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Assessment of Antimicrobial Resistance Genes and Pathobiome Diversity in Domestic Wastewater of a Tropical Country

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Abstract: Wastewater treatment plants (WWTPs) are recognised as key hotspots for the dissemination of antimicrobial-resistant bacteria and antimicrobial resistance genes (ARGs). However, studies addressing the role of domestic WWTPs in the spread of resistance traits in tropical regions remain limited. This study evaluated a domestic WWTP during dry and rainy seasons to examine its role as a reservoir and dissemination hub for ARGs and potential bacterial pathogens. The WWTP demonstrated stable physicochemical removal efficiencies, although its performance slightly decreased during the rainy season. Notably, the relative abundance of ARGs measured by qPCR was higher in the effluent than in the influent of the WWTP. Metagenomic analysis of activated sludge revealed that chromosomally encoded ARGs conferred resistance to macrolides, aminoglycosides, rifamycin, sulphonamides, and tetracyclines. In contrast, plasmid-associated ARGs were primarily linked to resistance against quaternary ammonium compounds (QACs), indicating the presence of a potential conjugative plasmid facilitating the mobility and persistence of QAC resistance genes within the microbial community. Furthermore, pathobiome analysis identified a high relative abundance of potential pathogens, including genera *Gordonia*, *Acidovorax*, *Pseudomonas*, and *Mycobacterium* members. These findings highlight the role of domestic WWTPs as reservoirs and potential amplifiers of antimicrobial resistance in tropical environments.

Keywords: antimicrobial resistance; horizontal gene transfer; plasmids; bacterial pathogens; removal efficiency

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1. Introduction

Antimicrobials revolutionised infection control, yet their widespread use has fuelled the emergence of resistance [1,2]. This issue is particularly pronounced in tropical regions, where elevated temperatures and weak regulatory enforcement may accelerate resistance dynamics [3,4]. Wastewater systems continuously receive antimicrobial residues and resistant bacteria from human and agricultural sources, making WWTPs key hotspots for ARGs and antimicrobial-resistant bacteria [5]. Within these plants, selective pressures drive the persistence and dissemination of resistance determinants through treated effluents, contributing to downstream environmental contamination [6]. For instance, fluctuating antimicrobial concentrations in sewage can enhance horizontal gene transfer, disrupt microbial communities, and promote ARG persistence in activated sludge [7].

Beyond their role in resistance dissemination, WWTPs provide favourable conditions for the survival of human pathogens such as *Arcobacter*, *Flavobacterium*, and *Aeromonas* [8]. These bacteria often carry ARGs, increasing the risk of releasing multidrug-resistant strains with pathogenic potential into the environment [9]. As a result, WWTPs contribute significantly to one of the most pressing global health challenges: infections caused by antimicrobial-resistant bacteria. Global analyses show that the burden of antimicrobial resistance is not evenly distributed. While antimicrobial resistance-related deaths are projected to rise from 4.71 million in 2021 to 8.22 million by 2050, low- and middle-income countries from tropical regions bear a disproportionate burden of antimicrobial resistance-related morbidity and mortality [10]. This geographic disparity underscores the need for region-specific studies to inform the implementation of targeted strategies.

Despite global concern over AMR, few studies have explored its dynamics in WWTPs under tropical climatic conditions [11]. Recent studies in Brazil, Thailand, and other tropical countries have highlighted unique ARG profiles and microbial communities in WWTPs compared to temperate systems, including the persistence of clinically relevant ARGs despite biological and disinfection treatment steps [11,12]. Warm temperatures (30–37 °C) in these regions can enhance horizontal gene transfer rates, further promoting ARG persistence [13]. These findings emphasise the need to investigate ARG prevalence, persistence, and transmission under such environmental conditions.

This study aims to evaluate the role of a domestic WWTP in Costa Rica as a reservoir and potential dissemination source for ARGs and pathogens under tropical environmental conditions. We analysed influent, effluent, and activated sludge to assess ARG removal efficiency, identify prevalent ARGs and pathogenic genera, and examine the genomic context of plasmid-borne *qac* genes linked to biocide resistance. These findings offer new insights into the persistence and dissemination of ARGs in tropical WWTPs, underscoring the challenges of mitigating antimicrobial resistance in such settings.

2. Materials and Methods

2.1. Wastewater Treatment Facilities

The studied WWTP is an activated sludge system at the Monserrat residential complex, a community of approximately 813 houses in Cartago, Costa Rica (9°55′25.7″ N, 84°00′17″ W). The plant operates using a sequential batch reactor configuration. First, raw wastewater passes through manually operated screens for preliminary treatment. Next, the water flows into a homogenisation tank, which regulates and stores the wastewater while each reactor completes its cycles. From there, the water is pumped into one of the four sequential batch reactors, each equipped with a bio-selector at the inlet, where a portion of sludge (6.44 g/L) is recirculated into the raw water. After treatment, the effluent is discharged into the María Aguilar River, while the excess sludge generated is directed to drying beds.

2.1.1. Sample Collection

A non-probabilistic composite sampling followed the guidelines established in the Standard Methods for the Examination of Water and Wastewater [14]. Three sampling events were performed for each season: the dry season (13 February, 19 February, 11 March 2024) and the rainy season (3 June, 10 June, 17 June 2024). Stratified sampling was carried out based on the time of day (8:00–10.30 a.m.) and the wastewater composition. For each sampling event, one homogenised biological sample was collected and analysed per sample type to represent the conditions corresponding to a specific hydraulic retention time. This approach ensured consistency and was appropriate given the system's operational stability and the study's focus on overall performance.

Approximately 1 L of influent wastewater was collected from the plant's homogenisation tank, 300 mL of sludge was sampled from the reaction tank during the aeration phase, and 1 L of effluent wastewater was collected at the tank's outlet. Polypropylene copolymer bottles and sterile plastic sampling bags were used to ensure sample integrity. The equipment was prepared and adapted according to the specific requirements for each analysis. Once collected, the samples were promptly transported to the Research and Services Centre for Chemistry and Microbiology and the Environmental Protection Research Centre, in the Technological Institute of Costa Rica, in a 40 L cooler below 10 °C and stored at (1–4) °C.

2.1.2. Physicochemical Characterisation

The wastewater samples' physicochemical characterisation was determined using either HACH® kits (HACH, Loveland, CO, USA) or Examination of Water and Wastewater methods. The parameters analysed included settleable solids, total suspended solids, chemical oxygen demand, nitrate (NO_3-N), ammonia (NH_3-N), phosphate (PO_4-P), and faecal coliforms. Settleable solids, total suspended solids, and faecal coliforms were analysed following Examination of Water and Wastewater methods 2540F, 2540D, and 9221B, respectively. The chemical oxygen demand and PO_4-P concentrations were measured using the HACH® kits with the 8178 and 8000 Reagent Solutions. Meanwhile, NO_3-N and NH_3–N concentrations were measured using HACH® kits with the 10,020 and 10,031 Test N'Tube TM Vials, respectively. NH_3-N results were converted and reported as NH_4-N.

2.2. Pretreatment of Wastewater Samples and DNA Extraction

The DNA extractions from the collected samples were conducted within 24 h of collection. S-Pak GSWG047S6 Millipore (Merck KGaA, Darmstadt, Germany) filters (0.45 µm) were used to filter 50 mL of the influent and effluent water samples, and the extraction was performed using the DNeasy® PowerWater kit from QIAGEN Group (Hilden, Germany). The QIAamp® PowerFecal® Pro-DNA kit from QIAGEN Group (Hilden, Germany) was used for the DNA extraction from 250 mg of sludge samples. After extraction, the recovered DNA was quantified using a NanoDrop 2000c microvolume spectrophotometer (ThermoFisher, Waltham, MA, USA) and stored at −20 °C in the Centre for Chemistry and Microbiology facilities. Afterwards, DNA extractions from samples collected during both study seasons were transported at temperatures below 10 °C to the Institute for Health Research at the University of Costa Rica, where they were stored at −80 °C for further analysis.

2.3. Detection and Quantification of ARGs

The ARGs selected for detection and quantification were prioritised based on their association with resistance to widely used antibiotic classes in clinical and environmental settings. These included tetracyclines (*tetA*), macrolides (*ermB*), sulphonamides (*sul1*, *sul2*, *dfrA*), and β-lactams (*blaTEM*). The inclusion of *qacE*/*qacE*Δ*1* and *intl1* further allows assessment of resistance to QACs and the presence of class 1 integrons, respectively, both of which are key indicators of anthropogenic pollution and horizontal gene transfer potential [15,16].

Quantitative PCR was performed using a StepOnePlus™ Real-Time PCR thermocycler (Thermo Fisher, Waltham, MA, USA) to quantify the relative abundance of target ARGs in all samples. Results were normalised by dividing the number of copies of each target gene by the number of copies of the *16sRNA* gene. Calibration curves were generated using a gBlock® (IDT®, Coralville, IA, USA) as a positive quantification control, with standard concentrations ranging from 10^6 to 10^1 copies per reaction. For the qPCR analyses, PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA, USA) was used according to the manufacturer's instructions with 5 µL of DNA from each sample and 5 µL of nuclease-free water for negative controls (three negative

controls per gene). Additionally, all qPCR reactions were set up in a dedicated clean area using filtered tips and sterile reagents to minimize the risk of cross-contamination. No amplification was observed in the controls across all assays. The PCR cycling consisted of an initial denaturation at 95 °C for 2 min, denaturation at 95 °C for 15 s, and annealing at 55 °C for 60 s. Details of primers are provided in Table S1.

2.4. Pathobiome Diversity Analyses

DNA extracts from three sludge samples per season (dry and rainy) were frozen and shipped to Macrogen Inc. (Seoul, Republic of Korea) for metagenomic shotgun sequencing. Library preparation and paired-end sequencing were performed using an Illumina NovaSeq6000 instrument (San Diego, CA, USA), obtaining approximately 2 × 2 GB of data per sample with a read length of 150 bp. Raw readings were processed for adapter detection, trimming, and quality filtering with the fastp tool [17] (v0.20.1) using default parameters (e-value of 25). Additionally, mapping to the human genome (hg19) was excluded using Bowtie2 [18] (v2.5.0) in very-sensitive-local mode.

High-quality readings were taxonomically classified with Kraken2 [19] (v2.1.2), applying a classification confidence threshold 0.5. Two separate databases were employed: one containing chromosomal sequences from the Genome Taxonomy DataBase r207 [20], and another containing only plasmid sequences available in the Genome Taxonomy DataBase r207. The readings were subsequently divided into two datasets: chromosomal readings (mapped to the chromosomal database) and plasmidic readings (mapped to the plasmidic database). Taxonomic reports generated by Kraken2 were processed with Bracken [21] (v2.8) to estimate the abundance of readings assigned to each taxon in both datasets.

2.5. ARG and Plasmid Sequences Identification

Filtered readings were co-assembled by season (dry and rainy) using MEGAHIT (v1.2.9) [22] with k-mer values of 33, 55, 77, 99, and 127. The resulting contigs were mapped to their respective filtered read files with Bowtie2 (v2.5.0), and only contigs longer than 500 bp were retained. Assembly quality was assessed with MetaQUAST [23] (v2.2). Contigs were further analysed with the PlasX pipeline (https://github.com/michaelkyu/PlasX, accessed on 21 January 2025) [24]. They were imported into Anvio [25] (v8.0) for coding sequences annotation with the COG [26] (v14) and Pfam [27] (v33.1) databases, followed by scoring using the PlasX tool. Contigs with scores above 0.5 were classified as plasmidic, and those below as chromosomal. We selected PlasX because this software has been shown to outperform other machine learning-based tools in plasmid identification [24]. In contrast, tools relying solely on database annotation are less accurate when applied to metagenomic datasets, where plasmid sequences may lack key features such as replicase or relaxase genes. ARGs were annotated using ABRicate [28] (v1.0.1) with the CARD (v4.0.0) [29] database. ARG coordinates within the contigs were mapped to the respective fastq files used in co-assembly with pysam (v.23.0) [30] and samtools [31] (v1.9). Readings aligning to the ARG region in the contigs were counted and then normalised by calculating Reads Per Kilobase per Million (RPKM). Contigs containing ARGs were taxonomically classified using Kaiju [32] (v 1.10.1) with the Swiss-Prot protein database. The main code used to analyse metagenomic data is available in the following repository: https://github.com/braddmg/Metagenomics (accessed on 21 January 2025).

2.6. Statistical Analysis

Data analysis and visualisations were performed using R version 4.2.3. Physicochemical and microbiological parameters of influent, effluent, and sludge samples were assessed for dry and rainy seasons. T-Student was applied to the microbiological data, which followed a normal distribution. The Wilcoxon test was employed for the physical-chemical pa-

rameters. Key indicators, including averages, standard deviations, and removal efficiencies, were calculated to evaluate the performance of the WWTP. The alpha level was set at 0.05.

Moreover, we determined the removal capacity of ARGs. The abundance and variability of the selected ARGs, obtained from the qPCR analyses, were visualised using box plots generated with the ggplot2 [33] package (v3.5.2).

Building on the previous data, the pathobiome diversity within the sludge samples was analysed to characterise the microbial diversity and assess the role of pathogen-related bacteria. The Bracken results were imported into R as a phyloseq object using the bracken2phyloseq (b2p) library (v1.0.0) (https://github.com/braddmg/b2p, accessed on 21 January 2025). Full taxonomic paths for each assigned taxon were retrieved from the NCBI taxonomy using the identifiers provided by Kraken2 (v2.1.2) and processed with the taxonomizr [34] package (0.11.1).

To ensure comparability across samples, the number of reads in each sample was rarefied to the minimum read count observed within each dataset. Subsets of the data were then generated, retaining only bacterial genera previously reported as human pathogens, based on [35]. These subsets were used to identify the potential chromosomal and plasmidic pathobiomes in the sludge. Microbial diversity within these pathobiomes was assessed by calculating taxonomic richness (number of distinct taxa) and the Shannon diversity index. Beta diversity was evaluated using a centred log-ratio data transformation.

To identify the most prevalent categories of ARGs in the sludge samples, ARGs were grouped based on their resistance phenotypes and their summed abundances (in RPKM values) visualised as a heatmap using the microViz (v0.12.6) [36] and ggplot2 (v3.5.2) libraries in R. ARGs were categorised as per ABRicate into the following antimicrobial groups: aminoglycoside, beta-lactam, diaminopyrimidine, glycopeptide, lincosamide, macrolide, streptogramin, phenicol, QACs, rifamycin, sulphonamide, tetracycline, and macrolide–lincosamide–streptogramin. Genes associated with resistance to two categories were combined into a single category (e.g., macrolide–streptogramin). In comparison, genes conferring resistance to three or more categories (excluding macrolide–lincosamide–streptogramin) were classified as "multidrug". Relevant plasmidic contigs were visualised with the PlasmidScope [37] web server (v1.2), which utilises the eggNOG-mapper (v2.1.12) [37] Tool for gene annotation.

3. Results

3.1. Removal of Physicochemical and Microbiological Contaminants

In Table 1, we show the results of the physicochemical parameters analysed in the influent and effluent of the WWTP, as well as the removal efficiencies, which are depicted for each season. High standard deviations were observed in the NO_3-N, NH_4-N, PO_4-P, and chemical oxygen demand measurements, suggesting variability in contaminant levels throughout the treatment process. Nonetheless, no significant differences (Wilcoxon test, $p \geq 0.05$) in the plant's inlet were found between seasons. Similarly, no significant differences (Wilcoxon test, $p \geq 0.05$) were found in the effluent between seasons. The respective *p*-values and t results are depicted in Table S2.

3.2. Quantification of the Relative Abundance of ARGs in Wastewater and Sludge

In Figure 1A, we illustrate the normalised abundance of various ARGs in wastewater samples, expressed as copies per *16sRNA* gene copies. Across all genes, concentrations in the effluent were consistently higher than in the influent during both seasons, with the highest abundances observed for *qacE*/*qacE*Δ, sul1, sul2, and *tetA*. Statistical analysis using t-Student revealed that, during the dry season, the abundance of *blaTEM*, *drfA12*, *intl1*, *qacE*/*qacE*Δ, and *sul1* genes in the effluent was significantly higher ($p < 0.05$, Figure 1A)

than in the influent. This trend was only observed during the rainy season for the genes *qacE*/*qacE*Δ and *sul1*. In contrast, we present the normalised abundance of genes in Figure 1B, where intl1, *qacE*/*qacE*Δ, *sul1*, *sul2*, and *tetA* exhibited the highest concentrations. When evaluating seasonal variations in the sludge matrix, t-Student indicated greater stability than wastewater, with no significant differences ($p \geq 0.05$). More detailed information is provided in Tables S4 and S5.

Table 1. Average values and standard deviations of physicochemical parameters from influent and effluent samples at the studied WWTP (February–June 2024, $n = 6$).

	Dry Season			Rainy Season			
Parameters	**Influent**	**Effluent**	**Efficiency (%)**	**Influent**	**Effluent**	**Efficiency (%)**	**Limits in National Regulation [38]**
Flow rate(m^3/d)	(415.59 ± 28.65)		N/A	(449.90 ± 30.77)		N/A	
Settleable solids (mL/L)	(0.30 ± 0.20)	(0.07 ± 0.06)	77.8	(0.20 ± 0.06)	(0.07 ± 0.06)	60.0	5.00
Total suspended solids (mg/L)	(82.43 ± 23.31)	(44.60 ± 1.98)	45.7	(57.00 ± 3.47)	(39.00 ± 0.99)	31.7	100.00
pH	(6.70 ± 0.58)	(6.70 ± 0.58)	N/A	(7 ± 0)	(7 ± 0)	N/A	6.00–9.00
Chemical oxygen demand (mg/L)	(133.60 ± 48.99)	(7.00 ± 9.64)	94.7	(180.00 ± 25.87)	(22.67 ± 21.78)	87.4	150.00
NO_3-N (mg/L)	(0.63 ± 0.71)	(5.63 ± 9.24)	N/A	(1.10 ± 1.91)	(8.17 ± 7.41)	N/A	N/A
NH_4-N (mg/L)	(31.36 ± 18.19)	(2.01 ± 2.18)	93.6	(26.90 ± 8.36)	(3.87 ± 3.40)	85.6	N/A
PO_4 -P(mg/L)	(20.37 ± 12.62)	(1.49 ± 1.88)	92.7	(25.50 ± 4.02)	(16.93 ± 12.77)	33.7	N/A
Faecal coliforms (log_{10}NMP/100 mL)	(5.40 ± 0.59)	(2.24 ± 1.28)	58.8	(6.60 ± 0.14)	(3.2 ± 0)	51.3	N/A

Notes: Median ± standard deviation; N/A: not applicable.

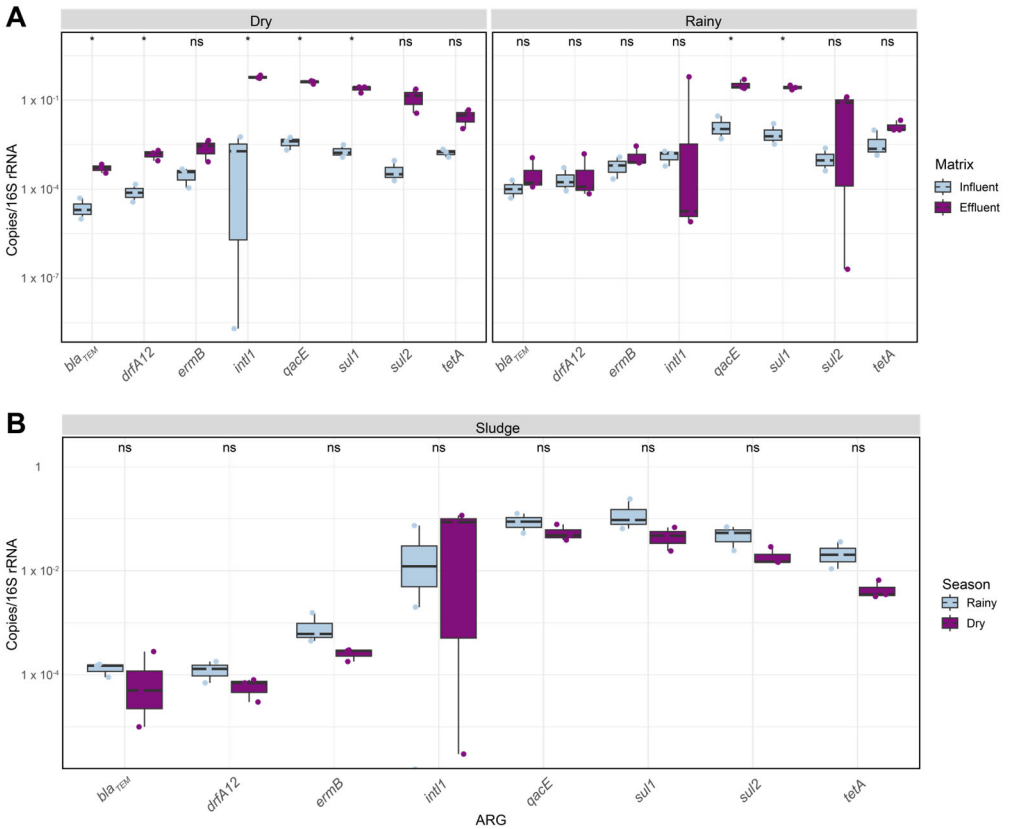

Figure 1. Relative abundance of normalised ARGs in (**A**) water and (**B**) sludge samples, February–June 2024, $n = 6$. * indicates statistical significance ($p < 0.05$).

3.3. Pathobiome Diversity Analyses of Sludge

In the chromosomal dataset, we identified 3628 taxa representing 996 genera. Of these, 1046 taxa (from 150 genera) were associated with potential pathogenic taxa. Conversely, the plasmidic dataset contained 141 taxa representing 83 genera, with 68 taxa (from 30 genera)

linked to potential pathogenic bacteria. Figure 2A presents the relative abundance of the top 10 pathobiome genera in the chromosomal dataset. *Gordonia* and *Acidovorax* were dominant throughout the year. Phocaeicola mainly appeared during the transition period, observed in the last sample of the dry season and the first sample of the rainy season. *Clostridium* and *Bacteroidetes* were detected at lower abundances, particularly in dry season samples (L2, L3, and L4). In contrast, we present the plasmidic dataset in Figure 2B, where *Gordonia*, *Pseudomonas*, *Acidovorax*, and *Mycobacterium* were the most abundant. However, *Mycobacterium* can also be found primarily in rainy season samples.

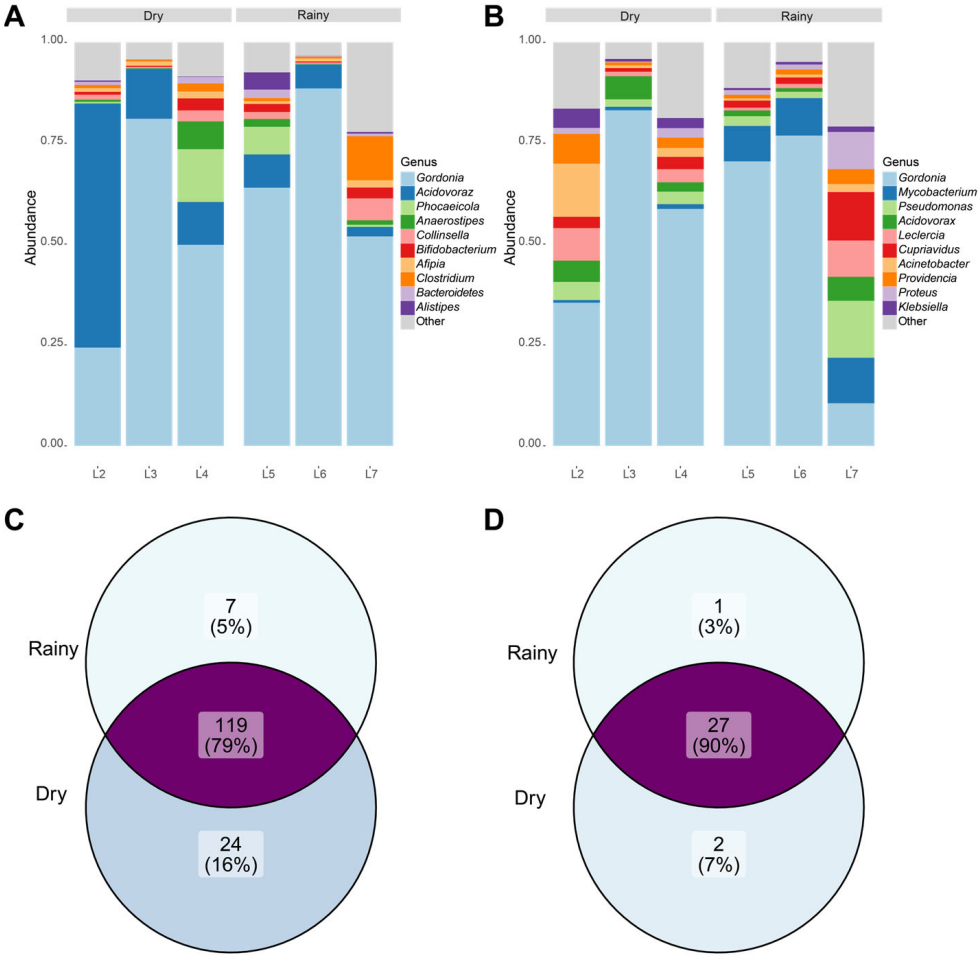

Figure 2. Relative abundance of the top 10 pathobiome genera for (**A**) chromosomal and (**B**) plasmid datasets, and the number of shared genera represented as a Venn Diagram between seasons for (**C**) chromosomal and (**D**) plasmidic datasets.

Gordonia was the dominant genus across most sludge samples except in sample L2 (dry season, Figure 2A), where *Acidovorax* prevailed, and sample L7 (rainy season, Figure 2B), which exhibited a more heterogeneous community. Through the Venn Diagram (Figure 2C), we show that the chromosomal communities shared 119 pathobiome genera (79%) between seasons, while with Figure 2D, we show that the plasmidic communities shared 27 pathobiome genera (90%). We did not observe statistically significant differences in richness or Shannon diversity values across seasons, either for the chromosomal (richness: $W = 8$, $p = 0.20$; Shannon: $W = 5$, $p = 1$) or for the plasmidic data (richness: $W = 6.5$, $p = 0.51$; Shannon: $W = 4$, $p = 1$). In terms of beta diversity, there were no significant differences in pathobiome composition across seasons in either the chromosomal ($F = 1.17$, $R^2 = 0.23$, $p = 0.20$; Figure S1) or the plasmidic communities ($F = 1.68$, $R^2 = 0.30$, $p = 0.10$; Figure S2).

3.4. ARG and Plasmid Sequences Identification in Sludge Samples

We annotated 69 ARGs associated with chromosomal contigs and grouped them into 15 categories based on their resistance phenotype. In plasmidic contigs, only five ARGs were found and grouped into four categories. The normalised abundances (in Reads Per Kilobase per Million) of ARGs in both chromosomes and plasmids from sludge samples across seasons are presented in Figure 3A,B. Chromosomal ARGs mainly conferred resistance to macrolide–streptogramin, aminoglycosides, rifamycin, sulphonamides, and tetracyclines. Plasmid-associated ARGs were primarily linked to resistance against QACs.

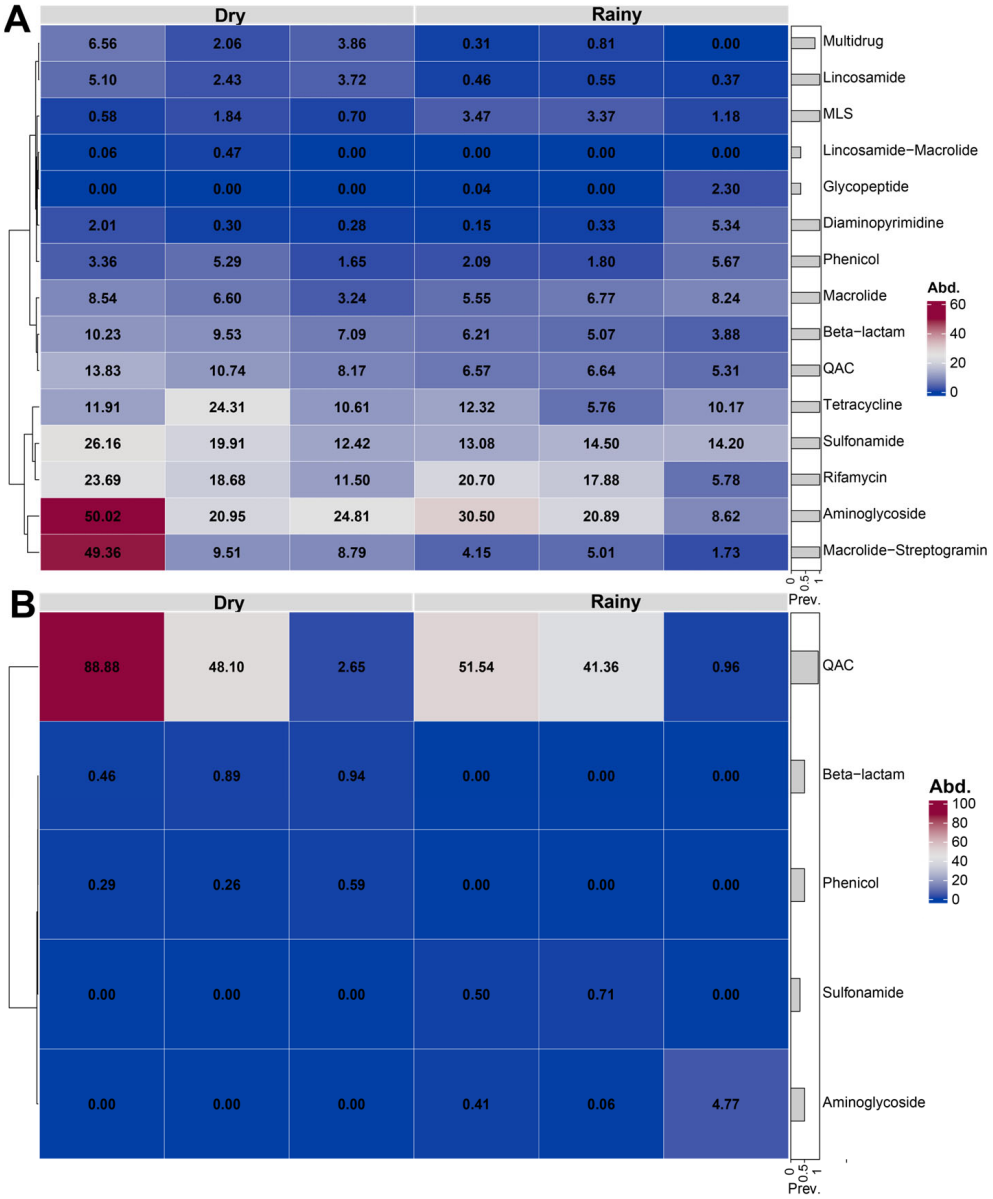

Figure 3. Seasonal distribution of ARGs across (**A**) chromosomes and (**B**) plasmids in sludge samples. The values within the boxes indicate the normalised amount of ARGs in Reads Per Kilobase per Million, with higher abundances shown in red and lower abundances in blue. The barplots on the right display the prevalence of ARGs associated with each resistance category across all samples.

Among plasmid-associated ARGs, *qacL*, a transporter gene potentially linked to QAC resistance, was detected in contigs from co-assemblies of both seasons. The dry season contig (dry_000000259481) carrying the *qacL* gene had a plasX score of 0.68, classifying it as

a plasmid, while the rainy season contig (rainy_000000407135) carrying the same gene had a plasX score of 0.45, initially classifying it as a chromosomal contig (score lower than 0.50).

Although contig rainy_000000407135 had a PlasX score below the classification threshold, we designated it plasmidic based on further alignment and comparison with contig dry_000000259481, revealing an identical gene arrangement. The only notable difference was a variant of the relaxase gene from the mobA/mobL family in rainy_000000407135. This variation likely affected the PlasX score, as the algorithm relies on a machine learning model trained on known plasmid-associated genes. The variant *mob* gene may not be represented in the PlasX training database, contributing to the lower score, especially considering that relaxase genes are key markers for plasmid identification. Nonetheless, given the high structural similarity and comparable gene content to the confirmed plasmidic contig dry_000000259481, along with the annotation of the relaxase gene in both contigs by PlasmidScope, we retained rainy_000000407135 as a potential plasmid. Both contigs were taxonomically classified as *Paracoccus aminovorans* with Kaiju software (v 1.10.1), and their complete structures and gene arrangements are shown in Figure 4. Additional details regarding all identified ARGs, including coverage, identity percentage, associated taxa, contigs, and coordinates, are provided in Table S6.

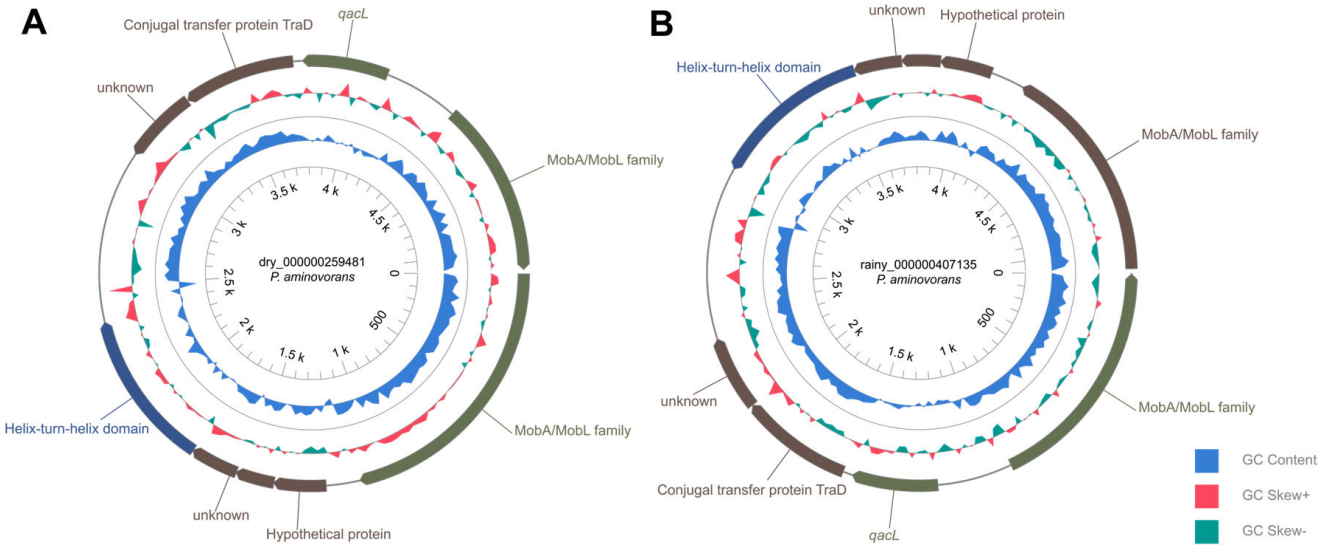

Figure 4. Visualisation of contigs with the plasmid-associated *qacL* gene in sludge samples from the studied WWTP during the (**A**) dry and (**B**) rainy season.

4. Discussion

4.1. WWTP Performance

The removal efficiency of a WWTP can be influenced by several factors, including influent composition variability, seasonal fluctuations (e.g., heavy rainfall), and microbiological dynamics within the treatment process [39]. The sequential batch reactor configuration, characterised by highly non-linear processes, is susceptible to significant variations in influent concentration, which can consequently affect effluent quality [40].

At the studied WWTP, influent conditions remained stable during the study period (Wilcoxon, $p \geq 0.05$, Table S2), supporting consistent removal of physicochemical parameters across seasons. Similarly, effluent medians showed no significant differences (Wilcoxon, $p \geq 0.05$, Table S2), indicating that seasonal climatic variation did not markedly impact plant performance. All effluent parameters complied with national regulatory standards (Table 1). However, a slight decline in removal efficiency was observed during the rainy season, suggesting that ongoing monitoring is needed to manage potential seasonal effects.

4.2. Antimicrobial Resistance Genes (ARGs) in Wastewater and Sludge Samples

Given the plant's stable physicochemical performance, we focused on microbiological indicators, particularly the abundance and behaviour of ARGs. Understanding ARG dynamics is essential for addressing resistance spread, as these genes encode mechanisms that enable bacterial survival in the presence of antimicrobials [41]. Influent ARG concentrations remained relatively stable across both seasons, with limited variation across individual samples (Figure 1A). In contrast, effluent samples showed larger interquartile ranges and greater variability, suggesting that internal treatment processes influence ARG levels.

Statistical analysis revealed significantly higher (*t*-test, $p < 0.05$) ARG concentrations in the effluent compared to the influent for bla_{TEM}, *drfA12*, *intI1*, *qacE/qacE*Δ, and *sul1* during the dry season, and for *qacE/qacE*Δ and *sul1* during the rainy season. Sludge samples exhibited more consistent ARG concentrations across seasons, with the highest levels observed for *intI1*, *qacE/qacE*Δ, *sul1*, *sul2*, and *tetA*. This may partly reflect the small number of sampling events and the inherent complexity of microbial communities in WWTPs. Nevertheless, the observed trends suggest that selective pressures within the treatment system can sustain ARGs over time, even without statistically significant seasonal differences.

The enrichment of ARGs, especially in the dry season, may reflect plant-level processes favouring gene persistence over removal. Similar patterns have been reported in other WWTPs [11,42,43], often linked to the extended sludge retention times typical of sequential batch reactor configurations [44] and to selective pressures within the system [45]. Embedded microorganisms found in WWTP biofilms have also been associated with elevated abundances of ARGs such as *sul1* and *intI1* [46]. These conditions facilitate bacterial interactions and promote horizontal gene transfer via mobile genetic elements such as plasmids and integrons [44,47].

Intl1-positive isolates are strongly associated with *qacE/qacE*Δ, a gene conferring resistance to QACs [48]. The high abundance of both genes in our study suggests that selective pressures, possibly linked to residual biocides, favour ARG-carrying bacteria [49]. Class 1 integrons, marked by the presence of *intI1*, function as genetic platforms for capturing and exchanging resistance gene cassettes. These integrons are often embedded in plasmids, enhancing their mobility and environmental dissemination [50]. Co-selection by contaminants such as heavy metals and QACs further promotes ARG stability even without antibiotics [51]. In this context, genes like *qacE/qacE*Δ*1* can indicate anthropogenic pollution and biocide pressure.

The elevated ARG levels in treated effluent underscore the role of WWTPs as significant contributors to the downstream dissemination of ARGs. These effluents, often released into rivers, lakes, or coastal waters, may carry ARG-harbouring bacteria into environments used for agriculture (e.g., irrigation), recreation (e.g., swimming), or even as sources of drinking water following further treatment [52]. This dissemination pathway heightens the potential human health risks associated with such water bodies.

Although antimicrobial-resistant bacteria may decrease due to environmental pressures in receiving water bodies, other factors may foster the persistence of antimicrobial-resistant bacteria and ARGs. For example, sediment particles or microplastics can serve as attachment sites for bacteria, acting as reservoirs of ARGs and promoting conjugation events [52,53]. During high river flows or tidal surges, particles may offer protection from ultraviolet radiation, enhancing the persistence of antimicrobial-resistant bacteria [54,55]. Additional environmental factors such as pH, nutrient levels, salinity, oxygen availability, and temperature also affect the survival, distribution, and potential for dissemination of bacteria in aquatic environments [56]. However, further studies are needed to understand how the tropical conditions may affect these dynamics.

4.3. Pathobiome Diversity Analysis in Sludge Samples

Analysis of the sludge pathobiome (Figure 2) showed similar community structures regarding richness and beta diversity in the dry and rainy seasons, with *Gordonia* and *Acidovorax* being consistently dominant. *Gordonia* is an opportunistic pathogen associated with infections in immunocompromised individuals [57], and it has been isolated from contaminated soils and WWTP sludge [58,59]. *Acidovorax* is known for multidrug resistance and the ability to metabolise diverse carbon sources in wastewater environments [60,61].

The limited seasonal variation in pathobiome composition may reflect the relatively stable temperatures in tropical regions, contrasting with seasonal microbial shifts observed in temperate zones. Studies have shown that tropical WWTPs harbour more phylogenetically diverse and unique microbial communities, shaped by stochastic processes and consistently high temperatures [46]. These factors influence microbial community structure and ARG persistence, which may explain the consistent seasonal taxonomic patterns.

Although the chemical oxygen demand concentrations in this WWTP were moderate (~200 mg/L, Table 1), they may still support the persistence of genera like *Gordonia*. Prior research links high chemical oxygen demand (>500 mg/L) to increased microbial activity and biofilm formation [62]. But even moderate levels can sustain resilient taxa in stable systems. The metabolic versatility and ability of *Gordonia* to thrive in wastewater environments [63,64], combined with the operational stability of the WWTP, likely contribute to the year-round resilience of this (and probably other genera) within the microbial community.

The sludge also harboured other genera, such as *Pseudomonas* and *Mycobacterium*, further underscoring its role as a reservoir for antimicrobial resistance. *Pseudomonas* species, including *P. putida*, are highly resistant and capable of degrading pharmaceuticals in high-phosphorus environments [65–67]. *Mycobacterium*, comprising both pathogenic and non-tuberculous species, can cause severe infections, especially in individuals with chronic conditions [68]. Other genera, including *Clostridium* and *Bacteroidetes*, were present in lower abundances, reflecting the dynamic microbial structure shaped by influent and nutrient variation [69].

These findings underscore the public health risks posed by WWTPs as potential sources of pathogenic bacteria. ARGs may be transmitted vertically or horizontally via mobile genetic elements, particularly plasmids, which facilitate horizontal gene transfer across bacterial taxa [70,71]. Therefore, monitoring the presence of potential pathogenic bacteria in WWTPs (hotspots for ARGs) could enhance strategies for tracking emerging multidrug-resistant pathogens released into natural environments, potentially linked to future clinical cases.

4.4. Predominant Chromosomal and Plasmid-Associated ARG Groups Found in Sludge

Metagenomic analysis revealed the dominant resistance features of the WWTPs' microbial community (Figure 4). Chromosomal ARGs were mainly linked to resistance against macrolide–streptogramin, aminoglycosides, rifamycin, sulphonamides, and tetracyclines. Plasmid-associated ARGs primarily conferred resistance to QACs, highlighting the role of plasmids in biocide resistance and the potential for horizontal gene transfer.

Macrolide–streptogramin and aminoglycosides are frequently used in human and veterinary medicine to treat Gram-positive bacterial infections and are commonly detected in wastewater [72]. Resistance mechanisms include antibiotic detoxification and target-site modification for macrolide–streptogramin [73]. Aminoglycosides, on the other hand, are bactericidal antibiotics that inhibit protein synthesis and are commonly used to treat acute hospital-acquired infections, such as those caused by *Pseudomonas aeruginosa* [74]. Bacterial resistance to aminoglycosides is diverse, but the most common mechanism involves enzymatic inactivation of the antimicrobial by aminoglycoside-modifying enzymes [75].

Rifamycin resistance genes were detected at higher levels than previously reported for domestic and hospital effluents [76,77]. These antibiotics are used to treat tuberculosis infections, and resistance is mainly conferred through RNA polymerase mutations and reduced membrane permeability [78]. The most abundant gene associated with rifamycin resistance was *rbpA* (Table S6), which encodes an RNA polymerase binding protein that protects the bacterial RNA polymerase. The high levels of ARGs conferring resistance to this antibiotic group, along with the presence of pathogenic genera such as *Mycobacterium*, which is associated with respiratory infections, emphasise the necessity of continuous surveillance of these and other WWTPs.

Among the other highly prevalent ARG groups, tetracyclines and sulphonamides have demonstrated similar tendencies, as reported in [79]. Tetracyclines are broad-spectrum antibiotics that inhibit bacterial protein synthesis. Although their use in human medicine is regulated, they remain widely used in veterinary medicine [80]. Resistance is typically mediated by efflux pumps, ribosomal protection, and enzymatic inactivation [81]. Sulphonamides account for up to 12% of global antibiotic use. They can represent up to 43% of pharmaceutical pollutants in Costa Rican waters [82,83], and their resistance is mainly due to dihydropteroate synthase variations [84]. Multiple *sul* genes were annotated in the metagenomic datasets. These genes are commonly observed in MGE as plasmids and integrons. Still, in our samples, most were located within chromosomal contigs, which may reflect the limitations of plasmid identification in metagenomic datasets or the integration of these dihydropteroate synthase variants into bacterial chromosomes.

QAC-related ARGs were most frequently plasmid-associated, reinforcing the concern over plasmid-mediated biocide resistance. QACs are widely used in household and industrial settings and are retained in WWTPs, where they exert intense selective pressure, eliminate susceptible strains, and promote resistant ones [85,86]. Resistance is often mediated by efflux pumps in the small multidrug resistance family encoded by *qac* genes [87].

In tropical low- and middle-income countries, widespread QAC use and limited regulation can intensify this selective pressure. These high-production chemicals persist in wastewater and sediments, contributing to ARG proliferation [88]. The consistent detection of *qacE/qacEΔ1* across sample types and seasons highlights its utility as a marker for anthropogenic contamination and antimicrobial resistance risk. Monitoring *qacE* can support efforts to evaluate biocide-related resistance in tropical wastewater systems [89].

4.5. Plasmid-Associated Resistance Genes in Metagenomic Sludge Samples

Given the predominance of plasmid-associated ARGs conferring resistance to QACs in our dataset, we further examined how their genomic structure influences resistance dissemination. Plasmid-borne ARGs evolve and accumulate differently from those located on chromosomes, often resulting in distinct phenotypic effects. Additionally, plasmids harbour significantly higher percentages of resistomes, with antibiotics facilitating the transposition and recombination of ARGs within plasmids [90]. By analysing the genomic structure of relevant plasmids in our study (Figure 4), we gained valuable insights into potential mechanisms of horizontal gene transfer and their role in ARG dissemination within the studied WWTP microbial community.

Among the plasmid-associated ARGs, those conferring resistance to QACs were predominant, leading to the detection of the *qacL* gene in contigs from both sampling seasons. In this case, the strong presence of plasmid-borne ARGs conferring resistance to QACs suggests that plasmids serve as key vectors for microbial adaptation to QAC exposure.

Furthermore, we identified a gene encoding the *MobA/MobL* family protein (Figure 4) in plasmids carrying the *qacL* gene during both seasons. This suggests that plasmids conferring QACs resistance possess mobilisation functions that enhance their transfer between

bacteria. *MobA*, a relaxase with both nicking and priming activities on single-stranded DNA, plays a crucial role in conjugative mobilisation [91]. Additionally, detecting the TraD conjugal transfer protein (Figure 4) in the same contig alongside *qacL* and the relaxase further supports the hypothesis that this plasmid has the potential for horizontal gene transfer via conjugation within the studied WWTP. Conjugation is a primary mechanism of horizontal gene transfer, enabling plasmid-mediated ARG dissemination through direct cell-to-cell contact and pilus formation [92]. This process has been widely documented in various environments, including wastewater ecosystems [93]. Additionally, QACs exert strong selective pressure in these settings and have been shown to enhance plasmid conjugation by altering cellular processes such as membrane permeability, extracellular polymeric substance production, and the expression of conjugation-related genes [94].

4.6. Limitations of Our Study and Future Directions

This study explored seasonal dynamics at a tropical WWTP by comparing two climatologically distinct periods—dry and rainy seasons—with sampling events strategically distributed to capture intra-seasonal variability. While each season included a limited number of sampling events (n = 3), spreading these across different weeks enhanced the representativeness of the data under each climatic condition.

Conducting the study at a single WWTP provided a focused, in-depth analysis of local processes, offering valuable insights into seasonal patterns. However, expanding future research to include multi-year datasets and a wider range of WWTPs employing different treatment technologies would greatly strengthen the temporal and spatial relevance of the findings.

The study also highlights a significant opportunity: the lack of national regulations requiring routine monitoring of fundamental contaminants, such as nutrients, and emerging concerns like ARGs underscores the need for more robust monitoring frameworks. Addressing this regulatory gap could improve data availability and support more comprehensive evaluations of wastewater impacts.

5. Conclusions

The studied WWTP operates effectively, maintaining consistent performance despite seasonal fluctuations. However, despite low and stable influent concentrations, the persistence and enrichment of ARGs in the effluent suggest that current treatment processes may inadvertently favour the survival and potential spread of antimicrobial-resistant bacteria. The high abundances of *intI* and *qacE*/*qacE*Δ genes further highlight the role of mobile genetic elements and selective pressure in propagating resistance traits.

The pathobiome analysis revealed the presence of potential pathogens from genera such as *Gordonia*, *Acidovorax*, *Pseudomonas*, and *Mycobacterium*, reinforcing the role of WWTPs as reservoirs for both ARGs and opportunistic pathogens. Additionally, detecting plasmid-associated ARGs—particularly QAC-related genes co-occurring with conjugative elements like *MobA* and *TraD*—indicates active horizontal gene transfer within the microbial community, facilitating the environmental dissemination of QAC resistance traits.

To address the environmental spread of antimicrobial resistance, enhancing tertiary disinfection processes, monitoring selective agents, and incorporating ARG and MGE surveillance into wastewater treatment performance metrics is recommended. Strengthening regulatory frameworks will be key to supporting these efforts.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/w17111574/s1: Table S1: Primers used for the qPCR analysis of specific ARGs. Table S2: Wilcoxon p-value for seasonal variations in influent and effluent of the physicochemical characterisation. Table S3: T-Student p-value for season variations in sludge characterisation.

Table S4: Comparison of samples between influent and effluent for each gene for both seasons (dry and rainy). Table S5: Comparison of sludge samples between seasons. Table S6: Additional details regarding the ARGs identified across samples. Figure S1: Principal Coordinate Analysis (PCoA) of beta diversity in the pathobiome composition across seasons, based on the chromosomal dataset. Figure S2: Principal Coordinate Analysis (PCoA) of beta diversity in the pathobiome composition across seasons, based on the plasmidic dataset.

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Abbreviations

The following abbreviations are used in this manuscript:

WWTPs	wastewater treatment plants
ARGs	antimicrobial resistance genes
QACs	quaternary ammonium compounds

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