably reduced in the effluent of both WWTPs. Pseudomonadota or Proteobacteria typically dominates the microorganisms comprising the samples of WWTPs, and it was also the predominant phyla at each stage of treatment in the effluents of both WWTPC and WWTPA (Fig. 1 and Fig. S1, S2 and S3) [31]. Proteobacteria are recognized for their significant contribution to the metabolic capacity for breaking down organic pollutants in bioreactors. Proteobacteria are the major carriers of ARGs in WWTPs effluents which was also true to our findings (Fig. 4). *Acinetobacter johnsonii* was identified as the predominant species which reduced with wastewater treatment. It has been earlier reported as the species thriving in WWTPs of warm areas and in extreme conditions such as Antarctica [13, 32]. The efflux pumps aid the adaptation of *A. johnsonii* to the challenging environment [33]. *Acinetobacter* spp. is associated with nosocomial infections but *A. johnsonii* is relatively rare with limited studies pertaining to antibiotic resistant strains [34]. It is prevalent in wastewater, as reported in WWTP

at Košice in Slovakia and South Korea [31, 35]. Recently, *A. johnsonii* carrying bla_{NDM-1}, bla_{OXA-58} and bla_{PER-1} was reported as an emerging high-risk clone with great resemblance to the global sewage strains [34]. The presence of the opportunistic pathogen *Pseudomonas* in effluents suggests the potential for carrying infections. Comparable microbiome richness and diversity between influent and effluent imply the inefficiency of WWTPs in bacterial filtration.

Remarkably, while the total number of ARGs decreased in both WWTPC and WWTPA effluents, the overall abundance of ARGs surged (Fig. 2). This increase is notably attributed to the heightened prevalence of *qacL* in the effluent WWTP-2, $bla_{OXA-900}$ in the samples WWTP-3 and 4, and *rsmA* in the samples WWTP-6 and WWTP-7. In the influent, *Lysobacter sp. H23M47* hosted *qacL*, while in the sample WWTP-2, *Stenotrophomonas sp. 610A2* carried this gene. *Shewanella putrefaciens* harbored $bla_{OXA-900}$. The transmission of *rsmA* involved multiple hosts, including *Rheinheimera sp. MM224* in the effluent WWTP-5, *Pseudomonas phenolilytica* in the sample WWTP-6, and *Stutzerimonas frequens* and unclassified *Pseudomonas* in the sample WWTP-7. Although *rsmA* wasn't detected in the influent, its persistence highlights its ability to traverse the filtration process, including UV disinfection (Fig. 3). It's crucial to note that the ARG mobilization from diverse origin species seems intricately linked to the presence of Mobile Integron-Associated Site Elements (MISE) in the surrounding environment. Research underscores a pronounced prevalence of numerous MISEs in wastewater treatment plant influents and hospital effluents when compared to other environments. This heightened occurrence may be attributed to the preference for mobile genetic elements that harbor MISE, particularly those linked to a diverse array of mobile ARGs. Additionally, the greater diversity of Proteobacterial species in wastewaters, as opposed to the human gut, likely contributes to this trend, given that Proteobacterial species are recognized as being disproportionately involved in carrying MISE [36].

In this study, several ARGs of clinical concern such as ARGs against aminoglycoside (*AAC(6')-Ib9*, *APH(3")-Ib*, *APH(6)-Id*), macrolide (*EreD*, *mphE*, *mphF*, *mphG*, *mphN*, *msrE*), lincosamide (*lnuG*), sulfonamide (*sul1*, *sul2*) and beta-lactamase (bla_{NDM-1}) persisted through both conventional and advanced treatment processes (Figs. 2 and 3). This is in congruence with other studies reporting prevalence of these genes in the effluents of WWTPs in Europe, South Korea, Sri Lanka and Tokyo [13, 36–38]. The prevalence of *sul1* and *sul2* is an indicator of anthropogenic activity. The *sul1* gene is consistently found in the 3'-conserved segment of class 1 integrons, emphasizing its significance in the capture and expression of gene cassettes [40]. The clinical class 1 integron-integrase gene stands out as a promising indicator for monitoring both the abundance and removal of antibiotic resistance genes in an urban wastewater treatment plant [41]. The acquisition and dissemination of ARGs in WWTP is a complex process influenced by multiple interconnected factors. While it is commonly believed that antibiotic residues play a crucial role in driving AMR in WWTP, a European surveillance study contradicts this notion, revealing no statistically significant correlation between antibiotic residues and AMR [13]. In WWTP, where the microenvironment is continuously altering with each successive step, predominance of microbial species signifies their adaptability and genome plasticity. Biofilm-forming antibiotic-resistant bacteria in WWTP effluent are the primary accumulators of ARGs [42]. The choice of filter media such as biologically activate carbon (BAC) can reduce the biofilm formation [43].

The prevalence of insertion sequences, such as IS_Pl3_3, ISPpu12, ISplu7D, and ISl2, are implicated in the dissemination of ARGs (Table S1). These elements serve as mobile genetic components, facilitating the transfer of genetic material, including ARGs, among bacteria. Their inherent ability to move within and between bacterial genomes is a pivotal factor in the widespread distribution of antibiotic resistance determinants within microbial communities [44]. This mobility occurs through processes such as transposition, horizontal gene transfer, and recombination, fostering the transfer and dissemination of ARGs and contributing to the emergence of antibiotic-resistant bacterial strains. The abundance of virulence factors (VFs) originating from *Pseudomonas aeruginosa* observed at every stage, including the effluent, signifies the robust adaptability and fitness of these factors within WWTPs (Table S1) [45].

With advancement in wastewater treatment technologies, pathogens and ARGs are supposed to reduce efficiently. We observed more pronounced reduction in ARGs, MGEs by WWTPA involving UASB, solar-powered anodic oxidation and UV reactors but still, it didn't remove all the ARGs. A concerning fact was the increased abundance of ARGs in the effluent and no significant change in microbiome abundance and diversity. A probable cause for the failure of UV irradiation in WWTPs is its dose which is generally lower in WWTPs [14]. It is crucial to account for seasonal variables such as spatiotemporal fluctuations, water temperature, and precipitation as they can significantly affect the destiny of antibiotic ARGs within aquatic ecosystems [46]. A limitation of our study which require future improvement is the inclusion of multiple sampling events across different seasons to further enhance the robustness of the study by accounting for seasonal variability.

Effective containment of ARGs necessitates vigilant surveillance of WWTP effluents. This study emphasizes

the critical need for establishing a standardized cut-off to govern acceptable ARG frequencies in WWTP effluents, particularly concerning their utilization in agriculture and other applications. Concurrently, advancements in technologies tailored for the comprehensive removal of both ARGs and ARB are indispensable. To address this, a multidisciplinary approach is proposed, encompassing studies that intricately investigate the interplay between ARGs, microbiome dynamics, MGEs, and VFs. Such investigations aim to identify robust indicators, quantifying the efficacy of ARB and ARG filtration processes. The proposed framework advocates for a holistic understanding of ARG dissemination, offering insights into developing strategies for mitigating the environmental impact of antibiotic resistance. Implementation of these measures will contribute to the optimization of WWTP operations, advancing our ability to curtail the proliferation of ARGs and combat the rising threat of antibiotic resistance in various sectors.

Conclusions

WWTPs are hotspots for AMR dissemination and an important link between human and the immediate environment. It is imperative to build a proper risk-assessment system and a cutoff value for ARGs and ARB content in the effluents of WWTPs before discharging them to the environment. Most of the treated water from the WWTPs are discharged into water bodies. Many at times, this water is sourced to irrigate agricultural farms or used for drinking water supply. This scenario increases the risk of introducing AMR in the food chain and exposing the community at higher risk of catching MDR and XDR (extremely drug-resistant) infections. In contrast, the advance WWTPs as used in this study that consist of UASB, Wetlands, UV disinfection and solar-powered anodic oxidation does remove more ARGs than conventional WWTPs but yet, there remains a crucial need for a filtration system equipped with absolute removal or significant reduction in the persistent ARGs.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

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Author contributions

AT: Methodology, Data curation, Analysis, Writing- Original draft preparation, Visualization, Investigation. YB: Analysis. CLB: MAG analysis. AUK: Conceptualization, Funding acquisition, Supervision, Writing- Reviewing and Editing. NK: Funding acquisition, monitoring and operation of the WWTPA, DG: Methodology.

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Data availability

"Data is provided within the manuscript or supplementary information files". The raw sequencing data generated in this study has been deposited at Sequence Read Archive, NCBI-NIH, USA under BioProject PRJNA862634, with accession numbers SRR20788541, SRR20788540, SRR20788539, SRR20788538, SRR20788537, SRR20788536, and SRR20788535.

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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