Science of the Total Environment 961 (2025) 178403

Contents lists available at ScienceDirect

Science of the Total Environment

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

Metagenomics unveils the role of hospitals and wastewater treatment plants on the environmental burden of antibiotic resistance genes and opportunistic pathogens

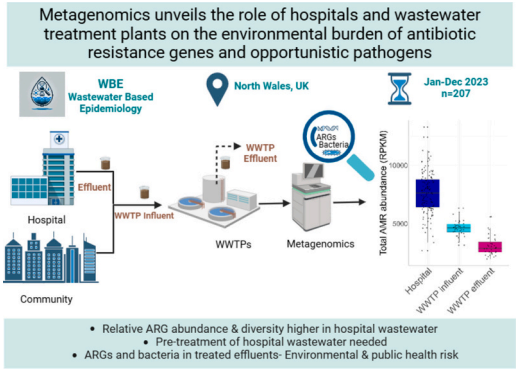
Reshma Silvester [a,*], William B. Perry [b], Gordon Webster [b], Laura Rushton [b], Amy Baldwin [b], Daniel A. Pass [c], Nathaniel Healey [a], Kata Farkas [a], Noel Craine [d], Gareth Cross [e], Peter Kille [b], Andrew J. Weightman [b], Davey L. Jones [a]

[a] School of Environmental and Natural Sciences, Bangor University, Bangor, Gwynedd Ll57 2UW, UK
[b] School of Biosciences, Cardiff University, Cardiff CF10 3AX, UK
[c] Compass Bioinformatics, 17 Habershon Street, Cardiff CF24 2DU, UK
[d] Public Health Wales, Microbiology Department, Ysbyty Gwynedd, Bangor LL57 2PW, UK
[e] Science Evidence Advice Division, Health and Social Services Group, Welsh Government, Cathays Park, Cardiff CF10 3NQ, UK

HIGHLIGHTS

- Higher diversity and abundance of ARGs in hospital effluents compared to WWTPs.
- ARGs conferring resistance to key clinical antibiotics common in hospital effluents.
- Spatio-temporal variations in ARG removal efficiency noted in the studied WWTPs.
- Advanced wastewater treatment in WWTPs and hospital-level control measures needed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Elodie Passeport

Keywords:
Antimicrobial resistance
Bacterial pathogens
Metagenomics
Public health surveillance
Wastewater-based epidemiology

ABSTRACT

Antimicrobial resistance (AMR) is a global health challenge, with hospitals and wastewater treatment plants (WWTPs) serving as significant pathways for the dissemination of antibiotic resistance genes (ARGs). This study investigates the potential of wastewater-based epidemiology (WBE) as an early warning system for assessing the burden of AMR at the population level. In this comprehensive year-long study, effluent was collected weekly from three large hospitals, and treated and untreated wastewater were collected monthly from three associated community WWTPs. Metagenomic analysis revealed a significantly higher relative abundance and diversity of ARGs in hospital wastewater than in WWTPs. Notably, ARGs conferring resistance to clinically significant

Abbreviations: ARB, antibiotic-resistant bacteria; ARG, antibiotic-resistance genes; AMR, antimicrobial resistance; CSO, combined sewer overflow; MLS, macrolide-lincosamide-streptogramin; MDR, multi-drug resistance; WBE, wastewater-based epidemiology; WWTP, wastewater treatment plant.

* Corresponding author.
E-mail address: r.silvester@bangor.ac.uk (R. Silvester).

https://doi.org/10.1016/j.scitotenv.2025.178403
Received 13 August 2024; Received in revised form 3 January 2025; Accepted 3 January 2025
Available online 10 January 2025

antibiotics such as β-lactams, aminoglycosides, sulfonamides, and tetracyclines were more prevalent in hospital effluents. Conversely, resistance genes associated with rifampicin and MLS (macrolides-lincosamide-streptogramin) were more commonly detected in the WWTPs, particularly in the treated effluent. Network analysis identified the potential bacterial hosts, which are the key carriers of these ARGs. The study further highlighted the variability in ARG removal efficiencies across the WWTPs, with none achieving complete elimination of ARGs or a significant reduction in bacterial diversity. Additionally, ARG profiles remained relatively consistent in hospital and community wastewater throughout the study, indicating a persistent release of a baseload of ARGs and pathogenic bacteria into surface waters, potentially polluting aquatic environments and entering the food chain. The study underscores the need for routine WBE surveillance, enhanced wastewater treatment strategies, and hospital-level source control measures to mitigate AMR dissemination into the environment.

1. Introduction

Antimicrobial resistance (AMR) represents one of the top ten global public health threats and is projected to cause nearly 39 million deaths between 2025 and 2050 (O'Neill, 2016; Sneka and Mahalakshmi, 2023; Naghavi et al., 2024). The world is on the verge of a post-antibiotic era, potentially pushing the healthcare system back by decades (Kwon and Powderly, 2021). According to a recent report, nearly 1.27 million deaths in 2019 were associated with antibiotic-resistant bacterial infections, much higher than the death rate ascribed to HIV or malaria (Murray et al., 2022). Heightened antibiotic usage during the COVID-19 pandemic to treat secondary bacterial infections has driven a global increase in AMR (Shomuyiwa et al., 2022; Dey et al., 2023). Furthermore, the latest report from the English Surveillance Programme for Antimicrobial Use and Resistance indicates a rise in deaths caused by antibiotic-resistant infections in the UK, alongside an 8 % spike in antibiotic usage in 2022 (Ashiru-Oredope et al., 2023). These reports underscore that AMR, the "silent pandemic," already has widespread impacts (Rayan, 2023).

Strengthening AMR surveillance to provide data for evidence-based decision-making is one of the eight pillars of action identified in the Antibiotic Resistance Coalition (ARC) policy briefing note to address the global issue of AMR (ARC, 2022). Wastewater-based epidemiology (WBE) is an innovative approach that provides data on the occurrence of AMR within the environment and populations feeding into the wastewater systems (van Leth and Schultsz, 2023). It can deliver a snapshot of community-level health and habits and be used as an early warning system to identify hotspots and trends for infectious diseases and AMR spread, thereby predicting public health risks (Sims et al., 2023). Metagenomics-based methods have already proven more effective than conventional methods for AMR surveillance, such as targeted qPCR assays and traditional culturing techniques (Munk et al., 2017; Hendriksen et al., 2019). Clinical-based surveillance methods are limited to specific drug-microbe combinations and are often impractical for sampling and studying AMR in a healthy community. By providing a comprehensive overview of the total resistome composition in samples, metagenomics captures both targeted and non-targeted antibiotic resistance genes (ARGs) and offers insights into newly emerging ARG variants. Through metagenomic analysis, suitable ARG targets can be identified for further detailed quantification using the targeted qPCR approach. Integrating WBE with metagenomics can serve as a powerful early warning tool for monitoring and predicting the emergence of pathogens and the dissemination of ARGs in the community (Kasprzyk-Hordern et al., 2014; Majeed et al., 2021).

At present, there is a critical knowledge gap concerning the types of AMR and bacterial pathogens in circulation within wastewater treatment systems and hospital settings in Wales. Unlike previous studies that primarily focus on hospitals or WWTPs in isolation, our research evaluates both these sources and their collective impact on environmental AMR. An improved understanding of the occurrence of AMR in the environment, alongside data on AMR within human and veterinary clinical settings, will support policymakers and public health authorities in devising effective strategies for mitigating the spread of AMR and related infections.

The primary objective of this study is to utilise WBE to predict the occurrence and assess the risk of ARGs and pathogenic bacteria released into the environment from hospitals via wastewater treatment plants (WWTPs). Specifically, we aim to employ a metagenomics approach to gain comprehensive insights into the diversity of ARGs and bacterial pathogens discharged through hospitals and municipal wastewaters. Additionally, we will evaluate the removal efficiency of WWTPs in reducing ARGs and assess the contribution of hospital effluents to environmental AMR. We hypothesise that hospital effluents play a significant role in contributing to AMR and bacterial pathogens present in the natural environment. The hypothesis will be tested by investigating combinations of three hospitals and their downstream WWTPs in Wales.

The findings from this study can be used to illustrate how WBE integrated with metagenomics can inform public health strategies and evidence-based policymaking, emphasising the real-world applicability of this surveillance method in tackling the AMR crisis.

2. Materials and methods

2.1. Sampling sites

Sampling was conducted for one year, from January to December 2023, as part of a wider national wastewater monitoring programme in Wales (Perry et al., 2024). A total of 1687 wastewater samples were collected during the study. Daily samples (Monday–Friday) of both influent and effluent wastewater were collected from three large municipal wastewater treatment plants (WWTPs) located in Wrexham (W-WWTP), Kinmel Bay (K-WWTP), and Bangor (B-WWTP) (Fig. 1). In addition, sampling was undertaken four days a week (Tuesday-Friday) at three hospitals located within the sewershed of each WWTP, namely Wrexham Hospital (W-H), Kinmel Bay Hospital (K-H) and Bangor Hospital (B-H) (Fig. 1).

The Bangor WWTP has an average inflow of 11,468 $m^3\ d^{-1}$ and serves a population of 36,400. The WWTP receives untreated effluent from Bangor Hospital, which has an average bed occupancy of 395.2 and serves the Northwest Wales region. The distance between the hospital and the WWTP is 1 km. The Kinmel Bay WWTP has an average inflow of 18,297 $m^3\ d^{-1}$, serving a population of 78,077. It receives untreated effluent from the Kinmel Bay Hospital, which serves the North Central Wales region with an average bed occupancy of 423.3. The hospital is located 3 km from the WWTP. The Wrexham WWTP has an average inflow of 29,927 $m^3\ d^{-1}$ and serves a catchment population of 129,824. It receives untreated effluent from the Wrexham Hospital, which serves Northeast Wales, with an average occupancy of 609 beds and is located 6 km from the WWTP. The Kinmel Bay WWTP utilises a secondary treatment process only, whereas the other two plants have implemented tertiary UV treatment. All three hospitals cover ca. 70 % of the hospital capacity within the region, while the three WWTPs capture wastewater from ca. 35 % of the region's population. Rhodamine-WT dye was introduced into the main sewer at each hospital site on multiple occasions, and its transit time to the WWTP was measured as described by Sonnenwald et al. (2023). For the Wrexham, Kinmel Bay, and Bangor sites, the transit times from the hospital to the WWTP were 86–109, 10–80, and 41–55 min, respectively, depending on the prevailing

weather conditions and the emptying frequency of the holding tanks (wet wells) at the hospitals.

Composite wastewater samples at each location were collected using refrigerated autosamplers, which take 50 ml samples at 15-min intervals throughout the day. These included samples from the main drain leaving each hospital site and the influent and effluent lines at each WWTP. Upon collection, one-litre samples were then transported refrigerated in clean 1-litre Nalgene® bottles to the central laboratory at Bangor and processed on the collection day.

2.2. Wastewater physicochemical analysis

Wastewater turbidity was measured on a HI-83414-02 turbidity meter (Hanna Instruments Ltd., UK). The pH and electrical conductivity (EC) were determined with a SevenCompact™ Duo S213 meter (Mettler Toledo INC, USA; Rhoades, 1996). Ammonium-N and phosphate were quantified colorimetrically using a SpectroSTAR nanoplate microreader (BGM Labtech, Germany) using the salicylic acid method of Mulvaney (1996) and molybdate blue method of Murphy and Riley (1962), respectively.

2.3. Wastewater sample processing

Composite daily wastewater samples (50 ml aliquots) were concentrated by centrifuging at 10,000 ×*g* for 30 min, and the supernatant was discarded. Wastewater biomass pellets were then pooled (n = 207) to represent weekly composite biomass samples for hospitals and monthly for WWTPs, respectively. They were suspended in 2 to 4 ml sodium phosphate buffer (pH 7.8–8.2; MP Biomedicals, Irvine, CA) and stored at −20 °C until required.

2.4. DNA extraction

Genomic DNA was extracted from pooled wastewater biomass samples (pooling samples weekly from hospitals and monthly from WWTPs) using a modified FastDNA™ SPIN Kit for Soil (MP Biomedicals) using a protocol routinely used for environmental samples (Webster et al., 2003). Essentially, the wastewater pellet was suspended in 1 ml of phosphate buffer (pH 7.0) and added to a 2 ml Lysing Matrix Tube E (MP Biomedicals) with 120 µl MT Buffer (MP Biomedicals) and homogenised for 2 × 30 s at a speed 5.5 m s^{-1} on a FastPrep-24TM 5G instrument (MP Biomedicals). Samples were centrifuged at 14,000 ×*g* for 8 min, and the supernatant was transferred to an APEX® 1.5 ml No-Stick microcentrifuge tube (Alpha Laboratories Ltd., Eastleigh, UK) with 250 µl PPS reagent (MP Biomedicals), mixed and centrifuged at 14,000 ×*g* for 5 min. The supernatant was then transferred to a 25 ml universal tube with 1 ml Binding Matrix suspension (MP Biomedicals), shaken for 3 min, and DNA allowed to bind for 30 min at room temperature. Bound DNA was captured and purified using a SPINTM Filter and eluted in 100 µl of DNase/RNase-free water (Severn Biotech Ltd., Kidderminster, UK). DNA samples were then stored at −20 °C until required.

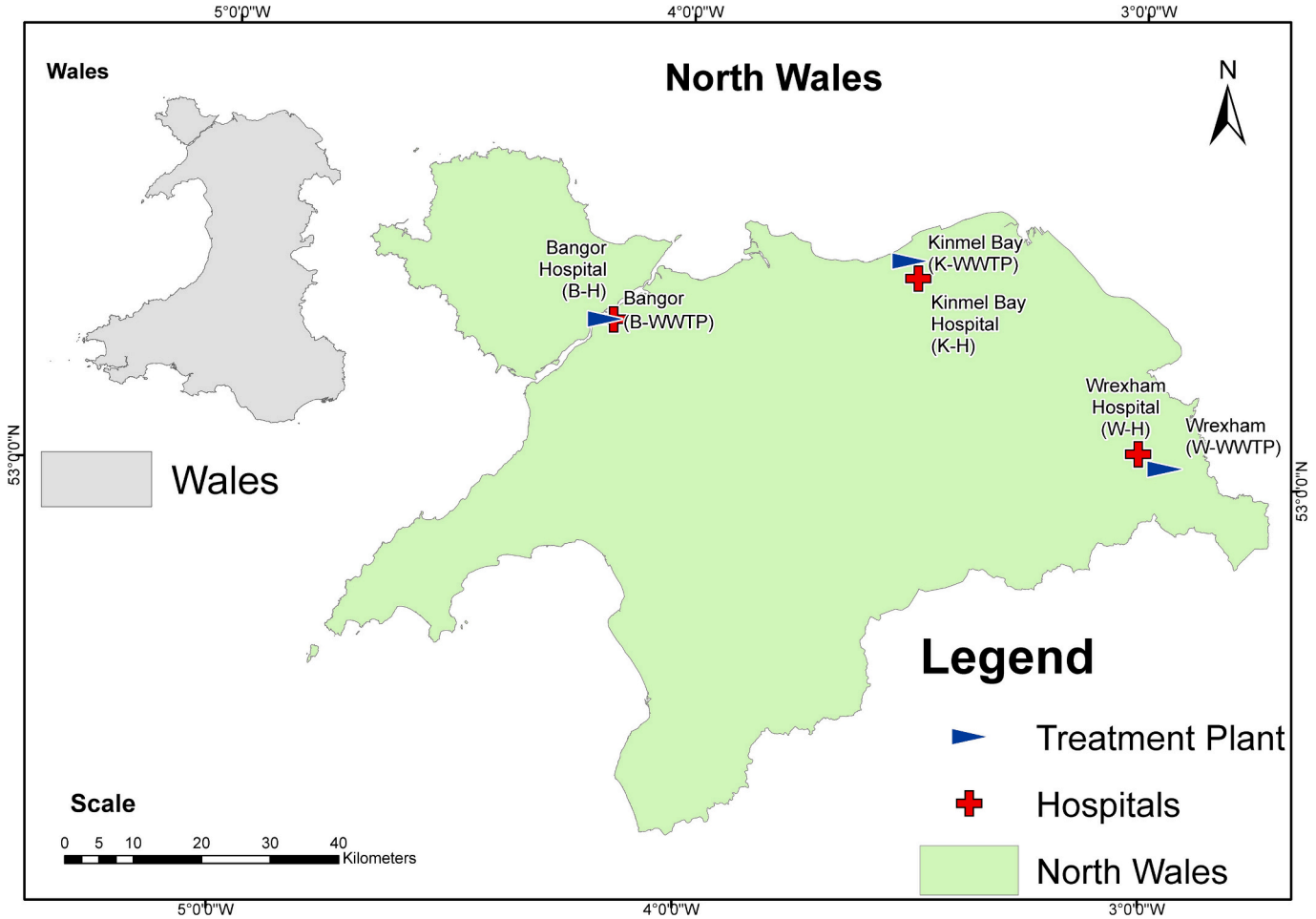


Fig. 1. Map showing the sampling locations of the three wastewater treatment plants (WWTP) and corresponding hospitals within North Wales. The population centres sampled were Bangor, Kinmel Bay, and Wrexham.

2.5. Metagenomic library construction and sequencing

Extracted DNA samples were diluted 1/10 in RSB resuspension buffer (Illumina Inc., San Diego, CA) and quantified using a Qubit 4 Fluorometer with a Qubit High Sensitivity dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA). Library preparation was then carried out using the standard input workflow on 200 ng DNA per sample using the Illumina DNA PCR-Free Prep, Tagmentation Kit and IDT for Illumina DNA/RNA UD Indexes (Sets A-D) as described in the Illumina reference guide. Dual-indexed paired-end single-stranded DNA Libraries (450 bp average library size) were then quantified using the CollibriTM Library Quantification kit (ThermoFisher Scientific) on a LightCycler® 96 Instrument (Roche Diagnostics Ltd.), adjusted to 2.0 nM and pooled. The pooled library was further checked by sequencing on a MiSeq 300 cycle cartridge (Illumina MiSeq System) and adjusted to 1.5 nM based on sequence reads before deep sequencing on an Illumina NovaSeq 6000 Sequencing System (paired-end 2 × 150 bp flow cell) with NovaSeq S4 cartridge v1.5 (300 cycles) and 1 % PhiX control at Wales Gene Park (www.walesgenepark.cardiff.ac.uk/).

2.6. Bioinformatic analysis of sequencing data

Metagenomic sequence reads were converted to FASTQ files from base call files (BCL), demultiplexed, and adapter trimmed using Illumina bcl2fastq2 conversion software v2.20. Sequence reads were quality-filtered using fastp v 0.20, checked with FastQC v 0.11.9, and compiled into a single report using MultiQC (Ewels et al., 2016). Reads representing host (human) DNA were then removed from all sequence files by mapping against the human genome using Samtools v 1.13 (Danecek et al., 2021) and Bowtie v2.4.1 (Langmead and Salzberg, 2012). High-quality non-host sequence reads were analysed for antimicrobial resistance genes using the AMR++ v3.0 bioinformatic pipeline implemented with NextFlow. The reads were aligned to the MEGAres v3 database and Kraken 2 for microbial taxonomy (Di Tommaso et al., 2017; Bonin et al., 2023).

To account for differences in the sample-wise sequencing depth and gene length, the obtained AMR gene reads were expressed as Reads Per Kilobase per Million mapped reads (RPKM) (Yin et al., 2023) using the following equation:

$$\mathrm{RPKM} = \frac{\mathrm{ARG\,reads} \times \ 1,000,000,000}{\mathrm{Total\ bacterial\ reads} \times \mathrm{ARG\,length\,(bp)}}$$

The bacterial read counts were normalised by dividing the abundance of each bacterial taxa by the total number of bacterial reads per sample. Only bacterial reads were used in our analysis as they were the dominant prokaryotic population responsible for AMR and archaea represented only 0.2 to 2.35 % of prokaryotic sequence reads.

2.7. Data analysis and visualisation

Data analysis and visualisation were performed using R version 4.2.3. The *pheatmap* package was used to plot heat maps (Kolde and Kolde, 2015). The diversity of bacterial species and AMR genes in the samples were analysed by calculating the Shannon diversity indexes using *vegan* package (Dixon, 2003). Principal coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity index was performed using *ape* package (Paradis et al., 2004). The correlation matrix between ARGs and bacterial genera was calculated based on the significant correlation of $p < 0.01$ and Pearson's $r > 0.70$ using *Hmisc* (Harrell Jr and Harrell Jr, 2019) and *igraph* (Csárdi et al., 2024) packages. Network analysis was then performed on Spearman's rank correlation coefficient to visualise the co-occurrence of ARGs and bacterial hosts using Gephi 0.10.1 (Bastian et al., 2009). The data did not follow a normal distribution; hence, a nonparametric statistical approach using Kruskal–Wallis was employed. This was followed by post-hoc multicomparisons using Dunn's test. The alpha level was set at 0.05.

ARG counts (RPKM) were also combined with water chemistry data and analysed using a linear model and analysis of variance. Two linear models were constructed, one for the hospitals and one for WWTPs. These were separated due to the differences in sample collection (near source and collection at a WWTP) and sample time window (weekly and monthly). RPKM was the response variable, with fixed variables including pH, conductivity, ammonium, phosphate, turbidity, sample site, season and, in the case of the samples from WWTP, sample type (effluent or influent). In addition to fixed effects, interaction terms between all fixed effects and site were included to better understand how relationships between water chemistry and RPKM differed between WWTPs and hospitals. Water chemistry measurements were taken multiple days a week and so to make them comparable with the hospital and WWTP wastewater RPKMs, a weekly and monthly average was calculated, respectively. Samples below the limit of detection for ammonium-N and phosphate were replaced with values half the recorded limit of detection for those assays, 0.066 mg/l and 0.0625 mg/l, respectively.

The flow diagram provided in the supplementary data (Fig. S1) provides an overview of the methodological steps.

3. Results

A total of 1687 wastewater samples were collected from 9 locations during the study period, giving rise to 207 pooled samples that were subjected to metagenomic analysis. The average (±SEM) number of raw reads generated by Illumina sequencing per sample was 157 (±4.1) million reads (range: 37–376 million reads). The sample metadata is provided in the supplementary file.

3.1. Prevalence and diversity of ARGs in hospital and community wastewater samples

Overall, a higher relative abundance (2–3-fold higher) of ARGs was detected in the hospital effluents as compared to those from the WWTPs to which the hospitals were directly connected and thereby linked by the sewage networks (Fig. 2a). Subsequent resistome analysis identified a diverse array of ARGs in both wastewater from hospitals and from WWTPs. Hospitals and WWTP influents had a higher ARG diversity than WWTP effluents (Fig. 2b).

The relative abundance of total ARGs (RPKM) among the hospital wastewaters ranged from 4607 to 12,424 at Bangor Hospital (B-H), 2682 to 13,290 in Kinmel Bay Hospital (K-H) and 3381 to 9246 in Wrexham Hospital (W-H) (Fig. S2a). Contrary to our expectation that hospitals with a larger number of beds would exhibit a higher relative abundance of ARGs, our study revealed a reverse trend. Specifically, K-H and B-H hospitals, which have fewer beds (n = 463), demonstrated a higher relative abundance than W-H with more beds (n = 804).

There was a statistically significant difference in the relative abundance of total ARGs across the three hospitals (Kruskal–Wallis $p = 0.0012$). A further post-hoc analysis showed no statistically significant difference between B-H and K-H (Dunn's test $p = 0.40$). However, there was a statistically significant difference between K-H and W-H (Dunn's test $p < 0.001$) and B-H and W-H hospitals (Dunn's test $p < 0.001$).

The relative abundance of total ARGs (RPKM) also varied among the three studied WWTPs (Fig. S2b); however, the variation was not statistically significant (Kruskal–Wallis $p > 0.05$). Specifically, the relative abundance ranged between 3154 and 5813 at Bangor WWTP (B-WWTP), 4472 and 5271 at Wrexham WWTP (W-WWTP), and 3407 and 6320 at the Kinmel Bay WWTP (K-WWTP).

Time series analysis revealed trends in the relative abundance of total ARGs (RPKM) in the WWTPs and hospitals across a 12-month sampling period (Figs. S3–S4). Each sample type exhibited distinct monthly and seasonal trends (Fig. S5). Monthly fluctuations were found in ARG counts in the B-WWTP and K-WWTP. At the B-WWTP, a notable spike in ARG counts occurred in April and July, while a drop in the levels

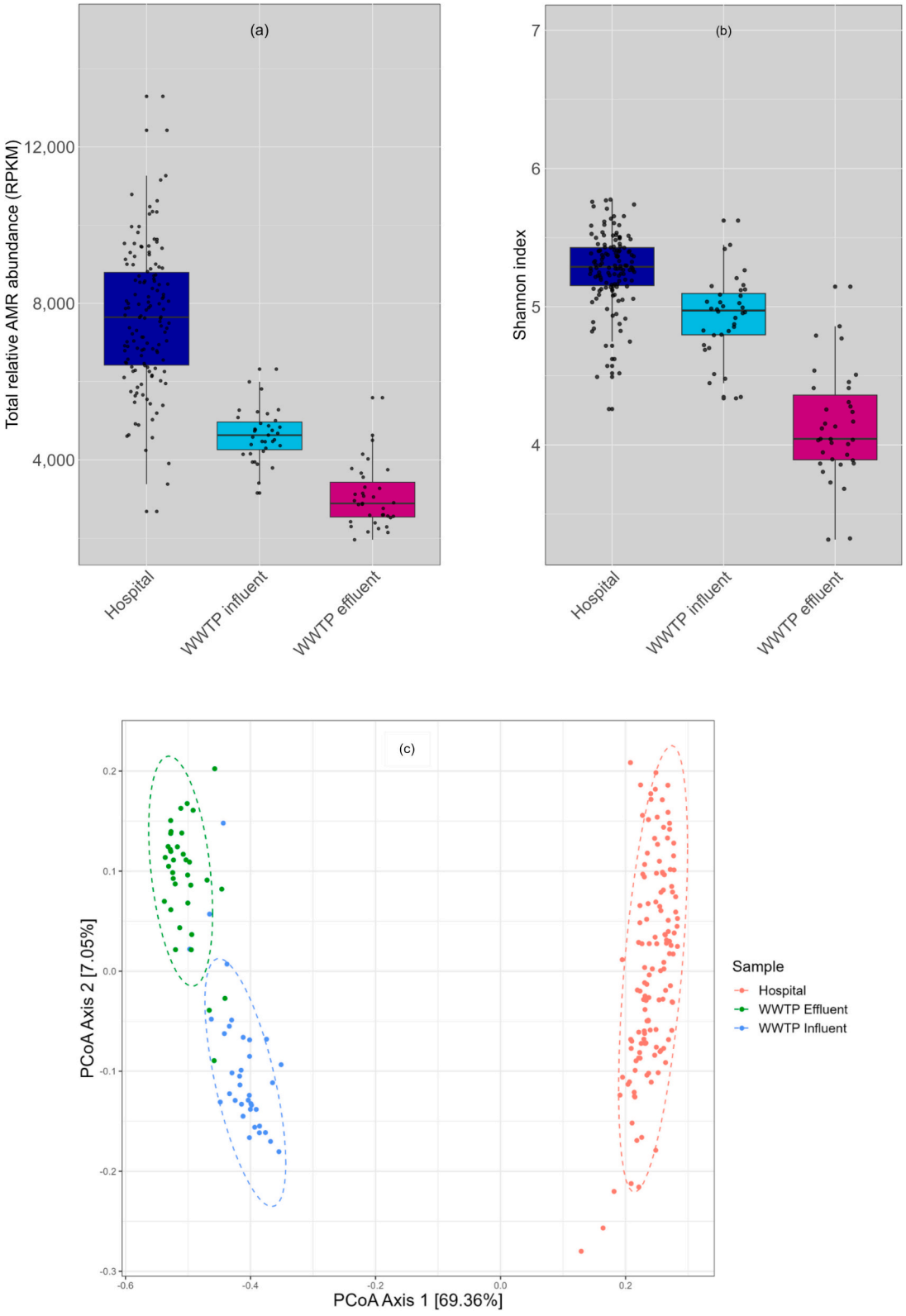


Fig. 2. (a) Boxplot displaying total AMR abundance (ARG reads per kilobase per-million mapped reads (RPKM)) per wastewater sample and (b) Boxplot displaying resistome diversity (Shannon index) stratified by sample (hospital wastewater, WWTP influent, and WWTP effluent) over a 1-year period. Each weekly (hospital) or monthly (WWTP) sample is represented by a dot with a horizontal jitter. The horizontal line shows the median and the whiskers represent the range of the data for three hospitals and three WWTPs, (c) principal coordinate analysis (PCoA) plot based on Bray-Curtis dissimilarity matrix showing compositional difference in the ARGs among hospital wastewater, WWTP influent, and effluent samples over a 1-year period. The plot considered both the presence/absence and the relative abundance of ARGs across the three sample types. Each dot represents an individual sample, with the spatial separation between dots reflecting the compositional dissimilarity between the samples.

of ARGs occurred in December in both K-WWTP and B-WWTP. The trend remained almost consistent at the W-WWTP, slightly fluctuating over time. The flow to the WWTPs (flow data is given in the supplementary file) did not appear to impact the monthly variation of the relative abundance of ARGs. Similarly, significant fluctuations in the ARG levels over time were observed in the B-H and K-H hospitals, whereas at W-H, only low fluctuations were noted.

3.2. Relationship between ARG prevalence and water chemistry

The physicochemical characteristics of hospital wastewater from the three study sites, including the influent and effluent properties at the corresponding wastewater treatment plants (WWTPs) and their temporal trends, are detailed in the supplementary data (Supplementary Table 1 and Fig. S6). A statistically significant effect of pH ($F(1, 49) = 10.45, p < 0.01$), conductivity ($F(1, 49) = 6.36, p = 0.02$), ammonium ($F(1, 49) = 48.11, p < 0.01$) and sample type (effluent or influent) ($F(1, 49) = 18.86, p < 0.01$) on relative abundance of AMR (RPKM) were observed in the WWTP linear model (Fig. 3). There were no significant interaction terms. In the hospital linear model, there was again a substantial effect of pH ($F(1, 109) = 9.86, p < 0.01$) as well as conductivity ($F(1, 109) = 4.71, p < 0.01$), ammonium ($F(1, 109) = 5.21, p = 0.02$), and site ($F(2, 109) = 8.35, p < 0.01$) on RPKM. A near-significant effect of season was detected ($F(3, 109) = 2.62, p = 0.05$). Unlike for the WWTPs, for the hospitals, there were significant interaction terms between site and ammonium ($F(2, 109) = 6.51, p < 0.01$) as well as site and turbidity ($F(1, 109) = 6.03, p < 0.01$). For ammonium, K-H showed a different trend to that of B-H and W-H, with a steep gradient of positive correlation between ammonium and RPKM (Fig. S7a). However, B-H showed a different trend for turbidity than the other two hospitals, with a positive correlation between turbidity and RPKM (Fig. S7b).

3.3. Analysis of class-level distribution of ARGs in hospital and community wastewater samples

The genes predicted to encode resistance towards aminoglycoside, β-lactam, MLS (macrolides-lincosamide-streptogramin), sulfonamide, and tetracycline classes were dominant in all three hospitals. However, their relative proportion varied according to sample type (Fig. 4). In the wastewater treatment plants, there has been a shift in the pattern, with rifampicin ARGs becoming dominant over sulphonamide ARGs.

The heatmap presented (Fig. 5) highlights the clustering of samples based on the distribution of the 50 most abundant ARG subtypes. The wastewater from hospitals and WWTPs formed two clusters based on their relative abundance. Most genes encoded resistance towards MLS (n = 10), followed by tetracycline (n = 9) and aminoglycoside and β-lactam (n = 7). The most abundant macrolide resistance ARG marker was associated with a mutation in 23S rRNA (MLS23S), the target site for macrolide drugs. Another abundant gene was *ermB*, which encodes the methylase enzyme. The bla_{OXA} gene, encoding oxacillinase, was the most abundant β-lactamase resistance encoding gene type. The *ant* gene variants, which encode aminoglycoside O-nucleotidyltransferases, and *aph* variants encoding aminoglycoside_O-phosphotransferases were the predominant genes associated with resistance to aminoglycosides. In this study, the most abundant *tet* variants coding ribosomal protection protein variants belonged to *tetM*, *tetA*, *tetC*, *tetR*, and *tetQ* types. In hospital wastewaters, genes responsible for tetracycline resistance predominantly belonged to *tetM* variants. The *tet39* and *tetR* variants were

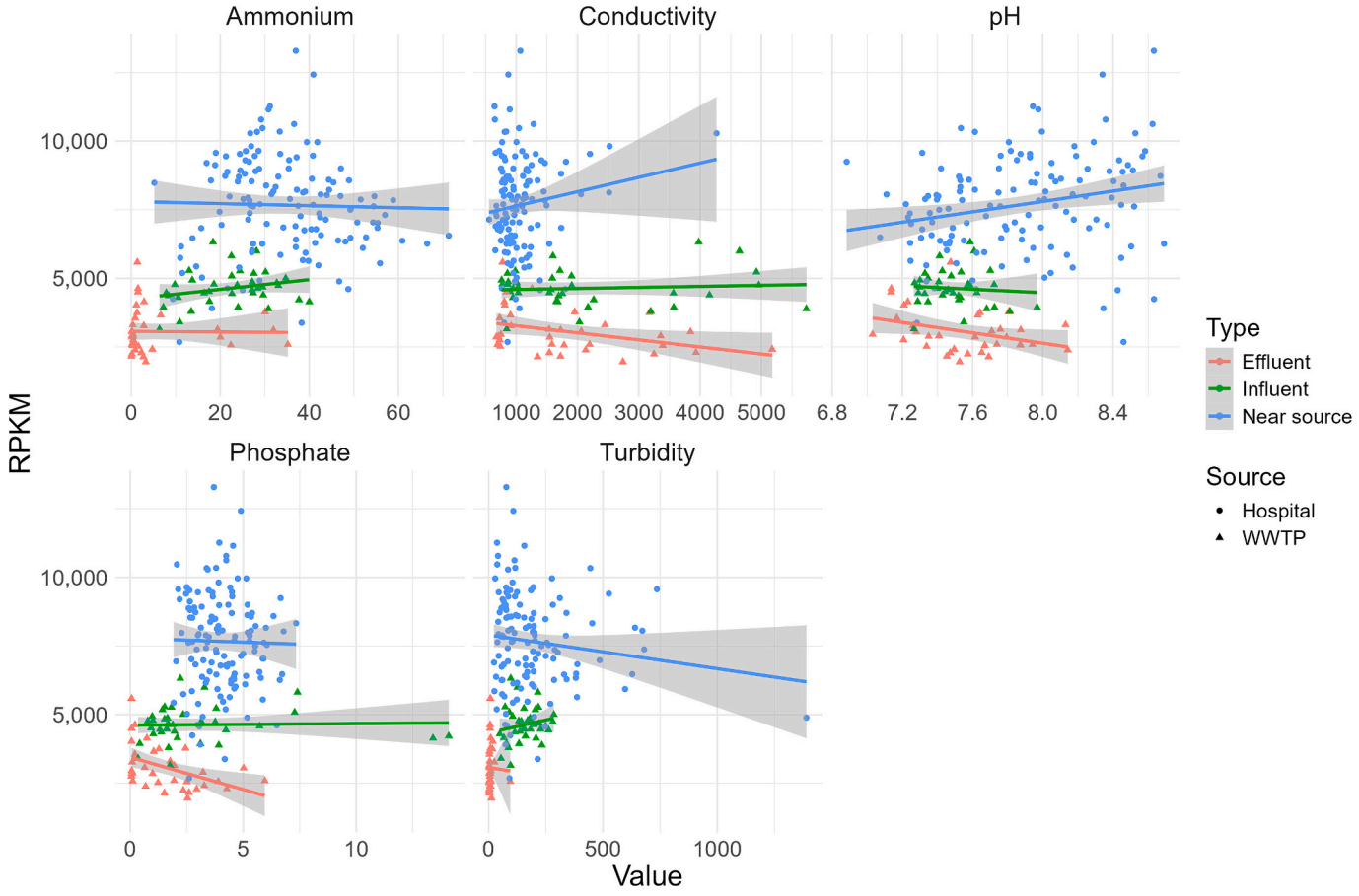


Fig. 3. Linear regressions between the different water chemistry measures assessed in this study (ammonium (mg/l), conductivity (mS/m), pH, phosphate (mg/l) and turbidity (NTU)) and ARG reads per kilobase per-million mapped reads (RPKM). Colours indicate whether the sample was taken at the WWTP effluent, WWTP influent or near source (i.e. the hospital wastewaters). Shapes indicate whether the sample is from a WWTP or a hospital.

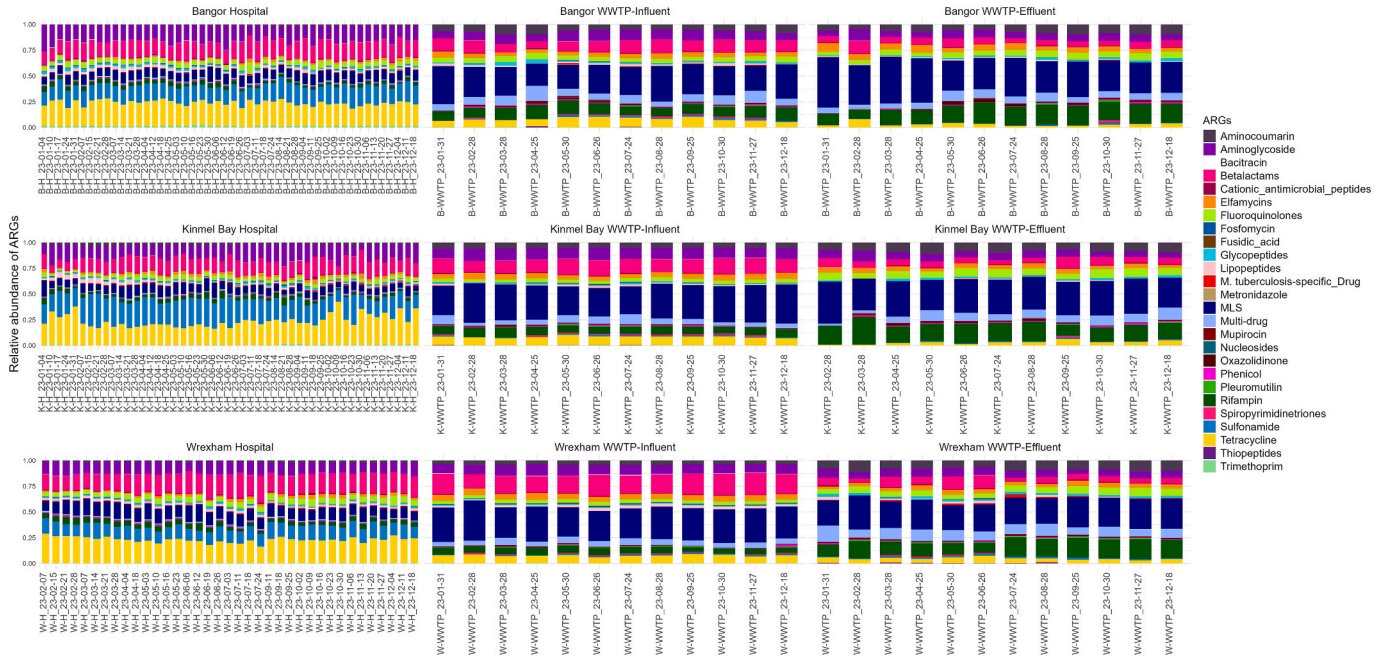


Fig. 4. Relative abundance of ARGs per class stratified by wastewater sampling site and date over a 1-year period. Hospital wastewater samples were collected weekly and those from the WWTPs monthly. The Bangor, Kinmel Bay and Wrexham WWTPs are denoted by B-WWTP, K-WWTP and W-WWTP, respectively, while the Bangor, Kinmel Bay and Wrexham hospitals are denoted by B-H, K-H and W-H, respectively. MLS, macrolide-lincosamide-streptogramin; MDR, multi-drug resistance.

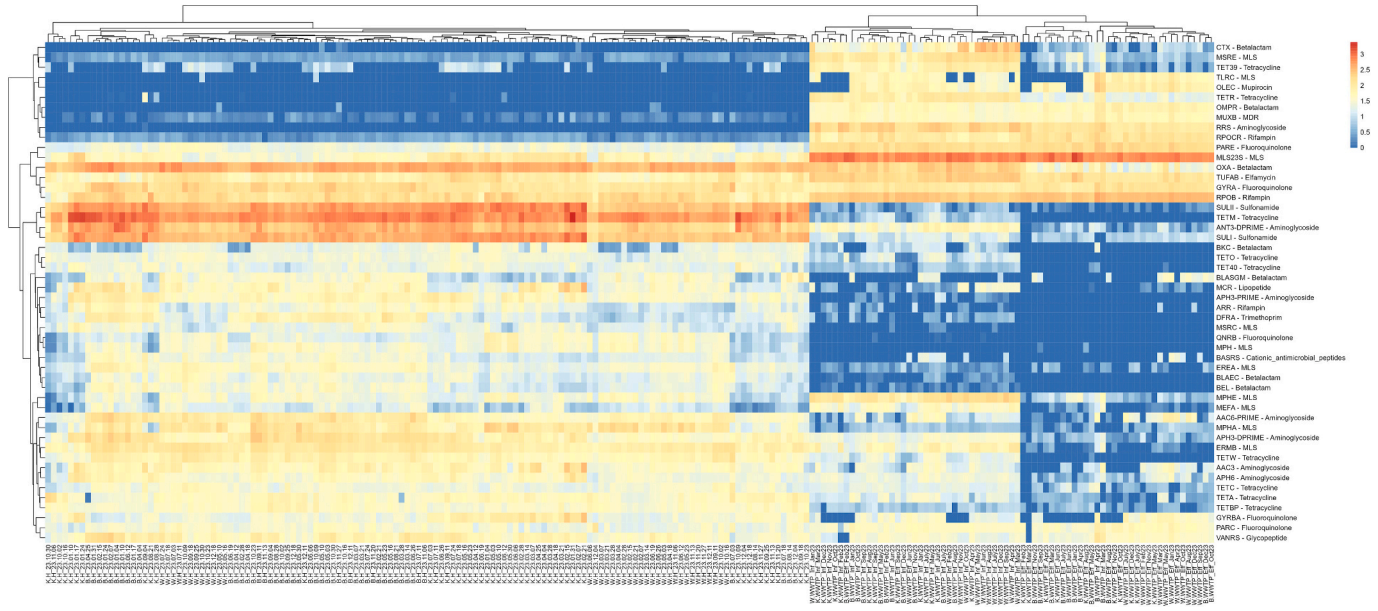


Fig. 5. Heatmap depicting clustering of the wastewater samples of the 50 most abundant ARG subtypes across three hospital and corresponding WWTP sites in North Wales, using log-transformed relative abundance RPKM values. Colour scale from red (high abundance) to blue (low abundance). Dark blue (0 on a scale) means no resistance gene detected. The Bangor, Kinmel Bay and Wrexham WWTPs are denoted by B-WWTP, K-WWTP and W-WWTP, respectively, while the Bangor, Kinmel Bay and Wrexham hospitals are denoted by B-H, K-H and W-H, respectively.

mainly confined to WWTPs and rarely found in the wastewater of the hospitals under study.

In some instances, genes encoding resistance to specific antibiotic classes were undetected or rare in certain samples. For example, genes conferring resistance to metronidazole and nucleosides were rarely observed in influent samples from the three WWTPs. Additionally, pleuromutilin resistance genes were infrequently detected in the samples tested. Many ARGs present in hospital effluents were either not detected or found in very low abundance in community wastewater. It should be noted that while the prevalence of glycopeptide-resistant genes was comparatively lower in the samples from WWTP, a significantly higher incidence of these genes was observed across all three hospital sites, with the highest observed in Kinmel Bay Hospital (Fig. 4). Similarly, phenicol, trimethoprim, and sulphonamide-resistance encoding genes had higher relative abundance in hospital wastewaters compared to WWTP influents and effluents.

PCoA analysis revealed compositional differences in resistome among the sample types (Fig. 2c). The hospital wastewaters clustered with no overlap with the samples from WWTP, indicating significant differences in their resistome composition. Overall, 255 ARG subtypes were shared between wastewater from hospitals and WWTPs (Fig. S8). However, five were unique to WWTP influents (bla_{FOX}, *dfrK*, *parEF*, *ptsL*, bla_{SCO}), and 2 were unique to effluent samples (bla_{PER}, *rm3*). These could have originated from the sewer effluent pipelines or biofilms formed inside the effluent pipes.

3.4. Analysis of the total bacterial composition and diversity in hospital and community wastewater samples

On average, the proportion of sequences attributed to bacterial genomes was higher in hospitals, accounting for 69 % of the total sequences. In contrast, the assignment of bacterial reads in WWTPs was considerably lower, with only 44 % and 36 % of sequences from influent and effluent samples, respectively, linked to bacterial genomic material. There was a statistically significant difference in the counts between hospital effluents and WWTP influents ($p < 0.001$) and no significant temporal or spatial variation in the overall abundance of bacterial phylum across the different sites and samples (Fig. 6, Fig. 7a; Kruskal-Wallis $p > 0.05$). The bacterial diversity was higher among the wastewater from WWTP compared to hospitals (Fig. 7b).

Pseudomonadota (formerly Proteobacteria) was the predominant phylum across all the samples throughout the study period, contributing to >50 % of the total bacterial reads. In the hospital settings, the most prevalent genera within the phylum were *Acidovorax*, *Acinetobacter*, *Pseudomonas*, and *Aeromonas*, whereas in WWTPs, *Acinetobacter*, *Acidovorax*, *Methylomonas*, and *Ralstonia* dominated in abundance.

Bacteroidota (formerly Bacteroidetes), Bacillota (formerly Firmicutes), and Actinomycetota (formerly Actinobacteria) were the other dominant phyla. Bacillota encompasses many genera, including *Clostridium*, *Bacillus*, *Enterococcus*, *Streptococcus*, and *Staphylococcus*, all of which are known to contain several pathogenic strains.

PCoA was performed to find differences in bacterial community (genus level) composition between wastewater from hospitals and WWTPs. Based on the proximity of ellipses, the bacterial community composition within hospital wastewater samples was notably different compared to WWTP effluent and influent samples (Fig. S9).

3.5. Co-occurrence patterns between ARG subtypes and bacterial genera: network analysis

Network analysis was performed to elucidate and visualise the co-occurrence patterns between different ARG subtypes and bacterial genera by selecting strong correlations (Spearman's $r > 0.70$, $p < 0.01$). The presence of similar abundance values between the ARGs and co-existing bacterial genera, as indicated by strong and significant correlation, can be used to predict the potential host of the ARGs. Most of the identified bacterial hosts belonged to the phylum Pseudomonadota and Actinomycetota. Most bacterial genera were speculated to carry genes resistant to MLS and aminoglycoside classes.

The network analysis of hospital wastewater samples generated 99 nodes and 188 edges, the WWTP influent samples network had 99 nodes and 202 edges, and effluents had 70 nodes and 223 edges. Network analysis of hospital wastewaters predicted 53 bacterial genera to be potential hosts to 46 ARG subtypes, conferring resistance to 11 antibiotic classes (Fig. 8, see Supplementary file 1 for correlation values and predicted hosts). *Enterococcus*, *Paracoccus*, and *Ottowia* were predicted to be potential hosts for the highest number of ARGs (n = 8) in hospital effluents. *Pseudomonas* and *Aeromonas* exhibited a strong correlation (Spearman's r > 0.7, $p < 0.01$) to ARGs encoding resistance to aminoglycosides, β-lactam and macrolides; *Streptococcus* to tetracycline ARGs (*tetO*, *tetQ*, *tetW*); *Yersinia* to trimethoprim (*dfrE*), vancomycin (*vanRA*, *vanYA*); *Enterococcus* to aminoglycosides (*aac3*, *aph2-D'*), β-lactams (bla_{TEM}, bla_{GES}, bla_{CTX}), tetracycline (*tetC*), macrolide (*mphA*), and quinolone (*qnrVC*); *Escherichia* to macrolides (*msrE*, *mphE*), β-lactams (bla_{MCA}), tetracycline (*tet39*, *tetR*), elfamycin (*tufAB*) and *Klebsiella* to tetracycline (*tetC*) and aminoglycoside (*ant6*).

Network analysis of WWTP influent samples predicted 50 bacterial genera to be potential hosts to 42 ARG subtypes, conferring resistance to nine antibiotic classes (Fig. 8b). In the influent samples, a strong correlation was found with clinically relevant ARGs and bacterial genera like *Acinetobacter*, *Pseudomonas*, *Aeromonas*, *Mycobacterium*, *Streptococcus*, *Klebsiella* and *Clostridium* (see Supplementary file). *Acinetobacter*, *Actinobacteria* and *Cloacibacterium* were predicted as hosts carrying the highest number of ARGs (n = 8) conferring resistance to aminoglycoside, tetracycline, macrolide, elfamycin, β-lactam, and colistin. *Bacteroides* were found to be hosts for seven ARGs. Other primary hosts were *Pseudomonas*, *Mycobacterium*, *Ottowia*, *Tessaracoccus*, *Clostridium*,

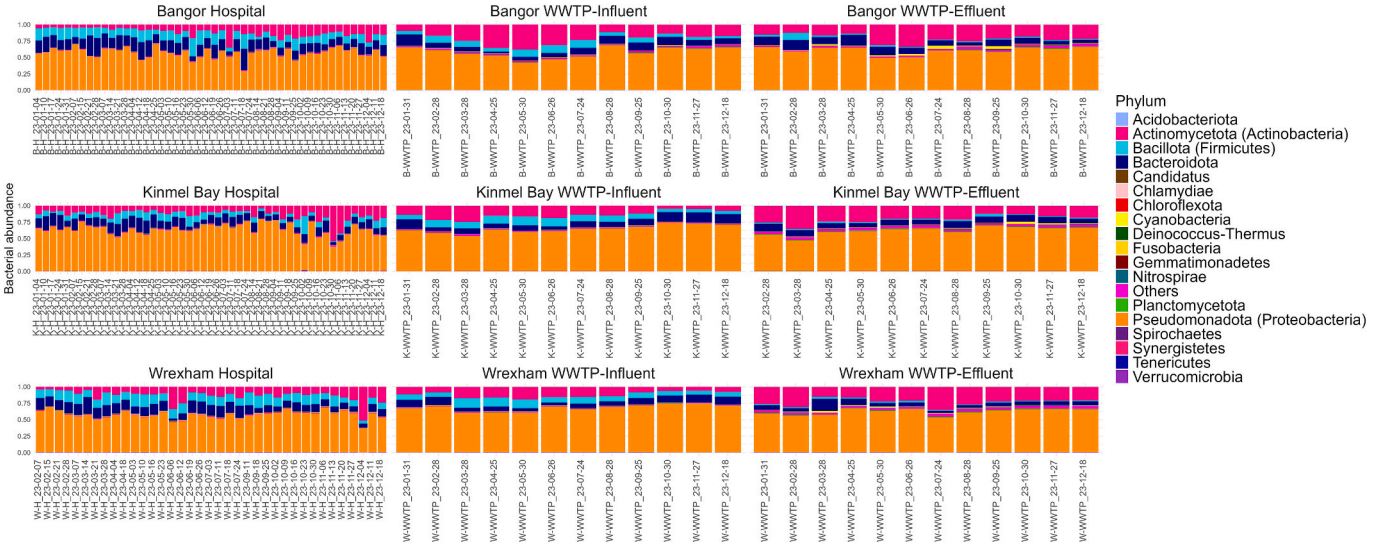


Fig. 6. Stacked column chart showing changes in bacterial diversity over time at the phylum level in wastewater over a 1-year period. Relative abundance of bacteria among the wastewater samples from three hospitals and corresponding WWTP sites based on abundance. Hospital wastewater samples were collected weekly and those from the WWTPs monthly. The Bangor, Kinmel Bay and Wrexham WWTPs are denoted by B-WWTP, K-WWTP and W-WWTP, respectively, while the Bangor, Kinmel Bay and Wrexham hospitals are denoted by B-H, K-H and W-H, respectively.

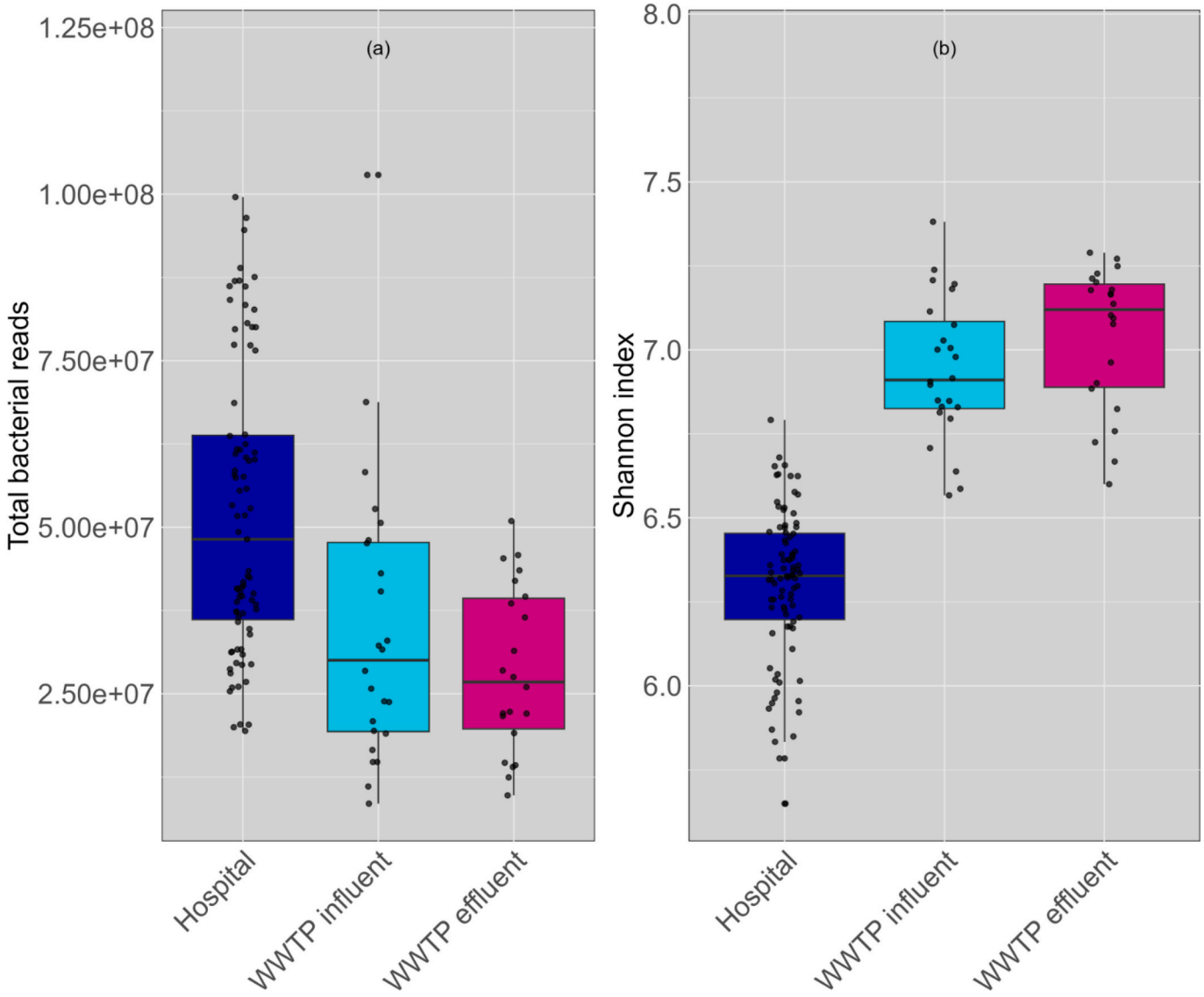


Fig. 7. Boxplots displaying (a) total bacterial reads per sample and (b) bacterial diversity (Shannon index) in wastewater stratified by sample type across three hospitals and WWTPs (untreated influent and treated effluent) over a 1-year period. Each sample is represented by a dot with a horizontal jitter. The horizontal line shows the median and the whiskers represent the range of the data.

Bifidobacterium, *Prevotella*, *Moraxella*, *Mycolicibacterium*, *Rhodoferax*, etc. *Mycobacterium* and *Clostridium* spp. exhibited a strong correlation with rifampicin, macrolide, and tetracycline ARGs.

Acinetobacter, *Arcobacter*, *Flavobacterium*, *Rhodococcus*, *Brevundimonas*, *Prevotella*, *Rhizobium*, *Rhizobacter*, *Streptomyces*, *Mycobacterium*, *Simplicispira*, *Blautia*, *Thaura*, *Variovorax*, and *Sphaerotilus* were the primary host genera in WWTP effluent samples. Notably, *Acinetobacter*, *Prevotella*, and *Mycobacterium* hosts were common in both WWTP influent and effluent wastewater. In the treated effluents, *Acinetobacter* was predicted to be the host with the highest number of ARGs (n = 15) conferring resistance to tetracycline (n = 4), aminoglycosides (n = 2), macrolide (n = 4), β-lactam (n = 3), rifampicin (n = 1) and elfamycin (n = 1) (Fig. 8c). *Rhodococcus* was also predicted to harbour 15 ARGs, followed by *Flavobacterium*, *Rhizobacter*, *Streptomyces*, etc. *Mycobacterium* showed a strong correlation with macrolide and tetracycline ARGs.

We also examined the correlation between ARGs and specific opportunistic human pathogens. *E. coli* exhibited strong correlations with many ARGs in hospital and WWTP influent samples (see Supplementary file). Opportunistic human pathogens such as *Klebsiella oxytoca* and *Aeromonas hydrophila* were also found to correlate with many ARGs in hospital wastewaters. In contrast, the bacterial host species identified in the WWTP effluents were primarily non-pathogenic.

3.6. Removal of ARGs and bacterial community in WWTPs: influent vs effluent comparative analysis

Both the treated (effluent) and untreated (influent) wastewater from WWTPs were compared to evaluate the efficiency of ARG removal in the wastewater treatment process. The ARG alpha diversity (Shannon index) decreased significantly ($p < 0.05$) in the treated effluents compared to the influents (Fig. 2b). Each of the three WWTPs exhibited distinct capabilities in removing ARGs, as demonstrated by varying removal efficiencies (Fig. S3).

In the K-WWTP and B-WWTP, there was a significant reduction in the detected relative ARG counts (RPKM) after treatment (Kruskal-Wallis test $p < 0.05$). However, the removal efficiency of studied WWTPs was not stable and there was temporal variation in the percentage removal of ARGs (Fig. S3). The removal efficiency of W-WWTP was significantly lower compared to the other WWTPs. In W-WWTP, the total ARG counts were reduced (ranging from 0.1 to 47 %) during all the months except

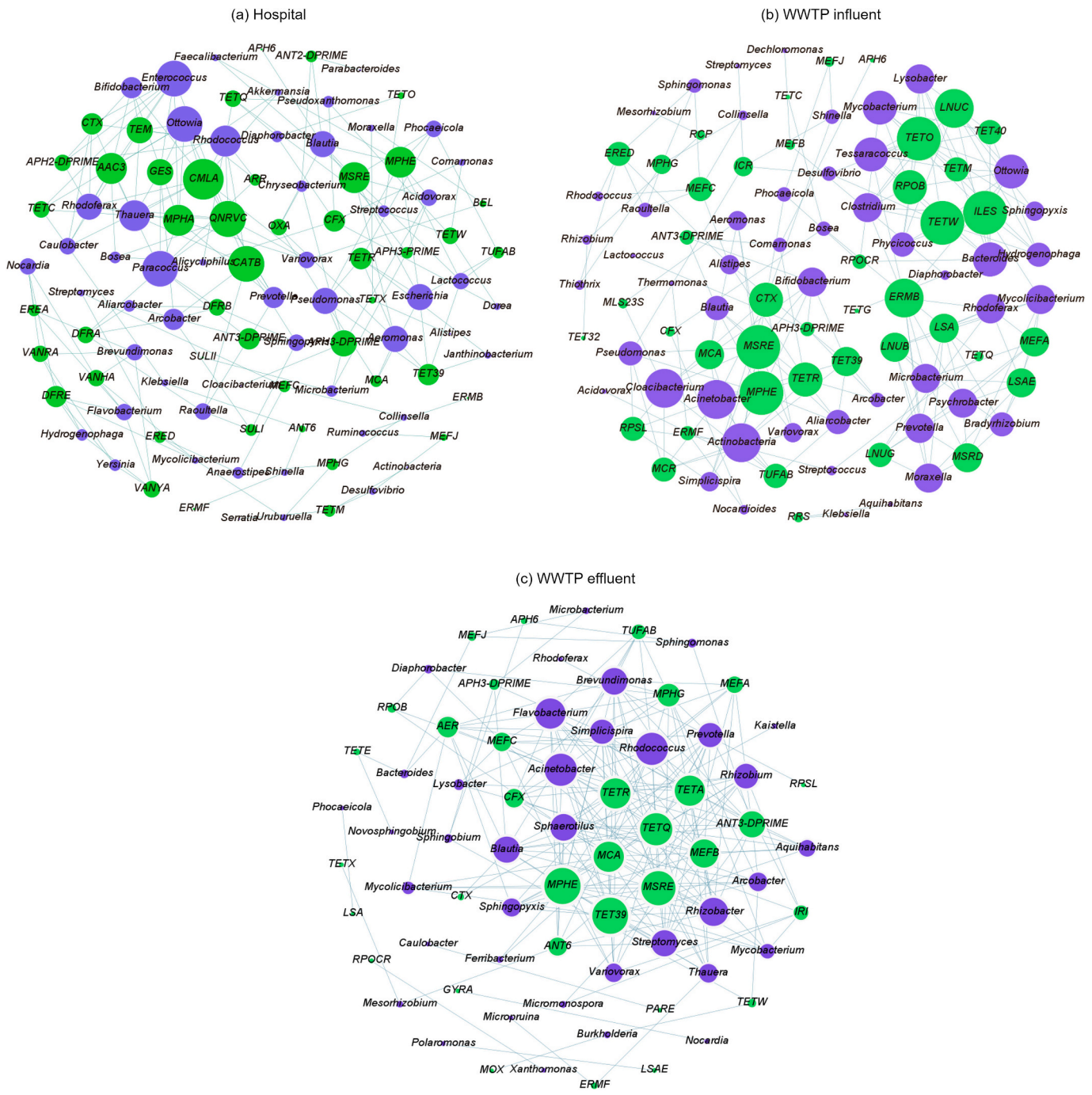


Fig. 8. Network analysis of (a) hospital (b) WWTP influent and (c) WWTP effluent wastewater samples displaying the co-occurrence pattern between bacterial genera and ARGs in the samples based on Spearman's correlation analysis. An edge represents a strong (Spearman's $r > 0.7$) and significant ($p < 0.01$) correlation. The size of each node is proportional to the number of connections. The purple nodes represent bacterial genera, while the green nodes represent ARG subtypes.

January, where only a 23 % enrichment (higher prevalence) of ARG counts was observed. In K-WWTP and B-WWTP, a reduction rate of total ARGs ranged from 10 to 62 % and 15 to 52 %, respectively. The highest reduction was seen during November in B-WWTP, October in W-WWTP, and June in K-WWTP.

An increased relative abundance of certain ARG classes during wastewater treatment was also observed, as indicated by higher levels in the effluents compared to influents. The overall class-wise removal efficiency was the least in W-WWTP, and there was an increased relative abundance of ARGs associated with aminocoumarins, fluoroquinolones, glycopeptides, rifampicin, sulfonamides, and MDR. An increase in the relative abundance of the above classes was also observed in K-WWTP-treated effluents.

The study also assessed the efficacy of WWTPs in reducing bacterial genera that include species with clinical significance, including the ESKAPE group (Fig. S10). There was a noticeable reduction in the relative *Acinetobacter* levels in all the effluents. There was an increase in *Mycobacterium* levels during most months across all three WWTPs. Additionally, the relative abundance of *Pseudomonas* increased in the B-WWTP effluents, while it remained unchanged in the influents and effluents of K-WWTP and W-WWTP.

4. Discussion

4.1. ARG composition and diversity in hospital and community wastewater samples

In this year-long, high-frequency study across multiple sites, hospital-derived effluents consistently showed a higher abundance of ARGs compared to WWTPs. These findings are supported by previous shorter-term studies by Rodriguez-Mozaz et al. (2015) and Kelly et al. (2023), who also reported high ARG loads in hospital wards and effluent and a qPCR-based study in Dutch cities where effluent from 2 hospitals had 0.4–1.8-fold higher ARG concentration compared to community wastewater receiving no hospital effluent (Paulus et al., 2019). Recent studies from Europe have also reported that hospital effluents harbour high levels of multi-drug resistant pathogens such as extended-spectrum beta-lactamases (ESBL)-producing coliforms, vancomycin-resistant *Enterococci* (VRE) (Lépesová et al., 2020) and carbapenem-resistant *Klebsiella pneumoniae* (David et al., 2019). The elevated presence of ARGs in hospital wastewaters supports a higher incidence of AMR within healthcare environments (Vaz-Moreira et al., 2016). The novelty of our metagenomic study is that the ARG trends are consistent over time, reflecting the endemic nature of high ARG loads in hospital wastewater. Considering the rapid transit times from the hospitals to the main WWTP and the potential for direct discharge to the environment via combined sewer overflows (CSOs), it also suggests that hospital effluent should be treated before entering the main sewage network. The most efficient methods and the associated costs involved in treating wastewater at healthcare facilities must be identified to alleviate the risk of contaminating coastal waters. Surface plasma treatment has proven highly effective (90 %) in eliminating ARGs and ARBs from hospital wastewater (Song et al., 2021), while cold plasma has shown significant success in degrading antibiotics in hospital wastewater in Vietnam (Nguyen et al., 2021).

Our study also highlighted that ARG loads varied among the hospitals and WWTPs, possibly reflecting differences in waste-water chemistry, antibiotic prescribing and stewardship practices, infection and control practices and local resistance patterns within each healthcare facility and the wider community. Specifically, our analysis identified a significant trend in which chemical markers in hospital wastewater and WWTPs exhibited opposing behaviours with AMR, though this pattern was not consistent across all parameters. These contrasting behaviours may primarily result from differences in wastewater composition. However, detailed studies are necessary to elucidate the factors driving this variation. Hospital wastewater is characterized by the presence of specialized contaminants such as antibiotics, heavy metals, biocides, and other cleaning agents, creating selection pressure for AMR (Pant et al., 2022) and diverse microbial communities that support ARG spread (Uluseker et al., 2021). The higher pH levels in hospital wastewater samples may be due to the flushing of disinfectants and detergents with alkaline properties into the wastewater system. While these elevated pH levels might suggest the possibility of a pre-treatment step, to the best of our knowledge, no on-site pre-treatment is conducted at the hospital facilities. In contrast, municipal wastewater encompasses a broader array of contaminants derived from domestic, industrial, and commercial sources. While it does contain pharmaceuticals and cleaning agents, the concentrations and diversity of these compounds are generally lower and more diluted compared to hospital effluents. Our findings indicate that, in hospital effluents, increased pH correlates with elevated ARG abundance. This may be attributed to the selective pressure exerted by compounds like biocides, which not only elevate pH but also select resistant bacteria (Chukwu et al., 2023). In WWTPs, an increased level of ammonia was found to be associated with higher ARG counts (RPKM). Ammonia stress has been reported to significantly enrich the horizontal gene transfer of ARGs in sludge (Zhang et al., 2020). Higher relative ARG counts at some sites may also be attributed to biofilm accumulation in sewer pipes, which serve as hotspots for ARGs and AMR bacteria, and also differences in effluent holding and pumping patterns (Ory et al., 2019; Luo et al., 2022).

The global sewage study aligned with our results, reporting high levels of ARGs conferring resistance towards aminoglycosides, β-lactams, and tetracyclines (Hendriksen et al., 2019). In Ireland, hospital wastewater showed high levels of tetracycline, macrolide, and β-lactam ARGs, though fluoroquinolone resistance was more prevalent than aminoglycosides likely due to differing antibiotic usage patterns (Kelly et al., 2023). Similarly, urban sewage from China found tetracycline, β-lactam, and aminoglycoside resistance genes to account for 50 % of the resistome (Su et al., 2017).

The prevalence of ARGs conferring resistance to clinically relevant antibiotic classes such as MLS, β-lactam, aminoglycoside, sulfonamide, and tetracycline in our hospital wastewater and samples from WWTPs poses a severe public health concern, given the critical role these antibiotics play in medical treatments and their potential for direct discharge into recreational waters. The latest culture-based antibiotic resistance report by Public Health Wales (2023a, 2023b) aligns with our metagenomic findings, which show a high prevalence of resistance towards beta-lactams, aminoglycosides, and MLS antibiotics in hospital settings in Wales. The report notes significant beta-lactam resistance in both *E. coli* and non-*E. coli* from urine samples, aminoglycoside resistance in blood and urine cultures, and MLS resistance in MRSA blood culture isolates. The *erm* gene, known for methylating 23S rRNA and conferring erythromycin resistance, was the abundant marker of macrolide resistance in wastewater (Ero et al., 2019). It is widely distributed in different environments and associated with mobile genetic elements (Araújo et al., 2010; Mutuku et al., 2022). Many ARGs in hospital effluents were absent or scarce in community wastewater, likely due to the dilution effect (Sims et al., 2023). This highlights the limitations of metagenomics in capturing the complete ARG profile at centralised WWTPs and the importance of near-source testing. Increasing sequencing depth could help but may not be cost-effective. The tetracycline resistance gene variant *tetM*, primarily found in hospitals, is common in Gram-positive pathogens (Doherty et al., 2000). Glycopeptide, phenicol, trimethoprim, and sulphonamide resistance genes were persistent in hospital wastewater, suggesting they have become endemic. Overall, our results support the use of metagenomic testing of hospital wastewater to (i) provide a comprehensive overview of the diversity and abundance of ARGs circulating within healthcare facilities, (ii) act as an early warning system to detect the emergence and spread of clinically relevant ARGs within hospitals; and (iii) identify the predominant bacterial hosts carrying ARGs in hospital wastewater to guide targeted surveillance efforts and inform evidence-based interventions to mitigate AMR spread.

4.2. Bacterial composition and diversity in hospital and community wastewater samples

Overall, bacterial diversity was consistently higher in the WWTPs wastewater than in hospitals. We ascribe this to the wider sources of inputs (e.g., industrial inputs, road-runoff) and greater population that contribute to urban wastewater compared to those from hospitals. Pseudomonadota (formerly Proteobacteria) was the dominant bacterial group within our study. This phylum includes the majority of the ESKAPE (i.e., *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) group of pathogens (Tacconelli et al., 2018). This bacterial group poses significant challenges for water disinfection due to their outer membranes and ability to migrate across environments (Bairán et al., 2020). They are prevalent throughout the wastewater treatment process (Che et al., 2019) and form a key part of the microbiome in activated sludge within WWTPs (Yoo and Lee, 2021). Other dominant bacterial phyla in our samples included Bacteroidota, Bacillota, and Actinomycetota, which are consistent with previous findings from hospital wastewater in Ireland (Kelly et al., 2023) and urban

sewage systems in the USA, South Korea, and China (Su et al., 2017; Baral et al., 2018; Yoo and Lee, 2021).

4.3. Co-occurrence between ARG subtypes and bacterial genera

It is crucial to predict the host bacterium associated with the ARGs to better assess the health risks posed by ARG-carrying organisms. Here, we capitalised on network analysis to track potential bacterial hosts for ARGs in wastewater by examining non-random co-occurrence patterns. Many studies have employed this approach to identify potential ARG hosts (Su et al., 2015; Pehrsson et al., 2016; Knight et al., 2024; Wang et al., 2024). While strong and significant correlations between ARGs and bacterial genera may suggest potential hosts, it is to be noted that this approach has limitations, particularly for plasmid-borne ARGs, which are more prone to horizontal gene transfer (HGT). It must also be emphasised that the correlations observed between ARGs and bacterial genera in this network analysis are not solely associated with pathogenic strains. Non-pathogenic strains from the same species or genus also contribute to these correlations. We showed that the composition of ARG-carrying bacteria in wastewater varied significantly between hospitals and WWTPs. *Enterococcus*, *Paracoccus*, and *Ottowia* were predicted to be among the top ARG hosts in hospital wastewater, whereas *Acinetobacter*, *Actinobacteria*, and *Cloacibacterium* predominated in WWTP influents. The WWTP-treated effluent samples also exhibited a shift in certain ARG hosts compared to untreated influent water. *Acinetobacter* was, however, identified as one of the common predominant hosts in both the WWTP influents and effluents in our study. In recent studies, *Acinetobacter*, *Pseudomonas*, and *Arcobacter* were the top ARG hosts in influent, while *Acinetobacter*, *Pseudomonas*, and *Enterobacter* spp. dominated both influent and effluent in China and Korea (Raza et al., 2022; Li et al., 2024). Network analysis is a prediction-based approach for identifying potential hosts of these ARGs. In the future, we plan to perform a detailed analysis to identify pathogens carrying ARGs by constructing metagenome-assembled genomes (MAGs).

We found an association of ARGs with genera containing opportunistic pathogens, including *Acinetobacter*, *Clostridium*, *Enterococcus*, *Mycobacterium*, *Pseudomonas*, and *Klebsiella* spp. Of these, *Acinetobacter*, *Pseudomonas*, and *Klebsiella* spp. are known to survive wastewater treatment and be detected in WWTP effluent, reflecting their ability to rapidly adapt to new environmental compartments (Bairán et al., 2020). *Acinetobacter* and *Pseudomonas* are known to exhibit intrinsically reduced susceptibility to several classes of antibiotics (Lupo et al., 2018). A recent study reported that six primary pathogens linked to resistance-related deaths; *E. coli*, *S. aureus*, *K. pneumoniae*, *S. pneumoniae*, *A. baumannii*, and *P. aeruginosa* accounted for approximately 929,000 deaths directly attributed to AMR, in 2019 alone (Murray et al., 2022). When the species level correlation was analysed, we found *E. coli* to be one of the host species for many ARGs in wastewater from hospitals and WWTPs. The presence of these pathogenic bacteria harbouring ARGs that can resist clinically relevant antibiotics in the wastewater poses a serious risk, particularly from a One Health perspective. Our data also suggest that these organisms could be specifically targeted within public health surveillance programmes due to their prevalence and high potential risk.

4.4. Removal of ARGs and bacterial community in WWTPs

Our study found that wastewater treatment only partially removed various ARG types, with efficiency varying between WWTPs and seasons. It is important to emphasise that our findings relate to the detection of distinct ARGs in hospitals and WWTPs rather than their quantitative levels. The reduction rates (23–67 %) were similar to those in the Netherlands (Paulus et al., 2019) but lower than other studies (Rafraf et al., 2016; Pallares-Vega et al., 2019). This variability is likely due to differences in plant design, treatment conditions, operational parameters, and ARG types (Rodriguez-Mozaz et al., 2015; Li et al., 2022; Wang and Chen, 2022). Despite tertiary UV treatment at Bangor and Wrexham WWTPs, ARGs persisted in effluents, potentially due to biofilms in sewer pipelines or microplastics that protect MDR bacteria from UV light (Luo et al., 2022; Manoli et al., 2022).

Our study observed an increased relative abundance of certain ARG classes, such as aminocoumarins, fluoroquinolones, glycopeptides, rifampicin, sulfonamides, and MDR, in effluents. Some other studies have also noted a relative enrichment of some ARGs in WWTPs (Rodriguez-Mozaz et al., 2015; Lee et al., 2017). The relative enrichment of ARGs in WWTPs can be linked to the presence of favourable conditions like temperature and pH for the persistence of microbes carrying these ARGs (Berendonk et al., 2015). Studies have shown that treatments, such as UV, can enrich specific resistance genes (Guo et al., 2013), while others report high levels of ARGs encoding MDR and bacitracin in effluents (Li et al., 2024). The persistence/enrichment of specific ARGs in effluents may also be attributed to their intrinsic role within the environmental microbial community, which is crucial for survival (Li et al., 2024). Genes related to MDR, responsible for encoding efflux pumps, are inherently present in bacteria (Webber et al., 2009). This intrinsic presence underlines their significant role in bacterial adaptability and persistence in various environments. Moreover, the nutrient-rich environment within a WWTP is ideal for microbial growth and may facilitate the exchange of resistance genes among the microbial communities in the wastewater (Amos et al., 2014).

We observed a reduction in certain clinically relevant bacterial genera, but *Mycobacterium* and *Pseudomonas* were relatively abundant in the effluents. Previous studies also highlight these genera as dominant in WWTP sludge and effluents (Yoo and Lee, 2021).

Based on our relative abundance data, while there is evidence of a reduction in ARGs and antibiotic-resistant bacteria (ARBs) during wastewater treatment, it is important to acknowledge that WWTPs may still discharge a wide range of microbial pollutants into the environment, contaminating the receiving water bodies. This is of significance given the proximity of the discharge points to major sites of recreational activity and commercial shellfish harvesting areas. This highlights the need for quaternary treatments like Fenton oxidation and plasma methods, which have been shown to effectively reduce ARBs and ARGs from wastewater (Hazra et al., 2024; Ferro et al., 2016; Nguyen et al., 2021; Song et al., 2021). Most conventional WWTPs do not fully remove contaminants, allowing ARBs and ARGs to be reintroduced into rivers, lakes, and coastal waters, contributing to ARGs and MGEs in the environment (Levallois and Villanueva, 2019; Quintela-Baluja et al., 2019). Monitoring ARGs and pathogens in effluents is hence crucial for health risk assessment, though DNA detection alone provides no indication of the viability of the organisms carrying the genes. Future studies should include viability assays to refine the potential risk. Our work suggests that the AMR profile was relatively consistent over a 1-year period, suggesting that this assessment could be done on a 6-month or annual basis.

4.5. Release of ARG carrying bacteria via combined sewer overflows

All of the WWTPs studied here release untreated wastewater into the environment during periods of high rainfall, providing a direct route for hospital effluent to enter the environment (WPCCEIC, 2022). Furthermore, the lack of treatment and rapid transit time through the sewer network from the hospital to the WWTP (0.1 to 2 h) means that a large proportion of the microorganisms present will still be viable. Currently, there is no statutory requirement for water companies to measure the volume of raw effluent discharged from WWTPs, thereby preventing a quantitative assessment of the importance of CSOs in ARG release to the environment. However, the number of CSO discharge events and their duration is recorded and was found to be 35, 65, and 99 events per year for the Wrexham, Kinmel Bay and Bangor WWTPs lasting 13.6, 30.1 and 39.1 cumulative discharge days, respectively (i.e. raw wastewater released for 3.7–10.7 % of the year) (The Rivers Trust, 2024). This high

frequency suggests that CSOs are likely to make a significant contribution to the environmental burden of ARGs. Our findings also support the future need to routinely monitor ARGs and ARBs in both WWTP influent (i.e., CSO) and effluent waters, alongside the introduction of flow gauges on CSOs to enable the actual ARG load to be quantified. This is especially important given that the frequency of CSO releases is predicted to increase with climate change, further contributing to AMR and pathogen release into the environment (Honda et al., 2020; Perry et al., 2023). It should also be noted that the introduction of quaternary treatment will have no impact on the ARGs and ARB release via CSO discharges, suggesting the need to introduce stormwater holding tanks or wastewater disinfection at hospitals to reduce the ARG volume and load in CSO discharges.

5. Conclusions

WBE, coupled with metagenomics, proved effective in providing a comprehensive overview of the diversity and type of ARGs circulating within the hospitals and the community at the regional level, as reflected in the analysis of wastewater at key WWTPs. Our findings suggest that WWTPs and hospital wastewaters are reservoirs of clinically relevant bacteria and ARGs, underscoring the relevance of routine wastewater surveillance as a critical component of environmental and public health risk evaluation. Data presented here can be used as evidence by public health organisations and government to draft necessary policy interventions. Implementation of pre-treatment measures for hospital waste before discharge into communal sewer systems should be a priority, to mitigate and reduce the spread of AMR into the natural environment. WWTPs were unable to completely remove ARGs and bacterial pathogens from wastewater, with variations in removal efficiency observed among WWTPs. Hence, it is imperative to thoroughly investigate the design of WWTPs and the treatment strategies adopted to identify if more effective treatment methods could be implemented in accordance with international guidelines already established for monitoring and managing AMR and pathogens in WWTPs.

CRediT authorship contribution statement

Reshma Silvester: Writing – review & editing, Visualization, Formal analysis. **William B. Perry:** Writing – review & editing, Formal analysis. **Gordon Webster:** Writing – review & editing, Methodology. **Laura Rushton:** Writing – review & editing, Methodology. **Amy Baldwin:** Writing – review & editing, Formal analysis. **Daniel A. Pass:** Writing – review & editing, Software. **Nathaniel Healey:** Data curation. **Kata Farkas:** Writing – review & editing, Conceptualization. **Noel Craine:** Writing – review & editing. **Gareth Cross:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Peter Kille:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Andrew J. Weightman:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Davey L. Jones:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Acknowledgements

The project was funded by the HM Treasury Shared Outcome Fund under the Pathogen Surveillance in Agriculture, Food and Environment (PATH-SAFE) program Workstream 2a. Funding for the sampling infrastructure was provided by the Welsh Government under the Wastewater Monitoring Programme in Wales programme (contract number C525/2021-2024). We thank Tony Harrington and Ian Trick at Dŵr Cymru Welsh Water for access to sampling sites and subsequent sample collection. We thank the team members of the Genome hub at Cardiff University and Wales Gene Park for help with metagenomic sequencing. This work would not have been possible without the help of the estates departments at the three hospitals evaluated in this study and the support of Robin Howe and Margaret Heginbothom from Public Health Wales. The graphical abstract was created with BioRender.com.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.178403.

Data availability

Data will be made available on request.

References

Amos, G.C., Hawkey, P.M., Gaze, W.H., Wellington, E.M., 2014. Wastewater effluent contributes to the dissemination of CTX-M-15 in the natural environment. J. Antimicrob. Chemother. 69, 1785–1791. https://doi.org/10.1093/jac/dku079.

Araújo, C., Torres, C., Silva, N., Carneiro, C., Gonçalves, A., Radhouani, H., Correia, S., da Costa, P.M., Paccheco, R., Zarazaga, M., Ruiz-Larrea, F., Poeta, P., Igrejas, G., 2010. Vancomycin-resistant enterococci from Portuguese wastewater treatment plants. J. Basic Microbiol. 50, 605–609. https://doi.org/10.1002/jobm.201000102.

ARC, 2022. Policy Briefing: The Global Action Plan on AMR at a Crossroads: Insights from the WHO's Comprehensive Review. Antibiotic Resistance Coalition, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA.

Ashiru-Oredope, D., Cunningham, N., Casale, E., Muller-Pebody, B., Hope, R., Brown, C. S., Hopkins, S., 2023. Reporting England's progress towards the ambitions in the UK action plan for antimicrobial resistance: the English surveillance programme for antimicrobial utilisation and resistance (ESPAUR). J. Antimicrob. Chemother. 78 (10), 2387–2391. https://doi.org/10.1093/jac/dkad248.

Bairán, G., Rebollar-Pérez, G., Chávez-Bravo, E., Torres, E., 2020. Treatment processes for microbial resistance mitigation: the technological contribution to tackle the problem of antibiotic resistance. Int. J. Environ. Res. Public Health 17, 8866. https://doi.org/10.3390/ijerph17238866.

Baral, D., Speicher, A., Dvorak, B., Admiraal, D., Li, X., 2018. Quantifying the relative contributions of environmental sources to the microbial community in an urban stream under dry and wet weather conditions. Appl. Environ. Microbiol. 84, e00896–e00918. https://doi.org/10.1128/AEM.00896-18.

Bastian, M., Heymann, S., Jacomy, M., 2009. Gephi: an open-source software for exploring and manipulating networks. In: Third International AAAI Conference on Weblogs and Social Media, vol. 3, pp. 361–362. https://doi.org/10.1609/icwsm.v3i1.13937.

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. https://doi.org/10.1038/nrmicro3439.

Bonin, N., Doster, E., Worley, H., Pinnell, L.J., Bravo, J.E., Ferm, P., Marini, S., Prosperi, M., Noyes, N., Morley, P.S., Boucher, C., 2023. MEGAREs and AMR++, v3.0: an updated comprehensive database of antimicrobial resistance determinants and an improved software pipeline for classification using high-throughput sequencing. Nucleic Acids Res. 51, D744–D752. https://doi.org/10.1093/nar/gkac1047.

Che, Y., Xia, Y., Liu, L., Li, A.D., Yang, Y., Zhang, T., 2019. Mobile antibiotic resistome in wastewater treatment plants revealed by nanopore metagenomic sequencing. Microbiome 7, 44. https://doi.org/10.1186/s40168-019-0663-0.

Chukwu, K.B., Abafe, O.A., Amoako, D.G., Essack, S.Y., Abia, A.L., 2023. Antibiotic, heavy metal, and biocide concentrations in a wastewater treatment plant and its receiving water body exceed PNEC limits: potential for antimicrobial resistance selective pressure. Antibiotics 12 (7), 1166. https://doi.org/10.3390/antibiotics12071166.

Csárdi, G., Nepusz, T., Traag, V., Horvát, S., Zanini, F., Noom, D., Müller, K., 2024. igraph: Network Analysis and Visualization in R. R Package Version 2.0.3. https://doi.org/10.5281/zenodo.7682609. https://CRAN.R-project.org/package=igraph.

Danecek, P., Bonfield, J.K., Liddle, J., Marshall, J., Ohan, V., Pollard, M.O., Li, H., 2021. Twelve years of SAMtools and BCFtools. Gigascience 10, giab008. https://doi.org/10.1093/gigascience/giab008.

David, S., Reuter, S., Harris, S.R., Glasner, C., Feltwell, T., Argimon, S., Abudahab, K., Goater, R., Giani, T., Errico, G., Aspbury, M., Sjunnebo, S., EuSCAPE Working Group, ESGEM Study Group, Feil, E.J., Rossolini, G.M., Aanensen, D.M., Grundmann, H., 2019. Epidemic of carbapenem-resistant *Klebsiella pneumoniae* in Europe is driven by nosocomial spread. Nat. Microbiol. 4, 1919–1929. https://doi.org/10.1038/s41564-019-0492-8.

Dey, P., Parai, D., Hossain, S.T., Mukherjee, S.K., 2023. The aftermath of corona virus disease on antimicrobial resistance across low- and middle-income countries. Univ. Sci. 28, 183–199. https://doi.org/10.11144/Javeriana.SC282.taoc.
Di Tommaso, P., Chatzou, M., Floden, E.W., Barja, P.P., Palumbo, E., Notredame, C., 2017. Nextflow enables 26 reproducible computational workflows. Nat. Biotechnol. 35, 316–319. https://doi.org/10.1038/nbt.3820.
Dixon, P., 2003. VEGAN, a package of R functions for community ecology. J. Veg. Sci. 14, 927–930. https://doi.org/10.1111/j.1654-1103.2003.tb02228.x.
Doherty, N., Trzcinski, K., Pickerill, P., Zawadzki, P., Dowson, C.G., 2000. Genetic diversity of the tet(M) gene in tetracycline-resistant clonal lineages of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. 44, 2979–2984. https://doi.org/10.1128/AAC.44.11.2979-2984.2000.
Ero, R., Kumar, V., Su, W., Gao, Y.G., 2019. Ribosome protection by ABC-F proteins-molecular mechanism and potential drug design. Protein Sci. 28, 684–693. https://doi.org/10.1002/pro.3589.
Ewels, P., Magnusson, M., Lundin, S., Käller, M., 2016. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics 32 (19), 3047–3048. https://doi.org/10.1093/bioinformatics/btw354.
Ferro, G., Guarino, F., Castiglione, S., Rizzo, L., 2016. Antibiotic resistance spread potential in urban wastewater effluents disinfected by UV/H_2O_2 process. Sci. Total Environ. 520, 29–35. https://doi.org/10.1016/j.scitotenv.2016.04.047.
Guo, M.T., Yuan, Q.B., Yang, J., 2013. Microbial selectivity of UV treatment on antibiotic-resistant heterotrophic bacteria in secondary effluents of a municipal wastewater treatment plant. Water Res. 47, 6388–6394. https://doi.org/10.1016/j.watres.2013.08.012.
Harrell Jr., F.E., Harrell Jr., M.F.E., 2019. Package 'hmisc'. In: CRAN2018, vol. 2019, pp. 235–236.
Hazra, M., Watts, J.E.M., Williams, J.B., Joshi, H., 2024. An evaluation of conventional and nature-based technologies for controlling antibiotic-resistant bacteria and antibiotic-resistant genes in wastewater treatment plants. Sci. Total Environ. 917, 170433. https://doi.org/10.1016/j.scitotenv.2024.170433.
Hendriksen, R.S., Munk, P., Njage, P., Van Bunnik, B., McNally, L., Lukjancenko, O., Aarestrup, F.M., 2019. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. Nat. Commun. 10, 1124. https://doi.org/10.1038/s41467-019-08853-3.
Honda, R., Tachi, C., Yasuda, K., Hirata, T., Noguchi, M., Hara-Yamamura, H., Watanabe, T., 2020. Estimated discharge of antibiotic-resistant bacteria from combined sewer overflows of urban sewage system. NPJ Clean Water 3, 15. https://doi.org/10.1038/s41545-020-0059-5.
Kasprzyk-Hordern, B., Bijlsma, L., Castiglioni, S., Covaci, A., de Voogt, P., Emke, E., Hernandez, F., Ort, C., Reid, M., van Nuijs, A., Thomas, K.V., 2014. Wastewater-based epidemiology for public health monitoring. Water Sewer. J. 4, 25–26.
Kelly, S.A., O'Connell, N.H., Thompson, T.P., Dillon, L., Wu, J., Creevey, C., Kiely, P., Slevin, B., Powell, J., Gilmore, B.F., Dunne, C.P., 2023. Large-scale characterization of hospital wastewater system microbiomes and clinical isolates from infected patients: profiling of multi-drug-resistant microbial species. J. Hosp. Infect. 141, 152–166. https://doi.org/10.1016/j.jhin.2023.09.001.
Knight, M.E., Webster, G., Perry, W.B., Baldwin, A., Rushton, L., Pass, D.A., Jones, D.L., 2024. National-scale antimicrobial resistance surveillance in wastewater: a comparative analysis of HT qPCR and metagenomic approaches. Water Res. 121989. https://doi.org/10.1016/j.watres.2024.121989.
Kolde, R., Kolde, M.R., 2015. Package 'pheatmap'. R Package 1, p. 790.
Kwon, J.H., Powderly, W.G., 2021. The post-antibiotic era is here. Science 373, 471. https://doi.org/10.1126/science.abl5997.
Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359. https://doi.org/10.1038/2Fnmeth.1923.
Lee, J., Jeon, J.H., Shin, J., Jang, H.M., Kim, S., Song, M.S., Kim, Y.M., 2017. Quantitative and qualitative changes in antibiotic resistance genes after passing through treatment processes in municipal wastewater treatment plants. Sci. Total Environ. 605, 906–914. https://doi.org/10.1016/j.scitotenv.2017.06.250.
Lépesová, K., Olejníková, P., Mackulák, T., Cverenkárová, K., Krahulcová, M., Bírošová, L., 2020. Hospital wastewater — important source of multidrug resistant coliform bacteria with ESBL-production. Int. J. Environ. Res. Public Health 17, 7827. https://doi.org/10.3390/ijerph17217827.
van Leth, F., Schultsz, C., 2023. Unbiased antimicrobial resistance prevalence estimates through population-based surveillance. Clin. Microbiol. Infect. 29, 429–433. https://doi.org/10.1016/j.cmi.2022.05.006.
Levallois, P., Villanueva, C.M., 2019. Drinking water quality and human health: an editorial. Int. J. Environ. Res. Public Health 16, 631. https://doi.org/10.3390/ijerph16040631.
Li, S., Ondon, B.S., Ho, S.H., Jiang, J., Li, F., 2022. Antibiotic resistant bacteria and genes in wastewater treatment plants: from occurrence to treatment strategies. Sci. Total Environ. 838, 156544. https://doi.org/10.1016/j.scitotenv.2022.156544.
Li, Z., Guo, X., Liu, B., Huang, T., Liu, R., Liu, X., 2024. Metagenome sequencing reveals shifts in phage-associated antibiotic resistance genes from influent to effluent in wastewater treatment plants. Water Res. 253, 121289. https://doi.org/10.1016/j.watres.2024.121289.
Luo, X., Zhang, B., Lu, Y., Mei, Y., Shen, L., 2022. Advances in application of ultraviolet irradiation for biofilm control in water and wastewater infrastructure. J. Hazard. Mater. 421, 126682. https://doi.org/10.1016/j.jhazmat.2021.126682.
Lupo, A., Haenni, M., Madec, J.Y., 2018. Antimicrobial resistance in *Acinetobacter* spp. and *Pseudomonas* spp. Microbiol. Spectr. 6 (3), 10–1128. https://doi.org/10.1128/microbiolspec.arba-0007-2017.
Majeed, H.J., Riquelme, M.V., Davis, B.C., Gupta, S., Angeles, L., Aga, D.S., Garner, E., Pruden, A., Vikesland, P.J., 2021. Evaluation of metagenomic-enabled antibiotic resistance surveillance at a conventional wastewater treatment plant. Front. Microbiol. 12, 657954. https://doi.org/10.3389/fmicb.2021.657954.
Manoli, K., Naziri, A., Ttofi, I., Michael, C., Allan, I.J., Fatta-Kassinos, D., 2022. Investigation of the effect of microplastics on the UV inactivation of antibiotic-resistant bacteria in water. Water Res. 222, 118906. https://doi.org/10.1016/j.watres.2022.118906.
Mulvaney, R.L., 1996. Nitrogen—inorganic forms. In: Methods of Soil Analysis. Part 3: Chemical Methods, vol. 5, pp. 1123–1184. https://doi.org/10.2136/SSSABOOKSER5.3.C38.
Munk, P., Andersen, V.D., de Knegt, L., Jensen, M.S., Knudsen, B.E., Lukjancenko, O., Mordhorst, H., Clasen, J., Agersø, Y., Folkesson, A., Pamp, S.J., Vigre, H., Aarestrup, F.M., 2017. A sampling and metagenomic sequencing-based methodology for monitoring antimicrobial resistance in swine herds. J. Antimicrob. Chemother. 72, 385–392. https://doi.org/10.1093/jac/dkw415.
Murphy, J., Riley, J.P., 1962. A modified single solution method for the determination of phosphate in natural waters. Anal. Chim. Acta 27, 31–36. https://doi.org/10.1016/S0003-2670(00)88444-5.
Murray, C.J., Ikuta, K.S., Sharara, F., Swetschinski, L., Aguilar, G.R., Gray, A., Tasak, N., 2022. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet 399, 629–655. https://doi.org/10.1016/S0140-6736(21)02724-0.
Mutuku, C., Gazdag, Z., Melegh, S., 2022. Occurrence of antibiotics and bacterial resistance genes in wastewater: resistance mechanisms and antimicrobial resistance control approaches. World J. Microbiol. Biotechnol. 38, 152. https://doi.org/10.1007/s11274-022-03334-0.
Naghavi, M., Vollset, S.E., Ikuta, K.S., Swetschinski, L.R., Gray, A.P., Wool, E.E., Dekker, D.M., 2024. Global burden of bacterial antimicrobial resistance 1990–2021: a systematic analysis with forecasts to 2050. Lancet 24, 01867–01871. https://www.thelancet.com/journals/lancet/article/PIIS0140-6736.
Nguyen, P.T.T., Nguyen, H.T., Tran, U.N.P., Manh Bui, H., 2021. Removal of antibiotics from real hospital wastewater by cold plasma technique. J. Chem. 2021, 1–13. https://www.hindawi.com/journals/jchem/2021/9981738.
O'Neill, J., 2016. Tackling Drug-resistant Infections Globally: Final Report and Recommendations. Wellcome Trust, London, UK. https://wellcomecollection.org/works/thvwsuba.
Ory, J., Bricheux, G., Robin, F., Togola, A., Forestier, C., Traore, O., 2019. Biofilms in hospital effluents as a potential crossroads for carbapenemase-encoding strains. Sci. Total Environ. 657, 7–15. https://doi.org/10.1016/j.scitotenv.2018.11.427.
Pallares-Vega, R., Blaak, H., van der Plaats, R., de Roda Husman, A.M., Hernandez Leal, L., van Loosdrecht, M.C.M., Weissbrodt, D.G., Schmitt, H., 2019. Determinants of presence and removal of antibiotic resistance genes during WWTP treatment: a cross-sectional study. Water Res. 161, 319–328. https://doi.org/10.1016/j.watres.2019.05.100.
Pant, A., Shahadat, M., Ali, S.W., Ahammad, S.Z., 2022. Removal of antimicrobial resistance from secondary treated wastewater—a review. J. Hazard. Mater. Adv. 8, 100189. https://doi.org/10.1016/j.hazadv.2022.100189.
Paradis, E., Claude, J., Strimmer, K., 2004. APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20, 289–290. https://doi.org/10.1093/bioinformatics/btg412.
Paulus, G.K., Hornstra, L.M., Alygizakis, N., Slobodnik, J., Thomaidis, N., Medema, G., 2019. The impact of on-site hospital wastewater treatment on the downstream communal wastewater system in terms of antibiotics and antibiotic resistance genes. Int. J. Hyg. Environ. Health 222, 635–644. https://doi.org/10.1016/j.ijheh.2019.01.004.
Pehrsson, E.C., Tsukayama, P., Patel, S., Mejía-Bautista, M., Sosa-Soto, G., Navarrete, K. M., Calderon, M., Cabrera, L., Hoyos-Arango, W., Bertoli, M.T., Berg, D.E., Gilman, R.H., Dantas, G., 2016. Interconnected microbiomes and resistomes in low-income human habitats. Nature 533, 212–216. https://doi.org/10.1038/nature17672.
Perry, W.B., Ahmadian, R., Munday, M., Jones, O., Ormerod, S.J., Durance, I., 2023. Addressing the challenges of combined sewer overflows. Environ. Pollut. 123225. https://doi.org/10.1016/j.envpol.2023.123225.
Perry, W.B., Chrispim, M.C., Barbosa, M.R.F., de Souza Lauretto, M., Razzolini, M.T.P., Nardocci, A.C., Jones, O., Jones, D.L., Weightman, A., Sato, M.I.Z., Montagner, C., Durance, I., 2024. Cross-continental comparative experiences of wastewater surveillance and a vision for the 21st century. Sci. Total Environ. 170842. https://doi.org/10.1016/j.scitotenv.2024.170842.
Public Health Wales, 2023a. Antimicrobial resistance in urine cultures in Wales in 2023. Retrieved from. https://phw.nhs.wales/services-and-teams/antibiotics-and-infections/antimicrobial-surveillance/all-wales-reports/antimicrobial-resistance-in-urine-cultures-in-wales-in-2023/.
Public Health Wales, 2023b. Antimicrobial resistance in blood cultures in Wales, 2023. Retrieved from. https://phw.nhs.wales/services-and-teams/antibiotics-and-infections/antimicrobial-surveillance/all-wales-reports/antimicrobial-resistance-in-blood-cultures-in-wales-2023/.
Quintela-Baluja, M., Abouelnaga, M., Romalde, J., Su, J.Q., Yu, Y., Gomez-Lopez, M., Smets, B., Zhu, Y.G., Graham, D.W., 2019. Spatial ecology of a wastewater network defines the antibiotic resistance genes in downstream receiving waters. Water Res. 162, 347–357. https://doi.org/10.1016/j.watres.2019.06.075.
Rafraf, I.D., Lekunberri, I., Sànchez-Melsió, A., Aouni, M., Borrego, C.M., Balcázar, J.L., 2016. Abundance of antibiotic resistance genes in five municipal wastewater treatment plants in the Monastir Governorate, Tunisia. Environ. Pollut. 219, 353–358. https://doi.org/10.1016/j.envpol.2016.10.062.
Rayan, R.A., 2023. Flare of the silent pandemic in the era of the COVID-19 pandemic: obstacles and opportunities. World J. Clin. Cases 11 (6), 1267. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10013119/.

Raza, S., Shin, H., Hur, H.G., Unno, T., 2022. Higher abundance of core antimicrobial resistant genes in effluent from wastewater treatment plants. Water Res. 208, 117882. https://doi.org/10.1016/j.watres.2021.117882.

Rhoades, J.D., 1996. Salinity: electrical conductivity and total dissolved solids. In: Methods of Soil Analysis: Part 3 Chemical Methods, 5, pp. 417–435.

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234–242. https://doi.org/10.1016/j.watres.2014.11.021.

Shomuyiwa, D.O., Lucero-Prisno III, D.E., Manirambona, E., Suleman, M.H., Rayan, R.A., Huang, J., Zaw, T.N., Babatunde, Y., Denkyira, S.A., Musa, S.S., 2022. Curbing antimicrobial resistance in post-COVID Africa: challenges, actions and recommendations. Health Sci. Rep. 5, e771. https://doi.org/10.1002/hsr2.771.

Sims, N., Kannan, A., Holton, E., Jagadeesan, K., Mageiros, L., Standerwick, R., Craft, T., Barden, R., Feil, E.J., Kasprzyk-Hordern, B., 2023. Antimicrobials and antimicrobial resistance genes in a one-year city metabolism longitudinal study using wastewater-based epidemiology. Environ. Pollut. 333, 122020. https://doi.org/10.1016/j.envpol.2023.122020.

Sneka, P., Mahalakshmi, K., 2023. Antimicrobial resistance—a silent pandemic. Natl. J. Commun. Med. 14, 71–72. https://doi.org/10.55489/njcm.140120232625.

Song, R., Li, H., Kang, Z., Zhong, R., Wang, Y., Zhang, Y., Wang, T., 2021. Surface plasma induced elimination of antibiotic-resistant *Escherichia coli* and resistance genes: antibiotic resistance, horizontal gene transfer, and mechanisms. Sep. Purif. Technol. 275, 119185. https://doi.org/10.1016/j.seppur.2021.119185.

Sonnenwald, F., Shuttleworth, J., Bailey, O., Williams, M., Frankland, J., Rhead, B., Mark, O., Wade, M.J., Guymer, I., 2023. Quantifying mixing in sewer networks for source localization. J. Environ. Eng. 149, 04023019. https://doi.org/10.1061/JOEEDU.EEENG-7134.

Su, J.Q., Wei, B., Ou-Yang, W.Y., Huang, F.Y., Zhao, Y., Xu, H.J., Zhu, Y.G., 2015. Antibiotic resistome and its association with bacterial communities during sewage sludge composting. Environ. Sci. Technol. 49, 7356–7363. https://doi.org/10.1021/acs.est.5b01012.

Su, J.Q., An, X.L., Li, B., Chen, Q.L., Gillings, M.R., Chen, H., Zhu, Y.G., 2017. Metagenomics of urban sewage identifies an extensively shared antibiotic resistome in China. Microbiome 5, 1–15. https://doi.org/10.1186/s40168-017-0298-y.

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D.L., Pulcini, C., Kahlmeter, G., Kluytmans, J., Carmeli, Y., Ouellette, M., Outterson, K., Patel, J., Cavaleri, M., Cox, E.M., Houchens, C.R., Grayson, M.L., Hansen, P., Singh, N., Theuretzbacher, U., WHO Pathogens Priority List Working Group, 2018. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect. Dis. 18, 318–327. https://doi.org/10.1016/S1473-3099(17)30753-3.

The Rivers Trust, 2024. Event duration monitoring – storm overflows – 2023 (England and Wales). In: Catchment Based Approach Data Hub, Callington, Cornwall, UK. https://data.catchmentbasedapproach.org/datasets/theriverstrust::event-duration-monitoring-storm-overflows-2022-england-and-wales/explore.

Uluseker, C., Thorsen, K., Basiry, D., Kommedal, R., Pala-Ozkok, I., 2021. A review on occurrence and spread of antibiotic resistance in wastewaters and in wastewater treatment plants: mechanisms and perspectives. Front. Microbiol. 12, 717809. https://doi.org/10.3389/fmicb.2021.717809.

Vaz-Moreira, I., Varela, A.R., Pereira, T.V., Fochat, R.C., Manaia, C.M., 2016. Multidrug resistance in quinolone-resistant Gram-negative bacteria isolated from hospital effluent and the municipal wastewater treatment plant. Microb. Drug Resist. 22, 155–163. https://doi.org/10.1089/mdr.2015.0118.

Wang, G., Gao, X., Cai, Y., Li, G., Ma, R., Yuan, J., 2024. Dynamics of antibiotic resistance genes during manure composting: reduction in herbivores manure and accumulation in carnivores. Environ. Int. 108900. https://doi.org/10.1016/j.envint.2024.108900.

Wang, J., Chen, X., 2022. Removal of antibiotic resistance genes (ARGs) in various wastewater treatment processes: an overview. Crit. Rev. Environ. Sci. Technol. 52, 571–630. https://doi.org/10.1080/10643389.2020.1835124.

Webber, M.A., Bailey, A.M., Blair, J.M., Morgan, E., Stevens, M.P., Hinton, J.C., Ivens, A., Wain, J., Piddock, L.J., 2009. The global consequence of disruption of the AcrAB-TolC efflux pump in *Salmonella enterica* includes reduced expression of SPI-1 and other attributes required to infect the host. J. Bacteriol. 191, 4276–4285. https://doi.org/10.1128/JB.00363-09.

Webster, G., Newberry, C.J., Fry, J.C., Weightman, A.J., 2003. Assessment of bacterial community structure in the deep sub-seafloor biosphere by 16S rDNA-based techniques: a cautionary tale. J. Microbiol. Methods 55, 155–164. https://doi.org/10.1016/s0167-7012(03)00140-4.

WPCCEIC, 2022. Report on Storm Overflows in Wales. Welsh Parliament Climate Change, Environment, and Infrastructure Committee, Welsh Parliament, Cardiff, UK. https://senedd.wales/media/v4apg5wb/cr-ld15015-e.pdf.

Yin, X., Chen, X., Jiang, X.T., Yang, Y., Li, B., Shum, M.H.H., Zhang, T., 2023. Toward a universal unit for quantification of antibiotic resistance genes in environmental samples. Environ. Sci. Technol. 57 (26), 9713–9721. https://doi.org/10.1021/acs.est.3c00159.

Yoo, K., Lee, G., 2021. Investigation of the prevalence of antibiotic resistance genes according to the wastewater treatment scale using metagenomic analysis. Antibiotics 10 (2), 188. https://doi.org/10.3390/antibiotics10020188.

Zhang, J., Buhe, C., Yu, D., Zhong, H., Wei, Y., 2020. Ammonia stress reduces antibiotic efflux but enriches horizontal gene transfer of antibiotic resistance genes in anaerobic digestion. Bioresour. Technol. 295, 122191. https://doi.org/10.1016/j.biortech.2019.122191.