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Bacteria and Bacterial Diseases

Metagenomic profiling of hospital wastewater: A comprehensive national scale analysis of antimicrobial 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], Neil Andrew Byrnes [d,e], Kata Farkas [a,f], Margaret Heginbothom [g], Noel Craine [h], Gareth Cross [i], Peter Kille [b], Barbara Kasprzyk-Hordern [d,j], Andrew J. Weightman [b], Davey L. Jones [a,f]

[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] Department of Chemistry, University of Bath, Bath BA2 7AY, UK
[e] Australian Centre for Research on Separation Science, School of Chemistry, Monash University, Wellington Road, Clayton, Victoria 3800, Australia
[f] Verily Life Sciences LLC., South San Francisco, CA 94080, United States
[g] Public Health Wales, Cardiff CF10 4BZ, UK
[h] Microbiology Department, Public Health Wales, Ysbyty Gwynedd, Bangor LL57 2PW, UK
[i] Science Evidence Advice Division, Health and Social Services Group, Welsh Government, Cathays Park, Cardiff CF10 3NQ, UK
[j] Centre of Excellence in Water-Based Early-Warning Systems for Health Protection, University of Bath, Bath BA2 7AY, UK

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SUMMARY

Background: Healthcare settings are recognised as potential hotspots for the emergence and spread of antimicrobial resistance (AMR).
Method: Metagenomic sequencing was conducted on a national scale using wastewater from hospitals across Wales to screen for antimicrobial resistance genes (ARGs) and opportunistic pathogens.
Results: The total abundance and diversity of ARGs varied significantly across the hospitals. Genes conferring resistance to aminoglycosides, beta-lactams, and Macrolide-Lincosamide-Streptogramin-class antibiotics were predominant, with distinct resistome patterns emerging spatially. OXA-type beta-lactamases were the dominant ARG types. Spatial variability was observed in the distribution of the "big five" carbapenemases (KPC, IMP, VIM, NDM, OXA-48-like) and mcr genes, as well as WHO-listed fungal priority pathogens and Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp., and Escherichia coli (ESKAPEE) pathogens. Furthermore, antibiotic concentrations in the effluents often exceeded risk quotients, posing a substantial risk for AMR emergence.
Conclusions: Overall, the study highlights the effectiveness of combining wastewater-based epidemiology with metagenomics to gain critical insights into the distinct resistome and microbiome profiles in hospital settings. Tailored strategies are essential to mitigate the spread of antibiotics, clinically relevant ARGs and pathogens in these settings. This study underscores the necessity of implementing pre-treatment processes for hospital effluents before release into community sewers and environmental waters to curb the spread of these micro-pollutants.

Abbreviations: AMR, antimicrobial resistance; ARGs, antibiotic-resistance genes; BL, beta-lactamase; ESKAPEE, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp., and *Escherichia coli*; HWW, Hospital wastewater; MDR, Multi-drug resistant; MGEs, Mobile genetic elements; MLS, Macrolide-Lincosamide-Streptogramin; WBE, wastewater-based epidemiology; WHO, World Health Organization; WWTP, wastewater treatment plant

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

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Introduction

Hospitals serve as critical epidemiological hubs with intensive antimicrobial use and are recognised as hot spots for the emergence and dissemination of antimicrobial resistance (AMR).[1] Studies highlight that hospitalisation and extended hospital stays significantly elevate the risk to individuals of acquiring AMR infections.[2,3] Moreover, nosocomial infections by antibiotic-resistant bacteria present a significant threat to public health.[4]

Hospital wastewaters (HWW) are highly complex effluents known to be an important source of antimicrobial resistance genes (ARGs) and pathogens in aquatic environments.[5] The COVID-19 pandemic has led to a global demand to monitor other infectious agents, such as antibiotic-resistant pathogens in HWW and their subsequent fate.[6,7] There is a growing interest in the contribution of HWW to the contamination of receiving environments, particularly from a One Health perspective.[8] Untreated/partially treated HWW released through combined sewer overflows[7] will disseminate multidrug-resistant (MDR) pathogens and ARGs into the environment, posing significant human and animal health risks.[9] HWW is also a major source of antibiotics, disinfectants, heavy metals and other drugs.[10] Discharging these contaminants from hospitals leads to further ARG selection and the emergence of AMR in sewer systems and receiving environments. Hence, studying the resistome and microbiome in clinical settings and gathering evidence-based data is essential for addressing the growing challenge of AMR. While there have been studies on monitoring ARGs and pathogens in hospitals from diverse locations,[10-14] more systematic studies across wider linked geographical ranges are needed, since resistome and microbiome profiles can differ significantly between regions and individual hospitals.[15]

Direct collection of patient samples from hospitals for research purposes presents significant ethical challenges. This can be overcome by employing wastewater-based epidemiology (WBE), a powerful tool to monitor and predict public health risks across different settings.[16] Clinical practices typically rely on targeted, traditional culture-based surveillance methods. These methods, though beneficial, may not capture the full spectrum of microbial diversity or the dynamics of ARGs, possibly overlooking emerging pathogens or genes. To overcome these limitations, advanced metagenomics approaches have been increasingly employed. The application of metagenomics to monitor HWW has become more common, providing valuable insights into antimicrobial resistance patterns and pathogen profiles within hospital environments.[17-19] A comprehensive data on the ARG signatures and pathogen profiles in hospital settings at a national scale is lacking in Wales. The novelty of this paper is its comprehensive national-scale analysis of ARGs, pathogens and antibiotics in HWW using large metagenomic datasets paired with antibiotic chemical profiling from the same wastewater samples. In addition, the most comprehensive data available for antibiotic prescribing within each hospital during the study period was analysed. This study provides the first detailed national scale mapping of resistome and microbiome profiles across multiple hospitals, offering data-driven insights to inform future policies and strategies to curb the spread of clinically relevant ARGs and pathogens in healthcare settings.

Materials and methods

Sampling and sample processing

Throughout April to July 2023, repeated 24-hour composite samples of hospital wastewater were collected (Tuesday to Friday) directly from the main sewage outlets at eight major hospitals across Wales, using refrigerated autosamplers (Teledyne ISCO and Aquamatic Ltd). Samples of one litre were transported in clean, refrigerated Nalgene® bottles to the central laboratory at Bangor and processed on the same day they were collected. Sampled hospitals include Bangor, B-H; Rhyl, R-H; Wrexham, W-H (North Wales), Llandough, L-H; Swansea, S-H; Glamorgan, G-H (South Wales), Carmarthen, C-H (West Wales), and Aberystwyth, A-H (Mid-Wales), chosen for their size and locations across five health boards (Fig. 1). Effluents from these hospitals are discharged directly to the local wastewater treatment plants (WWTPs) without any pre-treatment process. Data on the number of hospital beds and occupancy rates were obtained from Public Health Wales. The wastewater samples represented 30% of the total hospital patient bed occupancy in Wales. All hospitals are designated for antibiotic prescribing. The number of samples collected varied across hospitals and months due to logistical constraints. For instance, no samples were collected in April for C-H, and only one sample was collected for L-H during the same month. Samples (50 mL) were centrifuged at 10,000 × g at 4 °C for 30 min to create pellets of the daily samples, which were then pooled to make weekly composite samples (n = 112). Genomic DNA was extracted from pooled samples using a modified FastDNA™ SPIN Kit for Soil (MP Biomedicals) protocol.[20]

Metagenomic sequencing and bioinformatic analysis

A detailed methodology has been described previously.[21] Approximately 200 ng of DNA per sample was used for library construction following the Illumina DNA PCR-Free Prep and Tagmentation guidelines and using IDT for Illumina DNA/RNA UD Indexes (Sets A-D) as specified by the manufacturer. Sequencing was performed on an Illumina NovaSeq 6000 system using a paired-end

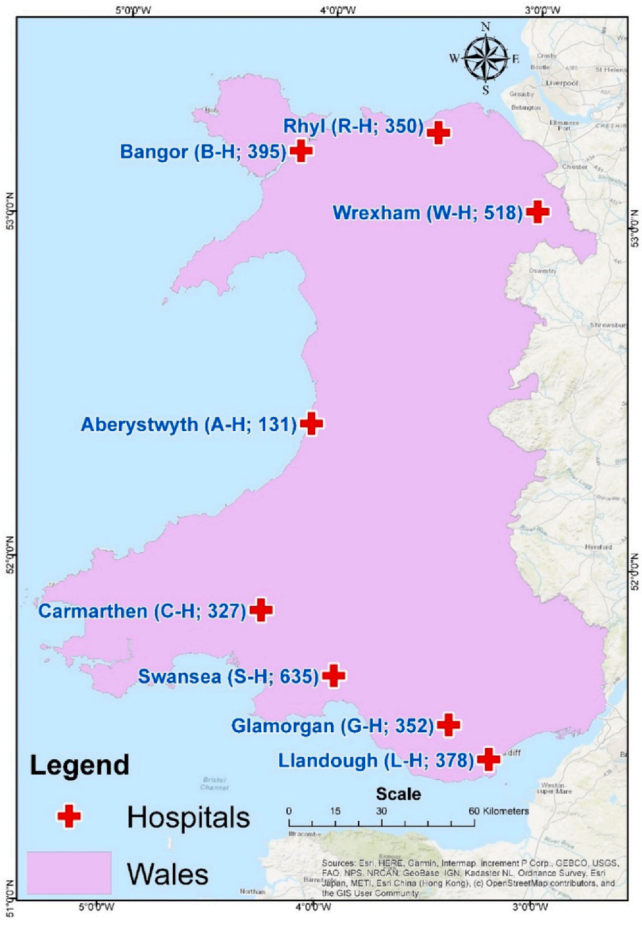

Fig. 1. Map displaying the locations of hospital sites across Wales. The site codes and average bed occupancy are presented in brackets.

2 × 150 bp S4 flow cell with a v1.5 NovaSeq S4 cartridge for 300 cycles at the Wales Gene Park, Cardiff. A 1% PhiX control was included.

Metagenomic sequence reads were converted into FASTQ files and processed for demultiplexing and adapter removal using the Illumina bcl2fastq2 software, version 2.20. Quality control was performed with fastp version 0.20, and results were assessed using FastQC version 0.11.9. Comprehensive quality reports were compiled using MultiQC software.[22] Host DNA removal was done by aligning against the human genome with Samtools version 1.13[23] and Bowtie version 2.4.1.[24] High-quality sequences were screened for antimicrobial resistance genes using the AMR++ pipeline v3 and for microbial taxonomy using Kraken 2[25,26] and Bracken.[27] The ARG reads were normalised as Reads Per Kilobase Million (RPKM) to account for variation in sequencing depth and length of the gene.[28]

Inpatient antibiotic usage data

The inpatient antibiotic usage data for secondary care was provided by Public Health Wales (PHW). The data originated from the hospital stock control systems, which were processed by Digital Health and Care Wales (DHCW) and analysed by PHW. It focused on antibiotics prescribed for systemic (oral and parenteral) and not topical usage. To standardise the values across hospitals of varying sizes and enable comparison with antibiotic chemistry data, usage was converted from Defined Daily Dose (DDD) to grams per 1000 bed days (grams/1000 BD) following World Health Organization Collaborating Centre (WHOCC) guidelines.[29] Bed days were calculated using in-patient activity (average daily occupied beds). In the UK, an adapted version of the World Health Organization (WHO) Access, Watch, Reserve, classification (AWaRe) of antibiotics has been adopted for evaluation and monitoring of the trends in antibiotic usage.[30]

Antibiotic detection in hospital wastewater and antibiotic resistance risk prediction

During the summer of May 2023, a comprehensive study monitoring the presence of antibiotics and their metabolites in wastewater from 15 sites across Wales was conducted, as discussed by Byrnes et al.,[31] using ultra-performance liquid chromatography-tandem mass spectrometry. This work includes results from the hospital sites studied here, except for C-H, where logistical constraints prevented sample collection, with a focus on the elucidation of AMR risk. Selected results from this research will be presented here to allow for comparisons between the patterns of antibiotic resistance genes and antimicrobial residue concentrations. Details of sample collection, sample preparation, chemical analysis, and determination of AMR risk are outlined in the previous publication.[31]

Data analysis and visualisation

The data analysis was conducted using R statistical software (version 4.1.2), employing packages such as *vegan* for diversity analyses, *ggplot2*, *pheatmap* for visualisation, and *dplyr* for data manipulation. The Kruskal-Wallis and Dunn's tests in the *ggpubr* package were employed to find the significant differences ($p < 0.05$) among the hospitals and to generate box plots. Principal coordinate analysis (PCoA) based on Bray-Curtis distances was performed with the *ape* package to visualise the differences in ARG and microbial composition across the hospital effluents.[32] Correlation analyses were carried out to investigate the relationships among numerous variables. We utilised the rcorr() function from the *Hmisc* package[33] to calculate Spearman's rank correlation coefficients to find the correlation between ARGs and bacteria and correlation graphs were generated using the *igraph* package.[34] Network diagrams were plotted using Gephi 0.10.1 to visualise the co-occurrence of ARGs and bacteria.[35] Pearson correlation and regression analysis assessed the relationship between ARGs and corresponding antibiotic concentrations. Pie charts were plotted using OriginPro 2024b (version 10.15).

Results

A total of 397 composite wastewater samples from hospital effluents were collected during the study period. They were pooled weekly and analysed for ARGs and microbial taxonomy by shotgun metagenomics, producing an average read depth of 143.2 ± 73.12 (mean ± SD) million sequence reads per sample (range = 27–328 million reads per sample).

Diversity and abundance of antimicrobial resistance genes (ARGs)

An average of 1.2% of the total reads were assigned to ARGs. There was a significant difference in total relative ARG abundance and diversity among the different hospitals (Kruskal-Wallis $p < 0.01$). Dunn's test highlighted specific inter-hospital differences (Fig. 2a, b). Despite being the smallest, A-H had the highest ARG reads, while G-H had the lowest (Fig. 2a). No significant differences were observed between K-H, B-H, W-H, S-H, and L-H ($p > 0.05$). G-H showed the lowest ARG diversity (Shannon index), whereas W-H and C-H had the highest (Fig. 2b).

Spatio-temporal variation was observed in the relative abundance of ARG classes. Genes encoding resistance towards the aminoglycoside, beta-lactam, Macrolide-Lincosamide-Streptogramin (MLS) and tetracycline drug classes dominated across the hospitals (Fig. 3). However, the overall resistome pattern varied. For instance, the relative abundance of glycopeptide (mainly vancomycin) resistance encoding genes was comparatively higher in R-H and W-H located in North Wales. Similarly, in G-H, the relative abundance of lipopeptide resistance genes was higher, and phenicol and sulphonamide resistance genes were much lower.

ARGs conferring resistance to WHO-listed critically important antibiotics for human medicine,[36] such as aminoglycosides, rifampin/rifampicin, macrolides, carbapenems, quinolones, tetracyclines, and vancomycin were among the top 50 abundant genes from the hospitals. OXA-type beta-lactamases were the most abundant (Fig. S1). Other dominant ones included the MLS23S, *msrE*, *mphE* genes, which encode macrolide resistance and bla_{CTX} types, which encode extended-spectrum beta-lactamases, *ant* and *aph* variants encoding aminoglycosidases, and *tetR* encoding tetracycline resistance genes.

The samples were specifically screened for the presence of the *mcr* gene variants, which encode resistance to colistin, a last-resort antibiotic and the "big five" carbapenemases, which include *Klebsiella pneumoniae* carbapenemase (KPC), imipenemases (IMP), Verona integron-encoded metallo-beta-lactamase (VIM), New Delhi metallo-beta-lactamase (NDM), and oxacillinase (OXA-48-like).[37] Their distribution varied distinctly across hospitals (Fig. S2a, b). In A-H, $bla_{OXA-48-like}$, bla_{IMP}, and bla_{VIM} were detected in all samples, while bla_{KPC} was absent, and bla_{NDM} rare. In R-H, bla_{IMP} was prevalent, while bla_{NDM}, bla_{KPC}, bla_{VIM}, and $bla_{OXA-48-like}$ were detected only once. In C-H, bla_{VIM} was not detected; however, bla_{IMP} and bla_{NDM} were present in all samples, with other genes being less frequent. The bla_{NDM} was undetected in B-H, while bla_{KPC} and bla_{IMP} types were predominant, with $bla_{OXA-48-like}$, bla_{VIM}, and bla_{NDM} less frequent. L-H had no detections of $bla_{OXA-48-like}$; bla_{KPC} was rare, but bla_{IMP} constituted half of the "big five" enzymes, with bla_{NDM} and bla_{VIM} consistently present. In W-H, $bla_{OXA-48-like}$ was absent; bla_{NDM} was rare, but all other genes were frequently detected. Similarly, in S-H, $bla_{OXA-48-like}$ was not detected, bla_{IMP} was rare, and bla_{KPC} was dominant, with other genes commonly found. The distribution of *mcr* gene variants varied widely across the hospitals (Fig. S2b).

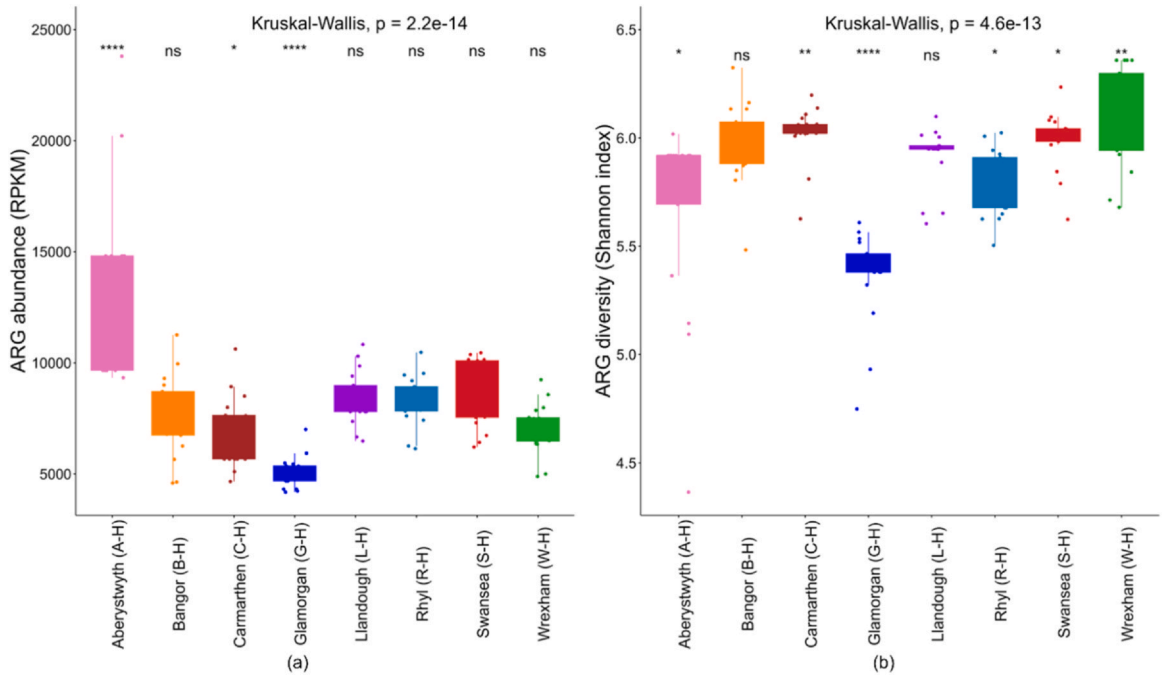

Fig. 2. Box plots displaying (a) total relative ARG abundance (RPKM) and (b) ARG diversity (Shannon index) in wastewater per hospital. The pairwise comparisons are annotated with significance levels: ns: Not significant ($p > 0.05$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

Notably, G-H showed a high relative abundance of the *mcr* gene despite a lower prevalence of the "big five" enzymes. G-H also exhibited the highest diversity of *mcr* gene variants, with *mcr 10.1* being relatively abundant, followed by *mcr-4.3* and *mcr-3.17*. The *mcr-4* variants were unique to G-H. In North Wales hospitals, *mcr-3.17* and *mcr-9.1* dominated, followed by *mcr-5* variants. MCR genes were not detected in any of the A-H samples, except for one detection of *mcr-9.1*.

PCoA highlighted similarities and variations in the ARG signatures across different hospitals (Fig. 4). G-H was distinct, isolated on Axis 1. This can be due to certain unique ARG types such as *vanSC*, *vanXYC*, *vanTC*, *vanC*, *mph*, *abaF*, *rahN*, *cmrA*, *mir*, and *qnrE*. S-H also displayed a unique ARG profile, with its ellipse mostly extending beyond the common overlap area. There was considerable overlap among most B-H, C-H, and L-H samples, indicating shared ARG profiles. Samples from North Wales hospitals showed some overlap,

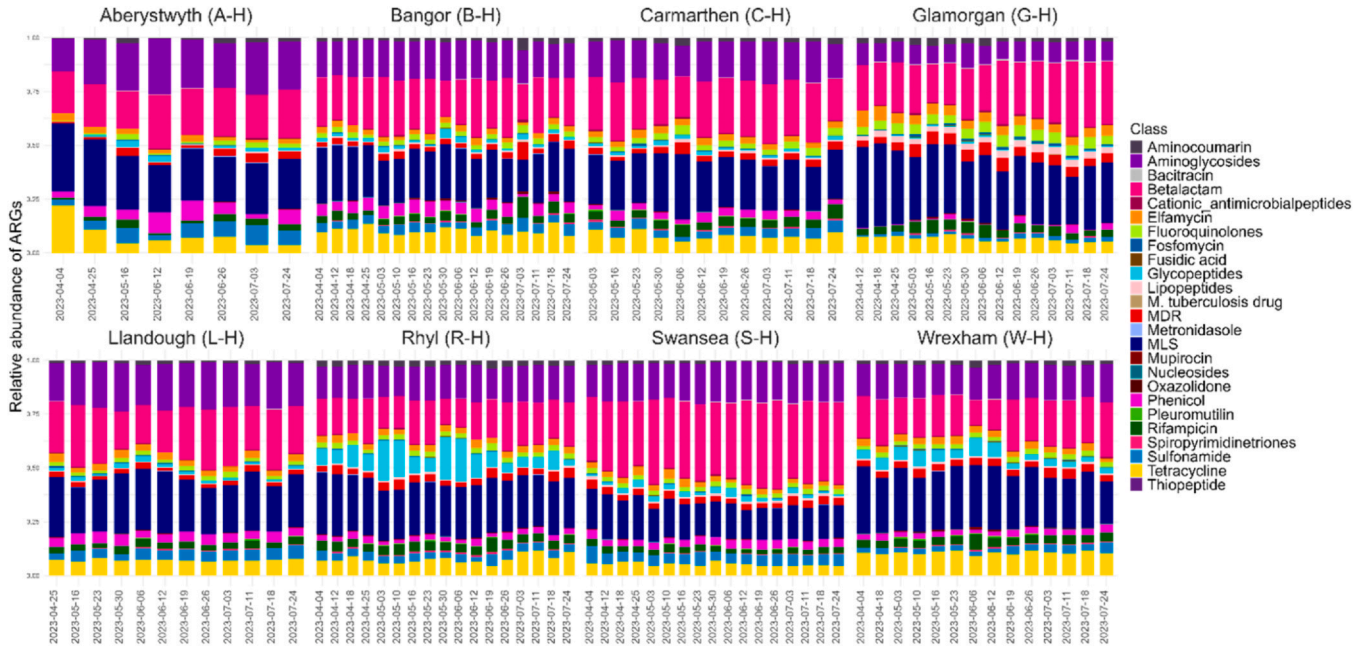

Fig. 3. Bar plots showing the relative abundance and temporal change of ARGs by antibiotic class in wastewater collected from 8 hospitals across Wales. All samples were collected during the period April-July 2023.

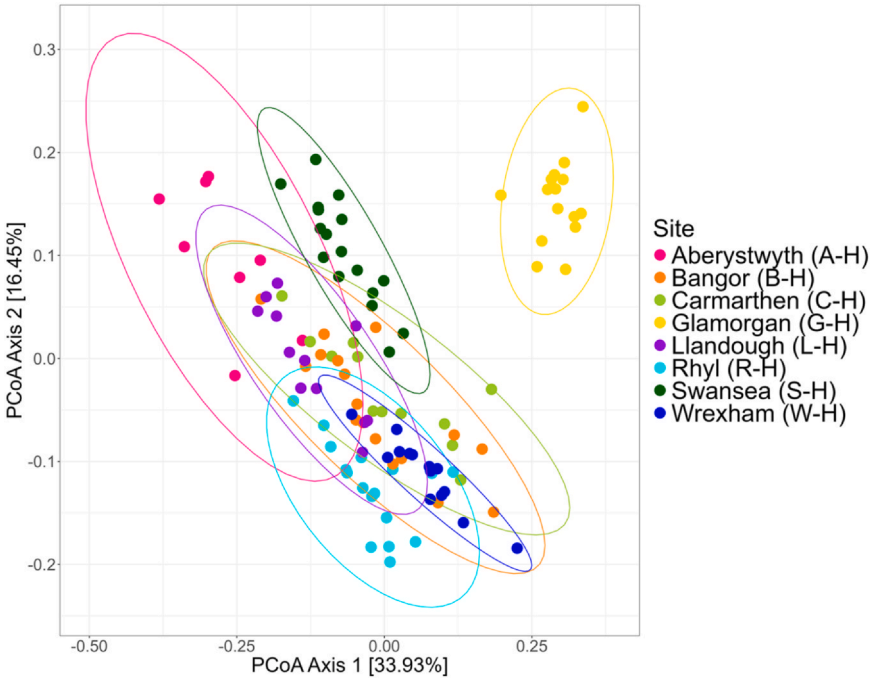

Fig. 4. Principal coordinate analysis (PCoA) plot illustrating the ordination of hospital wastewater samples according to their antimicrobial resistance gene (ARG) subtypes. Each point represents a sample, coloured by site, with the ellipses indicating the 95% confidence interval for each site group.

suggesting similarities in their ARGs. A-H's ellipse spanned a wider area on Axis 2, indicating more significant variability in ARG profiles.

Composition of bacterial phyla

On average, 51% of reads were assigned to bacteria from hospital effluents, identifying 36 phyla and 1827 genera. Pseudomonadota dominated, accounting for over 50% of total bacterial reads (Fig. 5a). Other key phyla included Bacteroidota, Bacillota, and Actinomycetota. Notably, the relative abundance of Bacillota decreased in A-H during the last three weeks of June. Higher bacterial diversity (genus level) was observed in hospitals from North Wales compared to other regions (Shannon index 4–4.8; Fig. S3a).

PCoA analysis revealed compositional differences in bacterial genera in the hospitals (Fig. S4a). However, the majority of the ellipses for hospital sites, except A-H, showed significant overlap, suggesting a shared bacterial community structure. A-H was distinct along both axes, indicating a unique bacterial community compared to other sites. However, a few samples from A-H overlapped with R-H, indicating some similarities in their bacterial profiles.

Fig. 5b shows the relative abundance of ESKAPEE bacteria in the samples. The proportion of reads of the ESKAPEE group of bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* species, and *Escherichia coli*) relative to the total bacterial population varied across the sampling periods and sites, ranging from 0.663 to 11.485. Weekly fluctuations were observed at A-H and G-H, with mean proportions of 2.574 and 2.113 and maximum values of 11.485 and 7.036, respectively. In contrast, sites like L-H and W-H displayed more consistent bacterial levels, with mean proportions of 1.182 and 1.078 and relatively narrow ranges. The remaining sites, including R-H, C-H, B-H, and S-H, exhibited moderate variability, with mean proportions ranging from 1.197 to 1.378. *Enterobacter* spp. and *P. aeruginosa* were particularly prevalent in G-H and R-H, respectively, indicating their relative dominance at these sites. In L-H and S-H, the patterns were consistent, with *A. baumanii* and *P. aeruginosa* being the predominant species, respectively. A-H exhibited significant weekly fluctuations, with spikes in *A. baumanii* in April and late June. During the first week of July, *E. coli* accounted for about 60% of the group in B-H. In contrast, W-H showed slight weekly variation, with *E. coli* and *Enterobacter* consistently dominant, except for a surge in *K. pneumoniae* in the second week of May. C-H also displayed weekly variation. Across all hospitals, *S. aureus* was the least abundant of the ESKAPEE group.

Abundance and diversity of fungi in the hospital samples

An average total of 0.03% of reads were mapped to fungi. The highest fungal diversity was observed in hospitals from North Wales (Shannon index range 3.7–4.3) and the least in G-H (Shannon index 1.1–3.2) (Fig. S3b). *Saccharomyces* spp. dominated the fungal community in G-H throughout the period, contributing to more than 50% of the total species (Fig. 6a). In the North Wales sites, the total fungal composition remained relatively constant and similar throughout the study period, except during the first two weeks of June in B-H, where there was a slight increase in the relative abundance of *Candida* spp. and *Nakaseomyces*. Similarly, in W-H, the relative abundance of *Yarrowia* increased slightly during the first week of June. In contrast, the relative abundance and total composition of fungi varied spatially and temporally in hospitals from other parts of Wales. The relative abundance of *Candida* spp. was comparatively much higher in S-H. During the second week of June, there was a sudden increase in *Candida* spp. relative abundance in A-H, contributing to more than 70% of the fungal community.

In the PCoA plots, G-H is positioned far from other sites, suggesting it hosts a distinct fungal community (Fig. S4b). Samples from other hospitals significantly overlapped, indicating the presence of shared fungal community structures.

Seven WHO-listed fungal priority pathogens[38] were detected in the samples (Fig. 6b). These included *Candida albicans, Aspergillus fumigatus*, and *Cryptococcus neoformans* from the critical priority group; *Nakaseomyces glabratus* (*Candida glabrata*) and *Fusarium* spp. from the high priority group; and *Cryptococcus gattii* and *Pichia kudriavzeveii* (*Candida krusei*) from the medium priority group. However, *C. auris*, also in the critical priority group, was not detected. *C. albicans*, *Fusarium* spp., and *N. glabratus* were the most prevalent

Fig. 5. Bar plots showing the relative abundance of (a) bacterial phylum and (b) ESKAPEE bacteria in wastewater collected from 8 hospitals across Wales.

fungi across all sites, while *A. fumigatus* and *C. gattii* were the least common. *Fusarium* spp. was consistently abundant in W-H and A-H, whereas *C. albicans* was notably higher in S-H. Spikes in *P. kudriavzevii* occurred in June across multiple hospitals, and a significant surge in *C. albicans* in the second week of June accounted for over 85% of the total detections.

Co-occurrence of bacterial genera and ARGs

Fig. 7a-h shows the network diagrams based on the co-occurrence of bacterial genera and ARGs by selecting strong correlations (Spearman's $r > 0.70$, $p < 0.01$). These diagrams visualise bacterial genera linked to specific ARGs, identifying potential resistance carriers. In B-H (Fig. 7a), *Acinetobacter* were predicted to host the majority of the ARGs (number of ARGs = 21) followed by *Aeromonas* (n = 16). *Enterobacter* and *Klebsiella* were shown to strongly correlate with bla_{CTX}. In R-H (Fig. 7b), *Acinetobacter* was host to a majority of ARGs (n = 11). *Enterococci* strongly correlated with 8 ARGs, including vancomycin resistance encoding genes. Other opportunistic pathogenic genera such as *Escherichia*, *Klebsiella* and *Pseudomonas* were also considered hosts to certain ARGs, including bla_{CTX}. In W-H (Fig. 7c), *Tolumonas* was predicted to host 16 ARG types, followed by *Alistipes* (n = 14). *Enterobacter* strongly correlated with 10 ARGs. In A-H (Fig. 7d), *Acinetobacter* was predicted to be the major host. *Enterobacter* and *Citrobacter* strongly correlated with bla_{CTX}. In C-H (Fig. 7e), *Aeromonas* (n = 16) and *Acinetobacter* (n =15) were predicted to host the majority of the ARGs. *Enterobacter* and *Klebsiella* were shown to strongly correlate with bla_{CTX}. In S-H (Fig. 7f), *Klebsiella* spp. was found to host the majority of ARGs (n = 11) and was strongly associated with many ARGs, including those encoding for extended-spectrum beta-lactamases (ESBLs) and carbapenemases. *Enterococci* and *Enterobacter* were also predicted as hosts for many ARGs. In G-H

Fig. 6. Bar plots showing the relative abundance of (a) Fungi (genus level) and (b) WHO-listed fungal priority pathogens in wastewater collected from 8 hospitals across Wales.

(Fig. 7g), *Acinetobacter* was the main host for ARGs (n = 10), followed by *Streptococcus. Enterococci* strongly correlated with 7 ARGs, including vancomycin resistance encoding genes. In L-H (Fig. 7e), *Aeromonas* was predicted to host the highest number of ARGs (n = 15), followed by *Tolumonas* (n = 12). *Acinetobacter* and *Klebsiella* were also predicted to harbour many ARGs. The results of the correlation analysis can be found in the Supplementary Data.

Inpatient antibacterial usage and detection of antibiotics in hospital wastewater

There was inter-hospital variability in the total and proportional antibiotic consumption (expressed in Grams/1000 BD) (Fig. S5). The inter-quarter consumption trend also varied within the hospitals. R-H (302,571.45 ± 12,772.57 gms/1000 BD) consistently had the

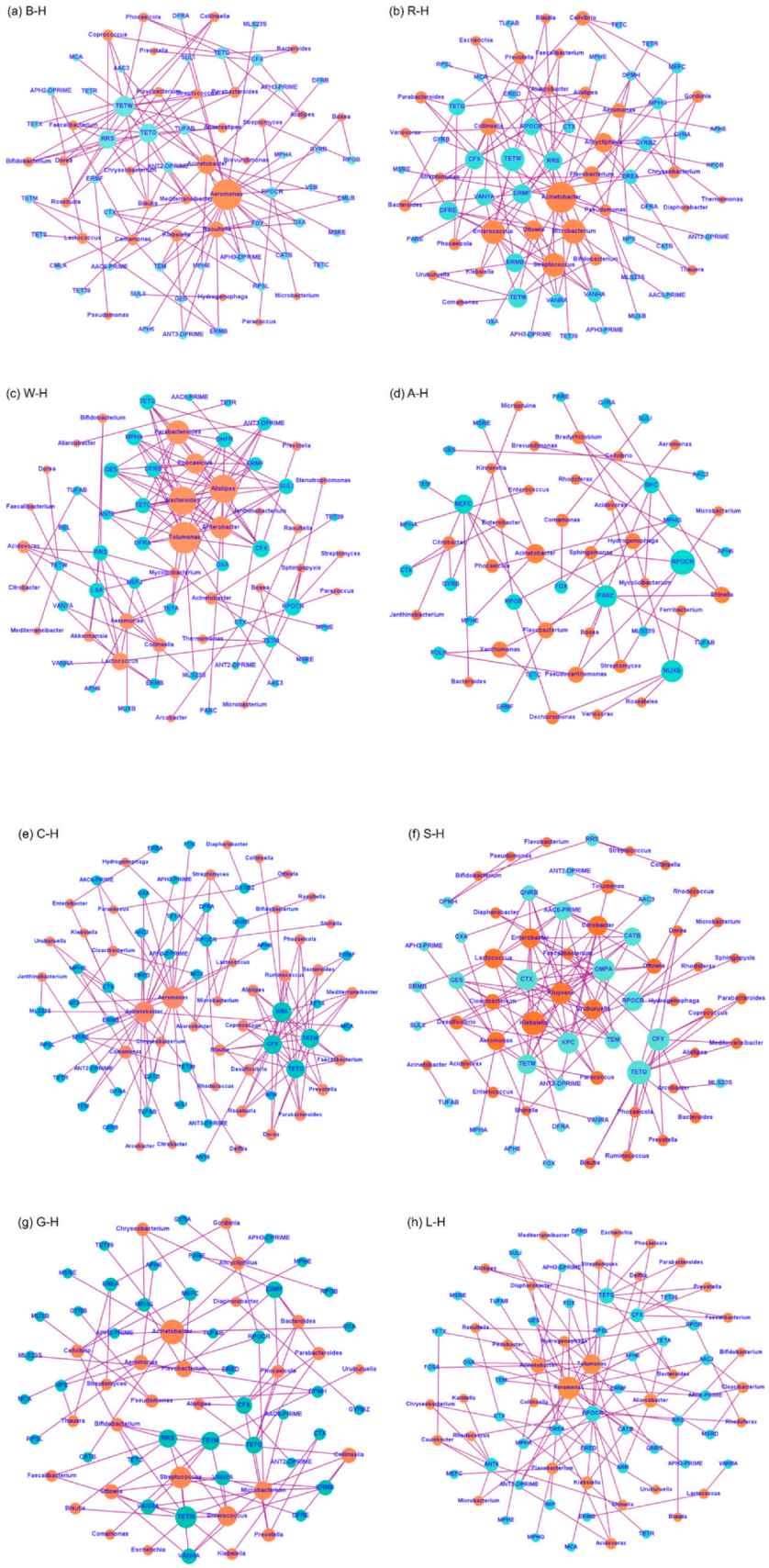

Fig. 7. Network graphs revealing the co-occurrence pattern of ARGs and bacterial hosts in 8 hospitals (a-h) based on Spearman's ($r > 0.7$) and significant ($P < 0.01$) correlations.

highest usage among the hospitals, with the peak during Q4 (autumn to early winter). The lowest usage was in L-H (136,718.76 ± 12,912.73) and W-H (148,432.85 ± 7900.86). Overall, consumption was highest during Q4 in most hospitals (C-H, L-H, R-H, S-H and W-H). Comparing our data with the England AWaRe classification, we found that Piperacillin/tazobactam (beta-lactam/beta-lactamase inhibitor combination) from the "Watch" group was the most commonly consumed (76,611.83 ± 20,584.13 gms/1000 BD) across hospitals in Wales. Antibiotics from the "Access" group: flucloxacillin (beta-lactamase resistant penicillin), amoxicillin (broad spectrum penicillin), metronidazole (imidazole), and co-trimoxazole (trimethoprim & sulphonamides) were the other commonly used. This reflects their broad application against various bacterial infections in clinical settings. From the "Watch" group azithromycin, clarithromycin, and erythromycin (macrolides) were consumed at 5074.79 ± 2170.55 gms/1000 BD, while teicoplanin and vancomycin (glycopeptides) were used at 4420.01 ± 1750.49 gms/1000 BD, with usage levels varying across the hospitals studied. Low usage was noted for "Watch" group drugs such as levofloxacin and ofloxacin (fluoroquinolones), and minocycline (tetracycline). Antibiotics categorised under the "Reserve" list, such as aztreonam (monobactam), ceftazidime/avibactam (third-generation cephalosporins), ceftolozane/tazobactam (fifth-generation cephalosporins), colistin (polymyxin), dalbavancin (glycopeptide), daptomycin (lipopeptide), linezolid (oxazolidinones) and tigecycline (glycylcycline) showed relatively lower usage levels. Detailed hospital-wise antibiotic usage data (gms/1000 BD) across all quarters is available in the Supplementary Sheet.

Chemical profiling of HWW revealed the presence of several important WHO-listed antibiotics, including meropenem, piperacillin, sulfamethoxazole, trimethoprim and vancomycin (Fig. S6). It is important to note that hospital antibiotic usage was not consistently reflected in the chemical data, as each antibiotic's stability in wastewater varied. Therefore, a high concentration of an antibiotic in wastewater does not necessarily indicate high usage. For example, beta-lactams are the most prescribed class, yet their poor stability affects the amount present in wastewater. Vancomycin had the highest relative concentration of quantified antimicrobials across the study period at A-H (52%), L-H (34.1%), R-H (30.1%), and S-H (41.2%), while piperacillin was prevalent at B-H (11.7%), G-H (28.7%), and R-H (22.6%). Sulfamethoxazole accounted for approximately 10–15% of the total relative antimicrobial concentration at the majority of sites, with the lowest proportion at A-H (2.2%) and the highest at W-H (22.8%). Trimethoprim contributed between 1.1% (A-H) to 21.2% (L-H) of antimicrobial residues across the summer campaign. Meropenem was quantified in wastewater effluent at all sites, except S-H, where it was consistently below the method quantification limit. Additionally, linezolid, nitrofurantoin, rifampicin and tetracycline were below the method quantification limit in samples from A-H. Rifampicin and tetracycline were not at quantifiable concentrations at G-H, while nitrofurantoin was not quantifiable at G-H and S-H. Although amoxicillin was below the method quantification limit in samples from A-H and S-H, its derivative, amoxicilloic acid, was present in some samples. Penicilloic G acid, a degradation product of penicillin G, had the highest mean concentrations at L-H and B-H across the sampling campaign.[31]

As discussed in detail in our previous paper,[31] the concentrations of many tracked in HWW posed a potential risk for the selection of antibiotic resistance. The degree of risk is defined by the risk quotient (RQ), which is calculated by dividing the measured antimicrobial concentration in wastewater by the predicted no-effect concentration for resistance selection. The risk of AMR emergence is then categorised into three groups based on the RQ: low (RQ < 0.1), moderate (0.1 ≤ RQ < 1) and high risk (RQ ≥ 1) (Table S1). The RQ was notably very high for antibiotics such as metronidazole, clarithromycin, ciprofloxacin, vancomycin, and trimethoprim, indicating a significant risk for the selection of antibiotic resistance at these hospital sites. Conversely, the RQ was comparatively lower for nitrofurantoin and linezolid at all sites.

Relationship between antibiotic concentration and ARG abundance

There was a statistically significant correlation between residual concentrations of antibiotics such as macrolide ($F(1, 18) = 64.17$, $p < 0.001$), oxazolidone ($F(1, 18) = 6.08$, $p < 0.05$) and vancomycin ($F(1, 18) = 11.8$, $p < 0.01$) and the relative abundance of the corresponding ARGs (Fig. S7). Concentrations of antibiotics such as beta-lactam, metronidazole, quinolone, tetracycline and sulphonamide were found to have no significant effect on corresponding ARG relative abundance ($p > 0.05$).

Discussion

ARG composition and diversity across hospitals

Hospital environments enriched with antibiotics, heavy metals, and disinfectants promote the emergence of antibiotic-resistant bacteria.[5,6] Metagenomic surveillance of hospital wastewater (HWW) using short-read sequencing has been proven as an efficient method for evaluating the overall AMR burden within hospital settings.[12] This national-scale study employed this approach and found a high relative abundance and diversity of ARGs in HWW effluents. Hospital communities generally exhibit a higher diversity and abundance of ARGs than local communities due to frequent exposure to last-line antibiotics.[21,39] In support of our approach for weekly monitoring, Lepper et al.[40] also showed that regular metagenomic analyses of HWW have the potential to reveal ARGs present in bacteria that colonise patients, the hospital environment, and its water system. Seasonal variability exists in resistance patterns in healthcare settings, suggesting the need for long-term surveillance to monitor trends in HWW.[11]

Interestingly, smaller hospitals like A-H had high relative ARG loads, similar to findings from India, where smaller hospitals had more ARGs than larger ones.[18] This may be due to effective antibiotic stewardship practices and proper waste disposal in larger hospitals.[18] The ARG signature varied across sites, indicating possible differences in ARG dynamics across hospitals. The inconsistency in sampling is a limitation of the study, as it may have impacted the representativeness and comparability of the data across sites. A study found that inpatient department-level antimicrobial usage positively correlates with the ARG pattern in HWW.[12] Certain ARGs may be prevalent in the local community and carried into hospitals by patients.[12] Further analysis should explore the factors influencing these differences in ARG dynamics across sites (e.g., geographical factors, hospital design, antimicrobial stewardship, patient loads, types of infections treated, and services provided, patient demographics) to inform the better design of targeted interventions for managing AMR in these hospitals. Patient demographics may include factors such as age, gender, underlying health conditions, hospital admission rates, and the proportion of patients receiving antibiotic treatments.

ARGs conferring resistance to aminoglycosides, beta-lactams, and MLS-were predominant across all hospital sites, aligning with findings from other studies.[18] The most recent report from Public Health Wales, based on phenotypic susceptibility testing on antibiotic resistance,[41,42] corroborated the observations on abundant ARGs made in our metagenomics study. For instance, high levels of beta-lactam-resistant *E. coli* and other bacterial isolates were found in both in-patient blood and in-patient and out-patient urine samples in hospitals in Wales. Medium level of aminoglycoside resistance was also observed among the isolates. There was a high level of macrolide (clarithromycin) resistant methicillin-resistant Staphylococcus

aureus (MRSA) blood-culture isolates. In addition, the widespread detection of mobile colistin resistance (*mcr*) genes and "big five" carbapenemases in the HWW samples in the study represents a critical challenge to public health, as colistin and carbapenems are among the last-resort options available for treating MDR infections. The *mcr-9* gene was highly prevalent in many hospitals. It often coexists with ESBL and carbapenemase genes.[43] The *mcr-10.1* was very prevalent in G-H. The infections caused by *mcr-10.1*-carrying bacteria are more challenging to treat.[44] However, not all *mcr* variants directly confer colistin resistance; for example, *mcr-9* does not confer resistance in the *Enterobacter cloacae* complex.[45] *Mcr-9* is transmitted via clonal spread, plasmids, or mobile genetic elements (MGEs), while *mcr 10* spreads mainly through clonal expansion and MGEs.[43]

Microbial community composition in hospital effluents

The bacterial community structure at the phylum level showed no significant spatial or temporal trends among hospitals, with Pseudomonadota and Bacteroidota dominating all sites. We ascribe this to their high relative abundance in the human gastrointestinal tract and adaptability to wastewater conditions.[46] These findings align with studies from other regions, where Pseudomonadota and Bacteroidota dominate hospital effluents.[15,18]

While the presence of ESKAPEE pathogens in HWW is expected, this study provides new insights into their spatial and temporal variability across different hospitals. The abundance of these pathogens fluctuated, suggesting that patient demographics, antibiotic usage, and infection control practices may have influenced the microbial community composition in hospital wastewater. Our findings reinforce previous reports indicating that HWW serves as a persistent reservoir for these high-risk pathogens, potentially contributing to their persistence and dissemination beyond healthcare settings. The detection of ESKAPEE pathogens in hospital effluents is particularly concerning, given their association with severe healthcare-associated infections. The WHO has classified *A. baumannii, P. aeruginosa*, and various Enterobacteriaceae as critical priority pathogens, recognising their significant role in hospital-acquired infections and antibiotic resistance.[47] ESKAPEE pathogens possess several key biological traits, including adaptive mechanisms for survival in hospital environments, efficient acquisition of resistance genes, and the global dissemination of high-risk clones.[48] These findings emphasise the need for tailored infection control strategies based on the specific pathogen profile of each hospital.

Regarding fungal pathogens, our study identified *C. albicans*, *Fusarium* spp., and *N. glabratus* as prominent WHO-listed fungal priority pathogens in hospital wastewater. This aligns with previous reports indicating that *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *Fusarium* spp., and *Penicillium* are among the most frequently detected fungi in hospital wastewaters.[49] *C. albicans* remains the leading cause of nosocomial fungal infections,[50] and our detection of *N. glabratus* aligns with the latest UK Health Security Agency[51] report, which identified *C. albicans* and *N. glabratus* as the most frequently isolated yeast species in hospital bloodstream infections.

Although Kraken was employed for bacterial species-level classification, it is essential to recognise the potential limitations of this method, as species-level assignments from metagenomic data may sometimes lead to over-interpretation, mainly when dealing with closely related species or gaps in the reference databases. Also, the fungal database within Kraken is less comprehensive than its bacterial counterpart, limiting the accuracy of fungal taxonomic assignments. This study acknowledges this limitation, suggesting that future work incorporates specialised fungal databases for improved accuracy.

Outbreaks of MDR microbial infections in hospitals have been linked to contaminated wastewater systems.[52,53] Blockages in hospital waste-pipe systems can cause backflow, leading to wastewater moving upstream and flooding outlets such as sinks or drains. Such incidents can heighten the risk of pathogens and AMR from the studied hospitals spreading into patient care areas, underscoring the critical need for effective wastewater management in the study areas.

Monitoring microbial communities using a WBE coupled with metagenomics approach, as demonstrated in our study, could potentially help predict and control the transmission of MDR infections within hospital settings and monitor the efficacy of interventions, thereby enhancing patient care and hygiene practices. Collaborative efforts among hospitals sharing similar bacterial and fungal profiles could enhance the management and prevention of hospital-acquired infections caused by bacteria and fungi. This can also be readily combined with surveillance for viral pathogens which can be simultaneously detected in hospital wastewater, and which have been implicated in ward closures (e.g., norovirus) or negative patient outcomes (e.g., SARS-CoV-2).[54] We recommend, therefore, that the hospital infection control teams implement tailored measures based on the microbial composition specific to each hospital's wastewater to help prevent the spread of infections and manage risks.

Predicting ARG hosts

Network analysis is widely used to predict potential hosts for ARGs based on the co-occurrence correlation between ARGs and bacterial genera in metagenomics-based studies.[55,56] In this study, *Acinetobacter* and *Aeromonas* were predicted to be key hosts for many ARGs across hospital effluents. Similar findings from Portugal and Croatia showed *Acinetobacter*, *Aeromonas*, and *Arcobacter* as major ARG carriers in hospital wastewater.[57,58] *A. baumannii*, a clinically significant pathogen, is known for its high levels of multidrug resistance[57] and association with nosocomial outbreaks and community-acquired infections.[59] *Aeromonas* spp. are already known to host a wide range of ARGs on MGEs, facilitating the exchange of ARGs with genetically diverse bacteria.[60] Many species of the genus, such as *A. hydrophila* and *A. caviae*, are recognised as emerging human pathogens responsible for gastrointestinal infections.[60] Many opportunistic pathogens, such as *Escherichia*, *Klebsiella*, *Enterococcus* and *Enterobacter*, were also found to be potential hosts for clinically relevant ARGs. *Enterococcus* was found to be significantly associated with genes encoding vancomycin resistance in certain hospitals studied. Infections caused by vancomycin-resistant enterococci (VRE) present considerable challenges in healthcare settings.[61] These findings suggest that efforts to reduce specific ARGs in hospital wastewater should target a wide range of bacteria, encompassing different genera and species, rather than focusing on a single type. Since network analysis is only a prediction-based method, for further validation, it is essential to construct metagenome-assembled genomes (MAGs) to identify potential bacterial hosts of ARGs. This can be complemented by conventional culture-based screening approaches for direct isolation and characterisation of resistant organisms. However, it is important to note that many environmental bacteria are not readily culturable under standard laboratory conditions, and hence, combining culture-dependent and culture-independent methods will provide a more comprehensive understanding of ARG distribution in wastewater.

Antibiotic usage and detection of antibiotic residues in hospital wastewater

Supporting our findings, a previous report on antimicrobial usage in secondary care in Wales also identified beta-lactam/beta-lactamase inhibitor combinations, beta-lactamase-resistant penicillins, broad-spectrum penicillins, imidazoles, macrolides, and tetracyclines as the most used antibacterials,[62] majority of which fall

under the “Access group” of AWaRe list. The usage data shows effective antibiotic stewardship practices across the hospitals in Wales. The data has some limitations because it is impossible to identify what is consumed within the hospital and what is taken home.

The interhospital variation in antibiotic consumption may be influenced by local prescribing policies and case mix. Antibiotic usage (gms/1000 BD) was generally higher during the late fall and early winter months in most hospitals studied, while a varied usage trend was found in others. This variation may be attributed to seasonal fluctuations in pathogens. While some bacterial infections, such as bacterial meningitis and respiratory infections, tend to follow a winter seasonal pattern in temperate climates,[63] infections like *Campylobacter* and *Salmonella* gastroenteritis are more prevalent during the warmer months.[64] Additionally, urinary tract infections (UTIs) typically peak in the UK between September and November in individuals aged 14–69.[65] Penicillins such as piperacillin-tazobactam and amoxicillin, commonly used to treat respiratory tract infections in the UK, were among the most widely administered antibiotics across all hospitals under study (gms/1000 BD). Other frequently used antibiotics include flucloxacillin, an anti-staphylococcal penicillin effective for treating skin and soft tissue infections caused by *Staphylococcus* and Group A *Streptococcus*. Another common antibiotic was metronidazole, prescribed to treat various bacterial infections, including *Clostridium difficile* infection (CDI), bacterial vaginosis, and pelvic inflammatory disease. Studies state that CDI rates are higher among the Welsh population than in England and Scotland.[66]

The variation in antibiotic concentrations detected across the hospital effluents could be due to differences in usage, with some hospitals potentially using certain antibiotics more frequently or in higher doses. Variations in antibiotic administration, bed count, water usage, and medical services across facilities can influence antibiotic levels observed in hospital wastewater.[67] Additionally, the low stability issue of certain antibiotics, such as beta-lactams, in wastewater,[68] made it challenging to establish clear, meaningful comparisons or trends between prescribed antibiotic quantities and those detected in wastewater. Hence, it is to be noted that a high concentration of an antibiotic in wastewater does not always indicate high usage. Our analysis was limited by the lack of flow data from each hospital, which restricted our ability to normalise antibiotic concentrations and conduct a standardised spatial comparison. Nonetheless, we were able to definitively confirm the presence of antibiotic residues in the effluents discharged. We collected quarterly data for antibiotic usage and daily data collected over a week for three months during the summer campaign for antibiotic residues. Future comparisons between antibiotic usage data and antibiotic detection in HWW during the same sampling period will offer a more precise understanding of the potential correlation between usage and detection in HWW. The levels of many antibiotics, such as metronidazole, clarithromycin, ciprofloxacin, vancomycin, and trimethoprim in the hospital wastewater under study represented a high risk of AMR selection; further actions are needed to prevent selection for AMR in wastewater. In a previous study, it was found that ciprofloxacin posed a high potential risk, while clarithromycin posed a low risk in wastewater from England and Wales.[69] To mitigate environmental impact and curb the emergence of AMR, physicians should prioritise prescribing more biodegradable antibiotics over those that persist longer in the environment.[12]

We found a significant correlation between the concentrations of certain antibiotics in wastewater and the relative abundance of their corresponding ARGs. It is important to note that this comparison was based on relative abundance data, not quantitative ARG measurements. Also, while beta-lactams were heavily prescribed in all hospitals, their rapid degradation in wastewater likely led to lower detectable concentrations, reducing their impact on ARGs. Therefore, the stability of antibiotics should also be considered when evaluating their influence on ARG prevalence. We could not establish a link between antibiotic usage and ARG due to the absence of longitudinal data. Longitudinal studies are necessary in these hospitals to monitor trends in antibiotic usage and ARGs, providing valuable insights into potential cause-and-effect relationships and guiding targeted interventions like antibiotic stewardship. There may be factors other than antimicrobial stewardship practices also contributing to a higher incidence of antimicrobial resistance and pathogens in specific hospitals, such as not adhering to strict hygiene, cleaning and disinfection practices and improper waste management.[70]

Environmental and public health risks of untreated hospital effluent and recommendations for improved management

Studies have identified HWW as a potential source of pathogens that can cause human infections acquired from the environment.[71,72]

In Wales, hospital and community wastewater are processed and treated together at the municipal wastewater treatment plant. Effluents from these hospitals are discharged directly to the local wastewater treatment plants (WWTPs) without any pretreatment (e.g., disinfection, retention tanks). Our findings indicate that clinically relevant ARGs, high-priority pathogens and critical antibiotics circulate in the HWW and, if not adequately treated, may end up in aquatic environments through untreated combined sewer overflows or improperly treated effluent.[7] The nutrient-rich environment fosters the emergence of "superbugs" through horizontal gene transfer.[73] Discharge of inadequately treated HWW into environmental waters has led to the emergence of resistance to last-resort drugs.[9] It is important to note that many of the hospitals in this study are located near the coast, where wastewater releases are expected to negatively impact both freshwater and near-shore coastal water quality. Their proximity to bathing waters, beaches, and shellfisheries also increases the risk of re-entry of contaminants into the food chain and human population.

Conventional WWTPs are not designed to remove micropollutants like antibiotics, AMR bacteria, and ARGs from HWW, making on-site pre-treatment essential to minimise environmental impacts.[74] Although these approaches are recommended by the WHO for the safe management of wastes from hospitals,[75] there are no established limits for indicator parameters in HWW prior to discharge. Moreover, regulations vary and rarely cover microbiological indicators.[76] Advanced Oxidation Processes like ozone, chlorination, and UV treatments are widely utilised in HWW management globally because of minimal environmental impact.[74] Ozone-based treatments targeting the WHO list of priority bacteria indicated a deactivation rate of over 99.9% for both antimicrobial-resistant bacteria and their genes.[77] Another study reported non-thermal plasma technology as an effective way to inactivate carbapenem-resistant *A. baumannii* from hospital wastewater.[78] Using bioactivated carbon is proven to be highly efficient for eliminating antibiotic residues from wastewater.[74] Certain antibiotics, such as beta-lactam, aminoglycoside, and vancomycin, are hydrophilic, whereas MLS and tetracycline are characterised as lipophilic.[79] Hence, the effectiveness of treatment is significantly influenced by factors such as the physico-chemical properties and stereochemical configuration of the antibiotic and hence, treatment strategies require a tailored approach. A comprehensive cost-benefit analysis should also be conducted to assess the economic viability and public health impact of implementing advanced wastewater treatment practices in these hospitals. This national-scale analysis should quantify both the potential reduction in hospital-acquired infections and the improvements in overall patient outcomes, weighing these benefits against the costs of implementation and maintenance.

Table 1
Summary of key findings from the national-scale hospital wastewater AMR study.

Hospital	Total ARG abundance (RPKM)	Total antibiotic usage (Average ± SD grams/1000 bed days)	Predominant ARG classes	Antibiotic concentrations exceeding Risk Quotients (RQ)	Distribution of ESKAPEE bacteria	Distribution of Fungi	Notable carbapenemases and *mcr* genes	Unique observations
Aberystwyth (A-H)	9333–23,800	180,827.33 ± 8715.56	Aminoglycoside, β-lactams and MLS	High RQ: Metronidazole, Clarithromycin, Vancomycin, Ciprofloxacin	Significant weekly fluctuations	Wide weekly variation, relatively higher abundance of *Fusarium spp.*	bla_{OXA-48}, $bla_{IMP,}$ bla_{VIM} detected; *mcr-* undetected except *mcr–9.1* in one sample	Higher relative abundance and diversity of total ARGs
Bangor (B-H)	4590–11,262	229,075.9 ± 6931.36	Aminoglycoside, β-lactams, MLS and tetracyclines	High RQ: Metronidazole, Clarithromycin, Ciprofloxacin, Trimethoprim	Significant weekly fluctuations	Relatively higher abundance of *Candida, Fusarium* and *Nakaseomyces* spp.	bla_{KPC}, bla_{IMP} dominant; *mcr- 9.1*, *mcr- 3.17* prevalent	-
Carmarthen (C-H)	4659–10,690	242,579.78 ± 7474.21	Aminoglycoside, β-lactams, MLS and tetracyclines	-	Significant weekly fluctuations	Relatively higher abundance of *Nakaseomyces* spp.	bla_{IMP}, bla_{NDM} prevalent, *mcr-9.1*, *mcr-3.17*, *mcr-5.1*, prevalent	-
Glamorgan (G-H)	6208–10,448	195,832.65 ± 14,412.64	Aminoglycoside, β-lactams, MDR and MLS	Moderate RQ: Metronidazole, Clarithromycin, Trimethoprim	Relatively higher abundance of *Enterobacter* spp.	Relatively higher abundance of *Saccharomyces spp.*	bla_{KPC} prevalent, *mcr-10.1* dominant	High diversity of *mcr* genes; relatively higher abundance of lipopeptide resistance encoding genes compared to other sites
Llandough (L-H)	6481–10,826	136,718.76 ± 12,912.73	Aminoglycoside, β-lactams and MLS	High RQ: Ciprofloxacin, Trimethoprim	Relatively higher abundance of *Acinetobacter baumanii*	Relatively higher abundance of *Fusarium* spp.	bla_{IMP} prevalent; bla_{NDM}, bla_{VIM} consistently present; *mcr-3.17* and *mcr-5* variants prevalent	Relatively lower usage of antibiotics (gm/1000 BD)
Rhyl (R-H)	6135– 10,470	302,571.45 ± 12,772.57	Aminoglycoside, β-lactams, MLS and vancomycin	High RQ: Metronidazole, Ciprofloxacin, Vancomycin, Trimethoprim	Relatively higher abundance of *E. coli* and *Pseudomonas aeruginosa*	Relatively higher abundance of *Candida, Fusarium* spp., and *Nakaseomyces* spp.	bla_{OXA-48} and bla_{IMP} prevalent, weekly variation of *mcr*-variants; *mcr-9.1* prevalent	Relatively high usage of antibiotics and higher abundance of ARGs conferring resistance to vancomycin
Swansea (S-H)	4180–7004	257,772.18 ± 8255.5	Aminoglycoside, β-lactams and MLS	Moderate RQ: Metronidazole, Ciprofloxacin, Trimethoprim, Vancomycin	Relatively higher abundance of *Acinetobacter baumanii* and *Enterobacter spp.*	Relative higher abundance of *Candida* spp.	bla_{KPC} dominant, *mcr-3.17*, *mcr-7.1*, *mcr-9.1* prevalent	Relatively higher abundance of *Candida* spp. (*C. albicans*)
Wrexham (W-H)	4888–9243	148,432.85 ± 7900.86	Aminoglycoside, β-lactams, MLS, tetracyclines and vancomycin	Moderate RQ: Metronidazole, Trimethoprim, Ciprofloxacin	Relatively higher abundance of *E. coli*	Not much weekly variation	bla_{KPC}, bla_{IMP}, bla_{VIM} dominant, *mcr-9.1*, *mcr-3.17* prevalent	Relatively higher abundance of ARGs conferring resistance to vancomycin

Conclusion

The present national-scale study strongly supports the applicability of wastewater-based epidemiology and metagenomics as robust tools to monitor and control antimicrobial resistance within healthcare environments. The key findings from the study are summarised in Table 1. This approach facilitates detailed tracking of microbial populations and antimicrobial resistance without the ethical concerns associated with direct patient sampling, offering a detailed view of the microbial and resistance profiles in hospital wastewater. Key findings and recommendations:

1. Distinct resistome and microbiome profiles in each hospital setting highlight the need for tailored strategies and region-specific policies, rather than a one-size-fits-all approach, to effectively mitigate the spread of clinically relevant ARGs and pathogens in these environments.
2. The study was limited by the fact that it only involved collecting wastewater samples during a single season, and hence, future studies should focus on the seasonal variations in hospital microbiome and resistome.
3. This comprehensive analysis not only maps the current state of AMR in hospitals across Wales but also provides crucial data-driven insights to guide future policymaking, enabling the mitigation of ARG release from healthcare settings by implementing on-site advanced wastewater treatment technologies (e.g. advanced oxidation processes, non-thermal plasma and biosorption techniques).
4. The findings also highlight the urgent need for policymakers to consider implementing regulations for the pre-treatment of hospital wastewater prior to its release into municipal sewage systems, particularly in areas where hospitals are located near coastal regions or recreational waters.
5. Policymakers should also consider mandating regular metagenomic surveillance of hospital wastewater as part of a comprehensive AMR monitoring strategy, integrating this approach into existing public health infrastructure.

Author contributions

Reshma Silvester: conceptualisation, formal analysis, writing – original draft, writing – review & editing, visualisation. William B. Perry and Amy Baldwin: data analysis, writing – review & editing. Gordon Webster, Laura Rushton and Neil Byrnes: laboratory analysis and writing – review & editing. Daniel Pass: bioinformatics. Kata Farkas, Gareth Cross, Noel Craine, Margaret Heginbothom and Barbara Kasprzyk-Hordern; writing – review & editing. Peter Kille, Andrew J. Weightman, and Davey L. Jones: conceptualisation, funding, supervision, writing – review & editing.

Availability of data

The data will be made available upon request.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2025.106503.

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