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Wastewater-based analysis of antimicrobial resistance at UK airports: Evaluating the potential opportunities and challenges

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ABSTRACT

With 40 million annual passenger flights, airports are key hubs for microbial communities from diverse geographic origins to converge, mix, and distribute. Wastewater derived from airports and aircraft represent both a potential route for the global dispersion of antimicrobial resistant (AMR) organisms and an under-utilised resource for strengthening global AMR surveillance. This study investigates the abundance and diversity of antimicrobial resistance genes (ARGs) in wastewater samples collected from airport terminals (n = 132), aircraft (n = 25), and a connected wastewater treatment plant (n = 11) at three international airports in the UK (London Heathrow, Edinburgh and Bristol). A total of 76 ARGs were quantified using high throughput qPCR (HT-qPCR) while a subset of samples (n = 30) was further analysed by metagenomic sequencing. Our findings reveal that aircraft wastewater resistomes were compositionally distinct from those observed at airport terminals, despite their similar diversity. Notably, flights originating from Asia and Africa carried a higher number of unique ARGs compared to those from Europe and North America. However, clustering of the ARG profile displayed no overall association with geography. Edinburgh terminal and pumping station wastewater had compositionally comparable resistomes to that of the connected urban wastewater treatment plant, though further research is needed to determine the relative contributions of the local population and international travellers. This study provides the first comprehensive investigation of AMR in wastewater from both aircraft and terminals across multiple international airports. Our results highlight aircraft wastewater as a potential route for cross-border AMR transmission and a valuable tool for global AMR surveillance. However, the findings also underscore the limitations and need for standardised approaches for AMR monitoring in airport environments, to effectively mitigate the global spread of AMR and enhance public health surveillance strategies.

1. Introduction

Antimicrobial resistance (AMR) represents a major global public health threat (Larsson et al., 2023; Tang et al., 2023). The distribution, abundance, and diversity of AMR-carrying bacteria vary significantly across geographic regions, influenced by a range of health, socioeconomic, and environmental factors (ARC, 2022; Hendriksen et al., 2019; Salam et al., 2023). This variability is further enhanced by growing global mobility, as cross-border travel and trade accelerate the worldwide exchange and spread of AMR bacteria (Frost et al., 2019). Consequently, there is an urgent need for a comprehensive understanding of the global distribution and international transmission

Abbreviations: AMR, Antimicrobial resistance; ARG, Antimicrobial resistance gene; MGE, Mobile genetic element; WWTP, Wastewater treatment plant; HT-qPCR, High-throughput quantitative polymerase chain reaction; MLS, Macrolide, lincosamide and streptogramine; ESBL, Extended-spectrum beta-lactamase; CT, Threshold cycle value; NMDS, Non-metric multidimensional scaling; dbRDA, Distance-based redundancy analysis; PERMANOVA, Permutational multivariate analysis of variance; WBE, Wastewater-based epidemiology.

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dynamics of AMR pathogens. Human-derived wastewater plays a critical role in this context, serving as an environmental reservoir for the dissemination and persistence of antimicrobial resistance genes (ARGs), while also providing a medium for horizontal gene transfer between bacterial species (Nguyen et al., 2021). However, implementing effective global AMR monitoring is challenging due to variations in testing methods, reporting standards, and resource availability between countries (O'Neill, 2016; WHO, 2021). These disparities can lead to gaps in the understanding of AMR prevalence and spread, particularly in low- and middle-income countries where surveillance systems may be less developed. In this context, airports emerge as strategic sites for AMR surveillance, acting as hubs where microbial communities from diverse geographic origins converge (Shingleton et al., 2023). Wastewater-based epidemiology (WBE), a method proven effective in monitoring other public health issues, such as illicit drug use and viral pathogens, therefore offers a novel and efficient approach to global AMR surveillance in these settings (Farkas et al., 2023; Qvesel et al., 2023; Tang et al., 2024; Tay et al., 2024).

With ca. 40 million passenger flights forecasted in 2024, global air travel is likely to make a major contribution to the spread of AMR, with both international visitors and returning residents potentially carrying resistant bacteria (Frost et al., 2019; Bokhary et al., 2021; Statista, 2023). For example, previous studies have shown that returning travellers have an increased likelihood of harbouring ESBL-producing *Escherichia coli* and to exhibit a more abundant and diverse gut resistome (Rodriguez-Molina et al., 2022; D'Souza et al., 2021). This risk is particularly pronounced for travellers visiting regions with high abundances of AMR pathogens, such as parts of Asia and Africa (D'Souza et al., 2021). Aircraft wastewater presents a unique opportunity for global AMR surveillance, capturing data from diverse international populations and linking it to countries of departure (Jin et al., 2024). This is particularly the case for international long-haul flights where there is greater likelihood of passengers using the aircraft toilets (Jones et al., 2023). This is exemplified by a metagenomic study of wastewater from 18 long-haul flights to Copenhagen which found clinically relevant ARGs, such as $bla_{\text{CTX-M}}$, in higher abundance on flights originating from South Asian countries (Nordahl Petersen et al., 2015). This study also highlighted aircraft wastewater as a potential route for 'exogenous' AMR bacteria to enter local water cycles, especially considering it having an observed richer resistome compared to municipal wastewater (Hess et al., 2019).

Despite the clear potential for AMR surveillance, research on aircraft wastewater remains in its infancy. This study aims to assess the utility of aircraft wastewater for global AMR surveillance and to evaluate the relative contribution of aircraft passenger waste to the resistome of local wastewater systems. We compared the wastewater resistome from three international airports of varying sizes in the UK (Bristol, Edinburgh, and London Heathrow). Samples were collected from sewers connected with airport terminals, vacuum trucks carrying wastewater from inbound aircraft, and a wastewater treatment plant (WWTP) proximal to Edinburgh airport. This multi-point sampling approach was designed to provide a better understanding of how AMR profiles change as wastewater moves through the airport system and into local treatment facilities. The resistomes were analysed using high-throughput qPCR for sensitive quantification of ARGs, with a subset of samples further examined by metagenomic sequencing for comprehensive resistome profiling. This dual-method approach provides both targeted quantification of known resistance genes and a broader view of the overall resistome composition. We hypothesised that (i) aircraft wastewater resistomes were compositionally distinct from those of airport terminals; (ii) the diversity and composition of ARGs in aircraft wastewater would vary based on the geographic origin of flights; and (iii) airport wastewater would introduce ARGs from aircraft passengers into the local wastewater treatment system.

2. Materials and methods

2.1. Sampling sites

Wastewater sampling was undertaken at three international airports in the UK, namely Bristol airport (51°23′08″N, 02°42′14″W), Edinburgh airport (55°57′08″N, 03°20′58″W), and London Heathrow airport (51°28′12″N, 00°27′15″W) (Table 1). The samples were taken during the COVID-19 pandemic, although no passenger COVID-19 tests were required for passenger entry across the study period, regardless of vaccination status and unvaccinated travellers no longer needed to take tests or quarantine upon arrival (i.e., unrestricted passenger entry to the UK). On-site wastewater sampling was conducted by external collaborators (2030 Labs, Veolia, and Aqua Enviro, UK) in coordination with local airport staff as detailed in Farkas et al. (2023). Authorisation for sampling was granted by Heathrow Airport Ltd, Bristol Airport Ltd, and Edinburgh Airport Ltd.

2.2. Sample collection and physicochemical analyses

Samples were collected from Bristol, Edinburgh and London Heathrow airports between the 8th and 31st March 2022 ($n = 158$, Table 1). At Bristol airport, samples were collected from manholes connected to the sewers of the arrival terminal (BRS-A) and the entire airport (BRS-WA). Grab samples were also collected from the manhole of a deposit site for vacuum trucks serving inbound aircraft (BRS-IA), for which an autosampler was programmed to take an individual sample during each wastewater discharge event. Each sample was a mixture of wastewater from 4 to 7 different inbound flights. At Edinburgh airport, samples were collected from manholes connected to the international terminal sewer (EDI-T), from a pumping station (PS) acting as a deposit site for vacuum trucks and from the WWTP where the airport wastewater is treated. At London Heathrow airport, wastewater samples were collected from a manhole connected to the sewer of the central terminal area (LHR-CT), capturing terminals T1, T2 and T3. Grab samples were also collected directly from vacuum trucks, which had collected wastewater from 10 individual aircraft (Table S1) on the day of sampling (LHA-IA). Wastewater electrical conductivity, pH, turbidity, ammonium, and orthophosphate concentrations were measured as previously described (Hillary et al., 2021).

Table 1
An overview of wastewater sampling from Bristol (BRS), Edinburgh (EDI) and London Heathrow (LHR) airports.

Airport	Passenger no. 2022	Sampling site	Sampling period	*n*	Sample type
Bristol	7.9 million	BRS-IA	25-28th March	7	Grab
		BRS-A	21-28th March	22	21-hour composite
		BRS-WA	21-29th March	28	21-hour composite
Edinburgh	11.2 million	EDI-T (MH1)	8-23rd March	43	2-hour composite
		EDI-T (MH2)	8-23rd March	16	24-hour composite
		EDI-T (MH3)	8-19th March	8	24-hour composite
		EDI-PS	8-23rd March	5	12-hour composite
		EDI-WWTP	8-24th March	11	24-hour composite
London Heathrow	61.6 million	LHR-CT	8-24th March	15	24-hour composite
		LHR-IA	16th March	10	Grab

IA, Inbound aircraft; A, Arrivals terminal; WA, whole airport; MH, Manhole; PS, Pumping station; WWTP, Local wastewater treatment plant; CT, Central terminal.

2.3. Wastewater DNA extraction

Wastewater samples were stored at −80 °C after collection and thawed when extracted. A 50 ml aliquot of the samples were centrifuged at 10,000xg at 4 °C for 30 min. DNA was extracted from the pellets using a modified FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) as described previously (Webster et al., 2003). The 100 µl DNA extracts were then stored at −20 °C until required.

2.4. High-throughput qPCR

A total of 96 primer sets were used in the HT-qPCR assay, targeting ARGs (76 genes), mobile genetic elements (MGEs, 10 genes), pathogens (5 genes), and 5 other genes including those conferring metal resistance and the 16S rRNA gene for normalisation (Table S2). These targets were chosen based on advice from the Specialist Antimicrobial Chemotherapy Unit at Public Health Wales to include a broad range of clinically relevant ARGs. DNA extract aliquots were sent to Resistomap Oy (Helsinki, Finland) for gene quantification using the SmartChip™ Real-Time PCR system (TakaraBio, CA, USA). The qPCR cycling conditions and raw data processing have been described previously (Lai et al., 2021; Muziasari et al., 2017). In brief, the protocol involved an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing at 60 °C for 30 s. Melting curve analysis was conducted on all primer sets, and amplicons exhibiting non-specific melting curves or multiple peaks were excluded from further analysis. A threshold cycle value (CT) of 27 was established as the limit of detection (Lai et al., 2021; Muziasari et al., 2017). Each sample was analysed in triplicate, and were a gene detected in at least two technical replicates, the mean C_t value was calculated. The minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin, et al, 2009) checklist can be found in Table S12.

2.5. Metagenomic library construction and sequencing

A subset of 30 wastewater samples derived from the LHR-IA, LHR-CT and BRS-IA (Table 1) underwent metagenomic analysis. The DNA extracts were diluted 1/10 in RSB resuspension buffer (Illumina Inc., San Diego, CA) and quantified using the Qubit High Sensitivity dsDNA Assay Kit on the Qubit 4 Fluorometer (ThermoFisher Scientific, Waltham, MA). Library preparation was then performed 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) following the protocol as described in the Illumina reference guide. Dual-indexed paired-end single-stranded DNA Libraries were then quantified using the CollibriTM Library Quantification kit (ThermoFisher Scientific) using a LightCycler® 96 Instrument (Roche Diagnostics Ltd., Rotkreuz, Switzerland) and concentrations were adjusted to 2.0 nM. The libraries were then pooled and quality assessed using a MiSeq 300 cycle cartridge (Illumina MiSeq System). Based on the sequence reads, the library concentration was adjusted to 1.5 nM and sequenced on an Illumina NovaSeq 6000 Sequencing System (paired end 2 x 150 bp flowcell) with NovaSeq S4 cartridge v1.5 (300 cycles) and 1 % PhiX control. Sequencing was conducted at the Wales Gene Park (https://www.walesgenepark.cardiff.ac.uk/).

2.6. Bioinformatic analysis of sequence data

Metagenomic sequence reads were converted from base call to FASTQ files, then demultiplexed and adapter trimmed using Illumina bcl2fastq2 conversion software v2.20. The reads were then quality filtered using fastp v 0.20, evaluated with fastqc v 0.11.8 and summarised in a report using MultiQC v1.9 (Ewels et al., 2016). Host removal was then conducted by mapping all reads against the human genome using Samtools v 1.15.1 and Bowtie v2.3.5. Non host reads were aligned against the MEGAres v3 database using the AMR++ v3.05 pipeline implemented with NextFlow v21.10, ARGs requiring SNP confirmation underwent verification using the SNP confirmation tool (Bonin et al., 2023). Reads were also aligned against the Kraken v2.1.2 database (Wood et al., 2019).

2.7. Statistical analysis

ARG counts derived from HT-qPCR analysis were expressed as ARG copy number /16S rRNA gene copy number and those derived from metagenomic analysis were expressed as Fragments Per Kilobase reference per Million bacterial fragments (Munk et al., 2022). Figure design and statistical analyses were performed on R 4.2.1 (R Core Team. 2022) using the following packages: phyloseq, vegan, ggplot2 and igraph. Metagenomic counts rarefied to the lowest sampling depth were used in the calculation of Shannon and Observed matrices. Bray-Curtis dissimilarity matrices of Hellinger-transformed ARG relative abundances were used in Non-metric Multidimensional Scaling (NMDS) and distance based Redundancy Analysis (dbRDA). Welch's two-sample t tests were used for the comparisons of two groups, $p < 0.05$ was considered significant.

Network analysis was performed on the correlated relative abundances of ARGs and bacterial genera detected in at least 15 of the 30 metagenomic samples. Spearman's rank correlation coefficient values with $\rho > 0.7$ and $p < 0.01$ were visualised using Gephi v0.10.1 (Bastian et al., 2009).

3. Results

3.1. The relative abundance of ARGs

The abundance of the HT qPCR target ARGs were quantified relative to the 16S rRNA gene in each sample (Fig. 1.A). Between the airport terminals, the mean total relative abundance of ARGs was significantly higher in BRS than EDI and LHR (One-Way ANOVA $p < 0.01$, $f = 9.57$). Comparing the terminals and aircraft, the LHR-IA samples carried a significantly higher relative abundance of ARGs than the terminals (Welch's *t*-test: $p < 0.05$), while no significant difference was observed between the BRS wastewater samples. Meanwhile, Edinburgh's WWTP carried a significantly lower relative abundance of ARGs than the airport samples (Welch's *t*-test: $p < 0.05$).

Comparing the airport terminal wastewater, HT-qPCR analysis found the macrolide, lincosamide and streptogramine (MLS) resistance genes in high abundance (Fig. 1.B), similar to levels observed at Edinburgh's WWTP. The MLS ARG, *ermB*, was especially abundant in the BRS-WA samples, whereas BRS-A carried a significantly higher level of *mrsE* (Welch's *t* test: $p < 0.01$). In contrast to those from the terminals, the Bristol and London aircraft wastewater samples carried a significantly higher abundance of the tetracycline resistance gene, *tetM* (Welch's *t* test: $p < 0.01$) and a lower abundance of MLS resistance genes (Welch's *t* test: $p < 0.01$). These distinct profiles were not observed in the EDI-PS, which serves as a repository for the aircraft vacuum truck waste. Indeed, all samples from Edinburgh displayed similar a ARG profile, with a comparatively high abundance of the *qacF* gene, encoding a multidrug and biocide efflux pump.

Metagenomic analysis on the subset of wastewater samples produced a mean output of 204,122 assigned reads per sample, 86 % of which conferred resistance against antimicrobials. The LHR-CT ARG profile was comparable to that determined by HT-qPCR (Fig. S1), while the aircraft resistomes were less alike. The metagenomic approach detected multiple MLS ARGs outside of the HT-qPCR target set, including the highly abundant *ermT*, such that they dominated the aircraft resistome similarly to the terminal wastewater. Nonetheless, hierarchical clustering analysis on the 50 most abundant ARGs found the LHR-CT and IA resistomes to form distinct clusters (Fig. 2). Differential expression (ALDEx2) analysis on centred log-ratio transformed counts confirmed *mefA* and *tet16A* were more abundant in the LHR-CT, whereas *tetM* and

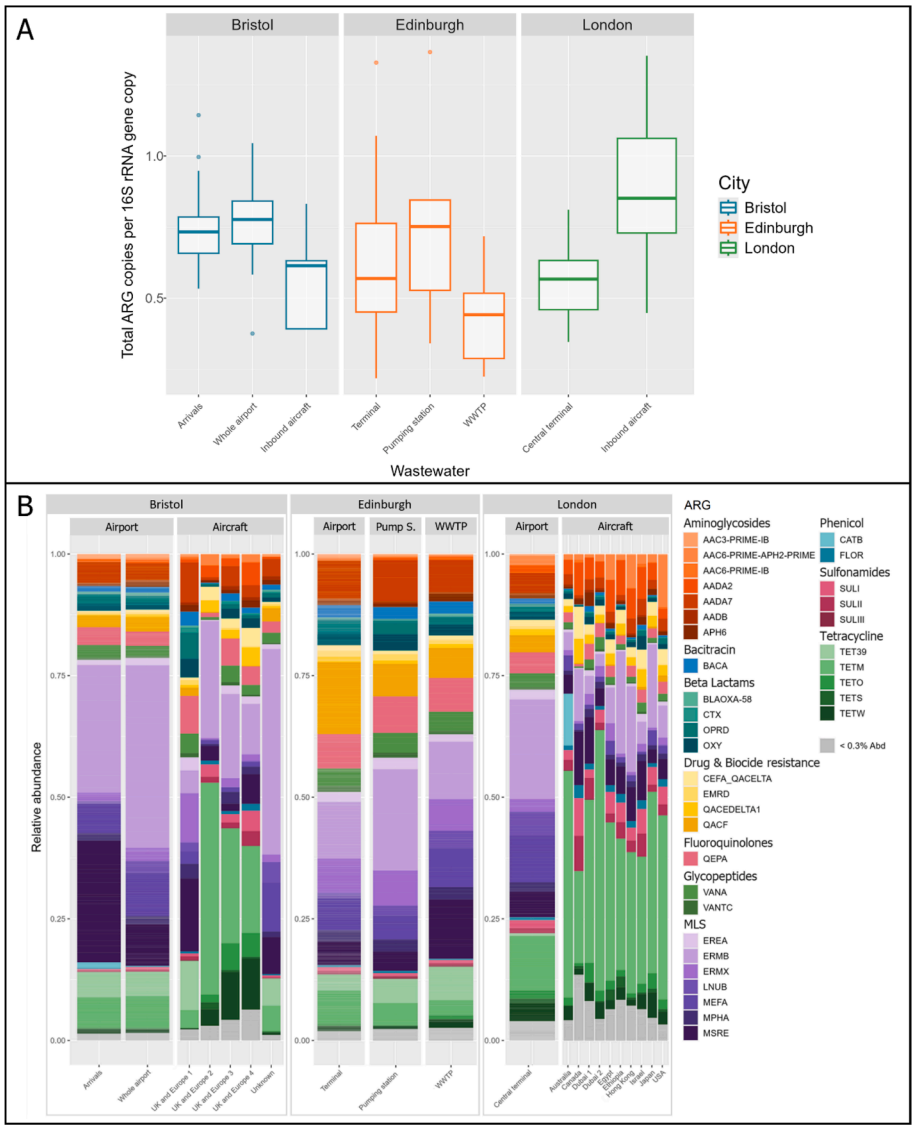

Fig. 1. (A) A boxplot showing the total ARG abundances relative to the 16S rRNA gene. (B) Stacked bar chart showing the relative abundances of HT-qPCR target antimicrobial resistance genes (ARGs) in aircraft and airport-related wastewater samples. Excluding the Inbound Aircraft, the relative abundances are a representative average of all samples from each wastewater source.

ermT were amongst those more abundant in the LHR-IA wastewater (Fig. S2). Among the aircraft samples, the clusters did not display any strong association with geography (Fig. 2).

The accessory resistome was also explored, comprising of genes which weren't detected across all metagenomic samples. The accessory resistome contributed a significantly higher proportion (Welch's *t* test: $p < 0.01$) to the total in the LHR-CT samples (19 ± 6.0 %) compared to the IA (11 ± 3.7 %) (Fig. S3.A). MLS and tetracycline resistance genes still dominated the LHR-CT and BRS-IA samples. However, LHR-IA samples saw a uniquely high relative abundance of phenicol resistance genes (Fig. S3.B).

3.2. Alpha diversity of ARGs

HT-qPCR analysis detected an average 66 of the 79 target ARGs within the wastewater resistomes (Fig. S4.A); only $bla_{\text{OXA-23}}$ and VEB were left undetected across all samples. Assessing the overall diversity of the aircraft samples by metagenomic analysis, the LHR-IA wastewater carried a significantly higher number of observed ARGs than BRS-IA (Welch's *t* test: $p < 0.01$), although it was not more diverse based on the Shannon index values (Welch's *t* test: $p = 0.1979$) (Fig. S4.B). Flights originating from Africa and Asia carried a higher number of observed ARGs than those from North America and Europe (Fig. 3). This difference was significant between those from Asia and Europe (Welch's *t* test: $p < 0.01$). There was no significant differences in diversity between the LHR CT and IA resistomes ($p = 0.8306$ and 0.7785 for Observed and Shannon indices, respectively). However, the overall number of unique ARGs (i.e., ARGs uniquely named at the "group" level of the MEGARes classification scheme; Bonin et al., 2023) detected in the LHR-IA samples was higher than those in the LHR-CT (202 and 182, respectively).

3.3. Beta diversity within the ARG profiles

The wastewater resistomes from Bristol and London clustered significantly (PERMANOVA $p < 0.01$) by source (Fig. 4.A and C). In contrast, such distinct clustering was not observed among the Edinburgh wastewater resistomes (PERMANOVA $p > 0.05$) (Fig. 4.B). Metagenomic analysis likewise found the London samples to cluster distinctly by source (Fig. S5.A), and both approaches saw a high degree of heterogeneity in the BRS-IA resistome compared to BRS A and WA (Fig. 4.A

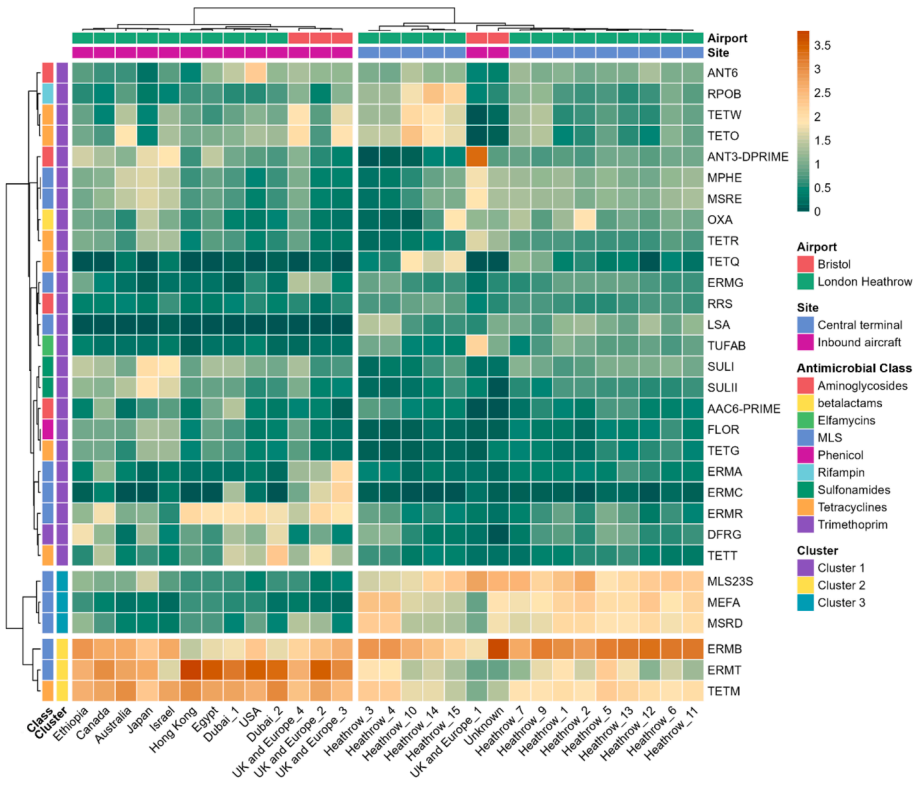

Fig. 2. Clustered resistome heatmap showing the log transformed abundance of the 50 most abundant antimicrobial resistance genes (ARGs) in the metagenomic wastewater sample subset.

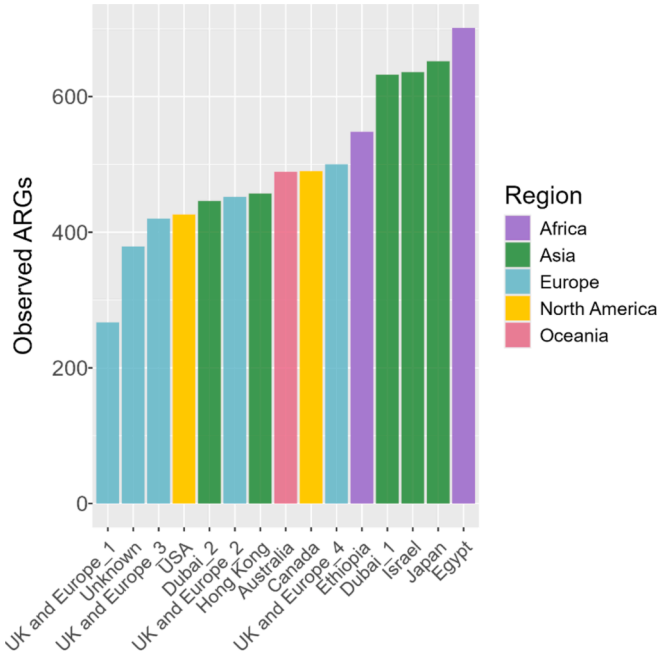

Fig. 3. Influence of global region on the total number of observed antimicrobial resistance genes (ARGs) identified in the metagenomic analysis of inbound aircraft wastewater.

and S5.B). Comparing terminal-derived wastewaters, the resistomes clustered significantly by source (PERMANOVA $p < 0.01$). Additionally, the aircraft resistomes from London and Bristol did not cluster by source (PERMANOVA $p = 0.06$) and were together distinct from that of Edinburgh's Pumping Station (PERMANOVA $p > 0.01$).

3.4. Correlations between ARGs and potential influencing factors

Distance-based redundancy analysis revealed that the wastewater physicochemical properties had a significant overall explanatory effect on the composition of the wastewater resistome (Fig. 5), with an adjusted R^2 of 0.38 ($p < 0.01$). Of the tested variables, ammonium concentrations contributed the largest influence (Table S4). Comparing the mean levels of each variable between wastewater samples (Table S5), ammonium differed between airports, being at their lowest in the samples from Edinburgh (e.g., 44.7 mg N l^{-1} for EDI T) and highest in the LHR-CT and BRS-A samples (127.9 mg N l^{-1}, and 122.3 mg N l^{-1}). In contrast, the explanatory effect issued by the other variables was shaped more by their differences between the sample sources. For example, the pH, electrical conductivity, phosphorus, and turbidity levels were all significantly higher in the LHR-IA compared to the LHR-CT samples (Welch's *t*-test: $p < 0.01$ for all comparisons).

At the level of individual genes, HT-qPCR target ARGs detected in at least 20 samples were correlated against physicochemical properties, mobile genetic elements, and HT-qPCR target pathogens (Fig. S6). The integrase genes, *intI3* and *intI1*, displayed the highest number of strong significant ($\rho > 0.7$, $p < 0.01$) correlations with ARGs. In contrast, few strong relationships were observed between ARGs and either the pathogens or physicochemical properties.

3.5. Microbial community and pathogen analysis

Kraken analysis of the metagenomic sample subset produced an output with a mean 39,990,814 taxa assigned reads per sample, 96 % of which were classified as bacteria. No significant difference was observed in the diversity of the microbial community between the LHR samples (Observed: $p = 0.5$, Shannon: $p = 0.5$) (Fig. S7). All samples were dominated by members of the Firmicutes phylum (Fig. 6.A), primarily within the *Enterococcaceae* family. However, microbial community composition of the LHR samples clustered significantly by source

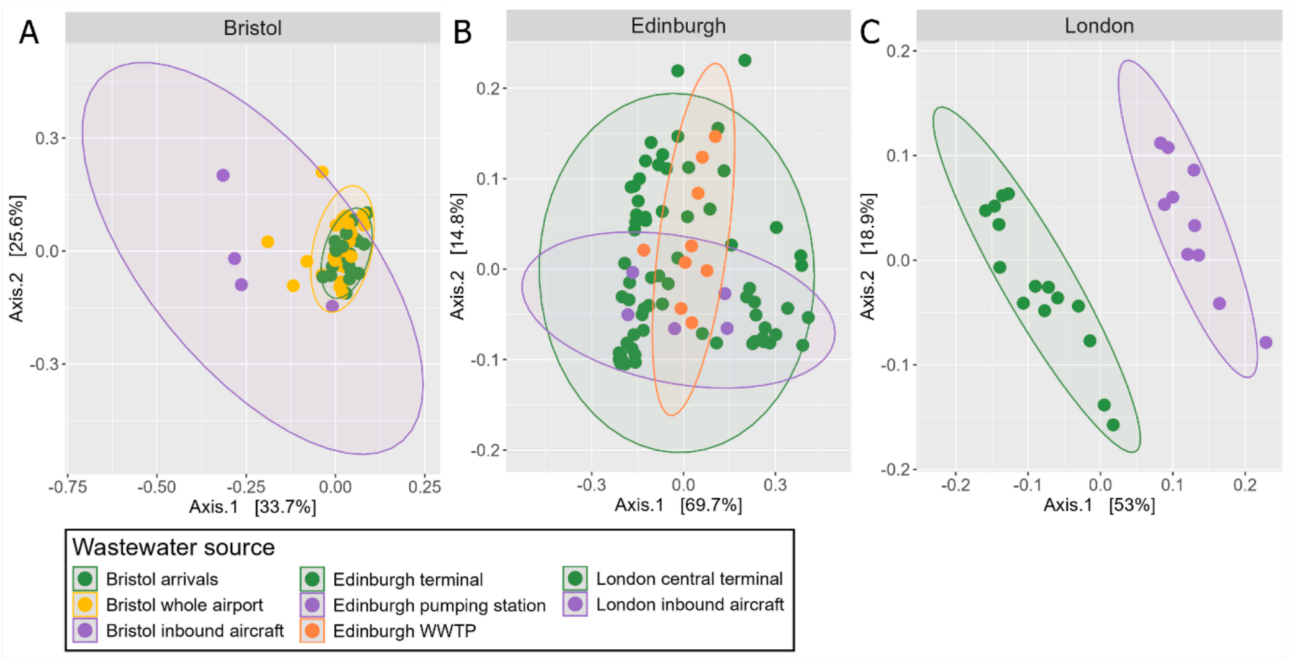

Fig. 4. PcoA ordination plots of Bray-Curtis dissimilarity matrices based on Hellinger-transformed ARG relative abundances, showing antimicrobial resistance gene (ARG) composition differences between wastewater samples from (A) Bristol, (B) Edinburgh, and (C) London Heathrow international airports.

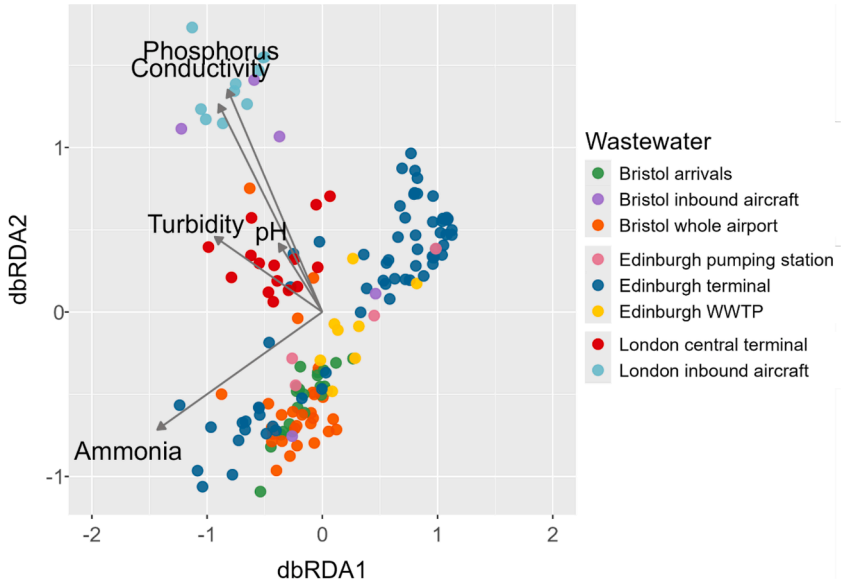

Fig. 5. Distance-based redundancy analysis (dbRDA) showing the influence of environmental variables over the antimicrobial resistance gene (ARG) profile in the airport-related wastewater samples.

(PERMANOVA $p < 0.01$) (Fig. 6.B). Proteobacteria were more abundant in the LHR-CT wastewater compared to the aircraft, owing to significantly higher abundance of *Comamonadaceae* (Welch's *t* test: $p < 0.01$) and other low abundance families.

Of the five qPCR target opportunistic pathogens, only *Staphylococcus aureus* was undetected across all samples (Table S3). *Acinetobacter baumannii, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were detected across all wastewater sample types, with *A. baumannii* detected at the highest rate (n = 141/180). The relative abundance of *P. aeruginosa* was not significantly different between airport terminals (One-Way ANOVA $p = 0.06$, $f = 2.794$; Fig. 7). In contrast, *A. baumannii* and *K. pneumoniae* did differ significantly (One-Way ANOVA $p < 0.01$, $f = 23.14$ and $p < 0.01$, $f = 14.01$, respectively). *A. baumannii* was most abundant in BRS, while *K. pneumoniae* was most abundant in LHR. Comparing aircraft and airport samples from BRS and LHR, the relative abundance of *A. baumannii* was significantly higher in the airport wastewater, whereas *P. aeruginosa* was significantly more abundant in the aircraft samples (Welch's *t*-test: $p < 0.05$ for both comparisons). *Enterococcus* species were only detected in the EDI samples, exhibiting significantly lower abundance in EDI-PS than EDI-T and EDI-WWTP (One-Way ANOVA $p < 0.01$, $f = 9.122$).

3.6. Network analysis of potential ARG hosts

Co-occurrence patterns between the 40 most abundant ARGs and bacterial genera were explored by network analysis, based on strong (ρ

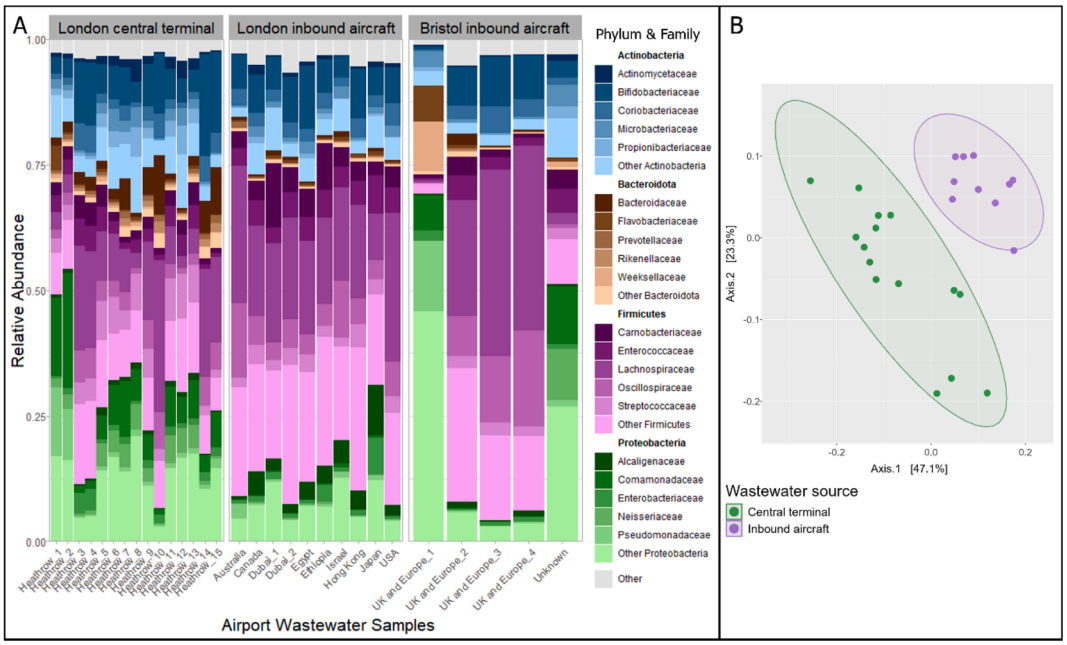

Fig. 6. (A) Stacked bar chart showing the relative abundances of taxa in the metagenomic wastewater sample subset (B) PcoA ordination plot of Bray–Curtis dissimilarity matrices based on Hellinger-transformed taxa relative abundances, showing taxonomic compositional differences between London Heathrow wastewater samples.

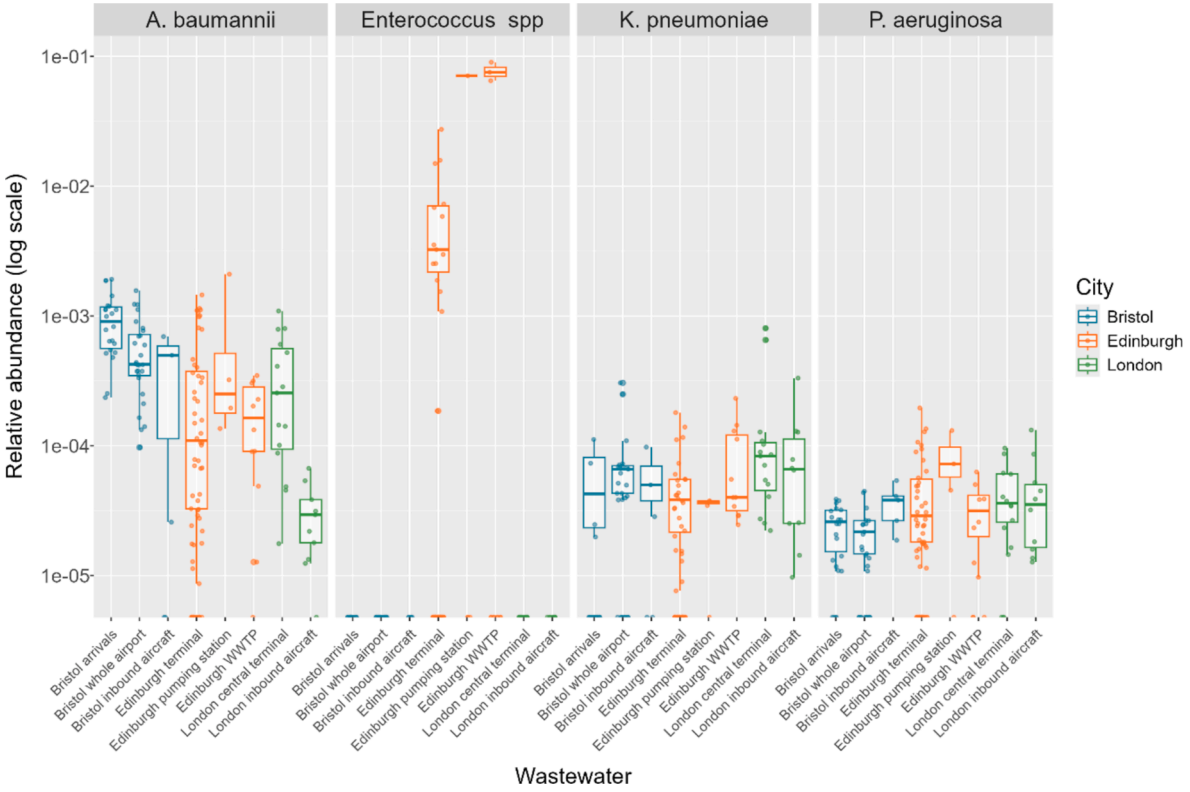

Fig. 7. Boxplots showing the relative abundances of HT-qPCR target pathogens in aircraft and airport-related wastewater samples.

> 0.7) and significant ($p < 0.01$) correlations (Fig. 8). The trimethoprim ARG, *dfrF*, and several tetracycline ARGs – *tetW*, *tetO* and *tet40* – correlated with multiple bacterial genera, including commensal intestinal flora, such as *Faecalibacterium*, *Dorea* and *Ruminococcus*. Multiple taxa correlated with highly abundant ARGs encoding resistance to various antimicrobials belonging to different classes (e.g., *sul1*, *catB*, *ermT*, *tetG* and *flor*), of which *Staphylococcus* correlated with the highest number.

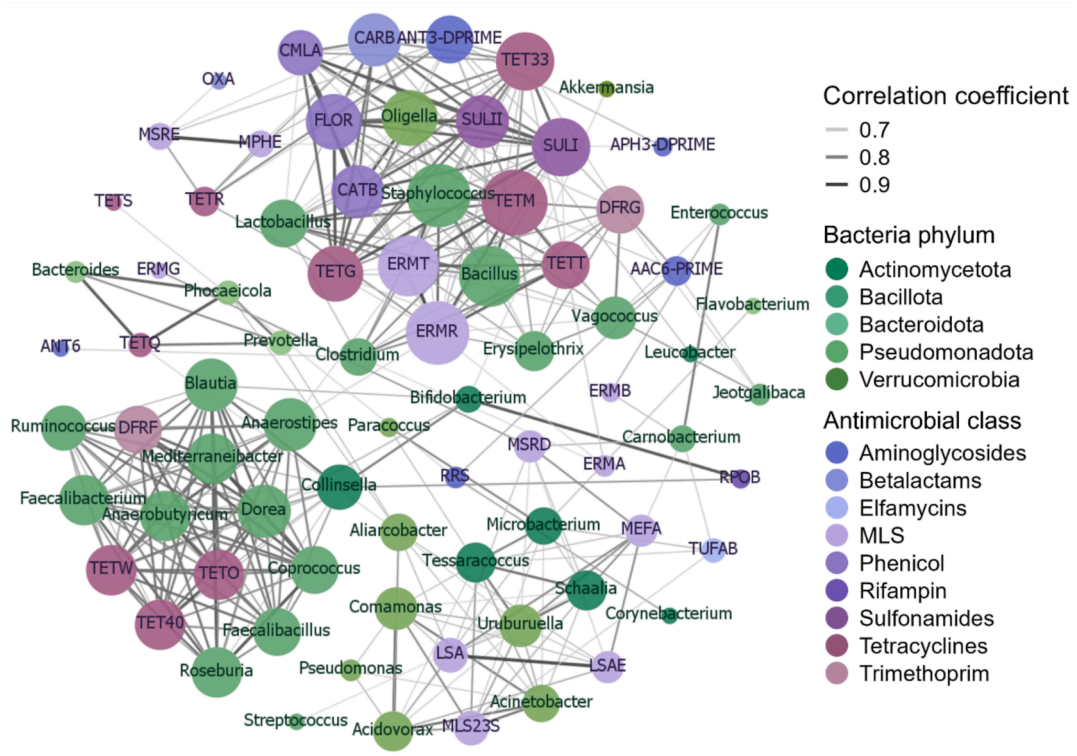

Fig. 8. Network analysis showing the relationships between the 40 most abundant antimicrobial resistance genes (ARGs) and bacterial genera in the metagenomic subset of aircraft wastewater samples. Connections represent strong, significant correlations (Spearman's correlation coefficient $\rho > 0.7$, $p < 0.01$).

4. Discussion

4.1. Aircraft wastewater as a tool for global AMR surveillance

Within the UK, aircraft passenger wastewater may serve as a conduit for AMR bacteria originating from diverse geographic sources to enter the local water cycle as most aircraft-derived wastewater is treated at the local urban WWTP. We predict that ARGs from inbound passengers may be introduced into local aquatic ecosystems within days of arrival. Though the wastewater treatment process reduces ARG abundances, effluent discharge can still act as a conduit for ARGs to enter into the environment, especially when released with untreated wastewater through combined sewer overflows (CSOs) (Perry et al., 2024; Sabri et al., 2020; Beltran de Heredia et al., 2023; Tipper et al., 2024). For example, there were 464,056 individual discharges of raw sewage into UK rivers, waterways and the coastal zone in 2023 (Environment Agency, 2023). This rapid transfer pathway highlights the potential for international travel to contribute to the rapid spread of AMR in the aquatic environment (Arcilla et al., 2017; Bengtsson-Palme et al., 2015). However, the analysis of aircraft wastewater also offers a mechanism to enhance global AMR surveillance by investigating the abundance and diversity of ARGs in wastewater sourced from the aircraft and terminals at three international airports in the UK. When sourced directly from vacuum trucks, into which aircraft wastewater is deposited, the microbial community and its resistome displayed distinct profiles to those from the air terminals and a local WWTP. Overall, higher numbers of observed ARGs were found in flights originating from Asia and Africa compared to those from Europe and North America. This finding may reflect the high resistance rates seen in low-middle income countries (Hou et al., 2023). On the other hand, clustering of the ARG profiles did not indicate a strong association with geography, contrasting observations made by Petersen et al. (2015). While their samples were collected directly from the aircraft, sampling the vacuum trucks may have introduced cross-contamination. There will, however, be other compounding factors, as discussed in Section 4.2. In addition, a higher diversity and overall number of ARGs were observed in the long-haul flights to London Heathrow than in the short haul flights to Bristol. This could be attributed to the strong relationship between flight duration and lavatory use (Jones et al., 2023). However, given the limited number of aircraft samples, it is difficult to draw definitive conclusions from these observations.

Our comparative analysis of ARG diversity across various wastewater sources yielded complex results. HT-qPCR analysis revealed no significant differences between wastewater sources. However, this method could only account for the 76 assayed genes, chosen for their clinical significance and high global prevalence (Feng et al., 2018). In contrast, the metagenomic analysis conducted by Hess et al. (2019) uncovered a richer resistome in aircraft wastewater compared to WWTPs. This discrepancy highlights the importance of methodology in resistome characterisation. Indeed, the metagenomic subset from this study exhibited far greater numbers of observed ARGs, better reflecting the resistome's true diversity. As such, metagenomics is advantageous for monitoring initiatives aiming for comprehensive resistome analysis. In contrast, HT-qPCR is more sensitive to low abundant genes and is capable of absolute gene quantification, making it suitable for initiatives requiring accurate measurements of specific clinically significant ARGs (Knight, et al, 2024).

The potential impact of aircraft wastewater on AMR transmission requires careful consideration. The volume of wastewater produced by aircraft is considerably smaller than other sources, suggesting a strong dilution effect when mixed into WWTP influent. Our analysis of Edinburgh airport's pumping station, which collects wastewater from multiple aircraft, revealed a resistome comparable to both the airport terminal and a local WWTP. This similarity could be ascribed to the convergence of diverse microbial communities from global travellers. The distinct resistome profiles observed in the terminal wastewater of the three airports further suggest that variations in passenger travel origins, demographics and volumes between airports influence the resistome. However, these differences could also reflect the resistomes of the local population who staff and traffic the airports. Furthermore,

other factors such as airport-specific hygiene practices and local climate may also play significant roles. Future studies should employ standardised, multi-method approaches across a broader range of airports and temporal scales to better elucidate these relationships.

The similarity between the Edinburgh airport and WWTP resistome is also likely influenced both by the input of travellers and the local population. Quantifying the relative contributions of these groups would provide valuable insights for targeted AMR mitigation strategies. Even if present at low abundance within the WWTP influent, ARGs from flight passengers can still contribute to the wider dissemination of resistance. If carried on mobile genetic elements, ARGs have the potential for horizontal transfer among the microbial community (Wei et al., 2021). With wastewater considered a major source of AMR contamination in the natural environment (Quintela-Baluja et al., 2019; Sabri et al., 2020) this raises significant concerns.

4.2. Uncertainties surrounding aircraft wastewater-based AMR surveillance

Despite the clear potential of aircraft-related wastewater for monitoring the import rate and global spread of pathogenic organisms, there are several limitations which need to be considered when interpreting the data. Firstly, a recent study assessing the bathroom habits of UK air passengers (n = 2103) reported a relatively low likelihood of people defecating during long-haul flights (ca. 20–40 %), with even lower estimates for short-haul flights (ca. 10–15 %; Jones et al., 2023). Additionally, some bias was also noted in the frequency of defecation in aircraft and airport terminals with both gender and age. As such, wastewater samples may not be truly representative of the AMR burden among flight passengers. Wastewater-based analysis of the AMR burden may also self-select for passengers with gastrointestinal infections and diarrhoea, which can be more common in travellers arriving from destinations with poor sanitation or on longer-haul flights (Steffen et al., 1983; Steffen, 2017; Holmes and Simmons, 2009; Graf et al., 2012). In addition, the likelihood of passenger urination on flights is very high in comparison to defecation. This may lead to the preferential detection of pathogens and ARGs associated with urinary tract infections (UTIs), including those from asymptomatic carriers (Alkhawaldeh et al., 2022; Mohapatra et al., 2022). Common uropathogens such as *E. coli*, *K. pneumoniae*, and *Enterococcus* spp. are frequently implicated in both symptomatic and asymptomatic bacteriuria, and these organisms often harbour clinically relevant ARGs (Cottalorda et al., 2022; Post et al., 2022). While this urine-centric sampling bias in aircraft wastewater may provide a unique window into the prevalence of UTI-associated ARGs in diverse populations, it may also skew overall AMR profiles when comparing to more faecal-dominated wastewater sources. Hand washing may also potentially introduce skin-borne AMR-carrying organisms into aircraft wastewater, although the quantitative importance of this pathway remains unknown (Tartari et al., 2024). Further work is therefore required to better understand passenger behaviour, the relative contribution of different sources of ARGs into the aircraft wastewater system and how this is influenced by cleaning regime.

Sample collection and processing methodologies may also significantly influence the recovery and detection of ARGs in water samples. Storage time and conditions can particularly impact the detection of low-abundance ARGs (Smith et al., 2024; Yan et al., 2024). Conversely, when accounting for the reduced flush volume and limited input of other liquids, aircraft wastewater is chemically distinct and highly concentrated compared to municipal urban wastewater. These differing characteristics can drive shifts in the microbial community, in turn reshaping the resistome. Consequently, interpreting results becomes more complex when comparing aircraft wastewater to municipal wastewater. The concentrated nature of wastewater might increase the probability of finding rare ARGs in aircraft wastewater, however, it may also increase the amount of potential qPCR inhibitors. In terms of sample recovery, care should also be taken given the highly concentrated, and unknown, pathogen load in aircraft wastewater (Lacey et al., 2010; Burton and McCleery, 2000). In terms of sample representativeness, it is also worth noting that in contrast to long-haul flights (> 6h), aircraft wastewater on short-haul flights (< 6h) typically reflects the accumulation of wastewater from several transboundary flights (e.g., emptied every 2–4 flights). Additionally, the origin of passengers may not correspond to the flight's origin, complicating geographical attribution of AMR patterns.

To contextualise the scale of air travel, approximately 0.53 million passengers arrive in the UK daily on ca. 5,200 flights (CAA, 2023; Eurocontrol, 2024). Given logistical constraints, it is only feasible to sample a small fraction of these flights. Consequently, aircraft-based surveillance is valuable for estimating general ARG import rates and prevalence but may lack the sensitivity to detect the introduction of novel pathogens or ARGs. This limitation is supported by previous studies on SARS-CoV-2 variant monitoring using aircraft wastewater which showed that such surveillance was only effective when COVID-19 case numbers in the country of origin were relatively high (Ahmed et al., 2022; Farkas et al., 2023).

Air travellers are expected to have different socioeconomic backgrounds, health statuses, and exposure risks compared to the general population (Gössling and Humpe, 2020; AA, 2024). Temporal variations also play a significant role in wastewater composition and AMR profiles. For example, day versus night flights may carry different passenger demographics (e.g., business versus leisure travellers), while seasonal variations can affect travel patterns, passenger health (e.g., flu season), and even antibiotic use in different regions (Büchs and Mattioli, 2021). These factors are collectively likely to influence the representativeness and interpretation of AMR data obtained from aircraft wastewater, necessitating careful consideration in surveillance programs.

4.3. Ethical considerations

The utilisation of aircraft wastewater for global pathogen surveillance presents several ethical challenges, balancing public health benefits against individual privacy rights. In terms of informed consent, passengers in this study were unaware that their waste was being analysed, which could be seen as a violation of personal autonomy, especially on flights with limited passenger numbers (Armitage et al., 2002). Although wastewater is generally considered 'abandoned' material, the targeted nature of aircraft sampling might be perceived differently from municipal wastewater surveillance where much larger populations are typically sampled (i.e. 10^4-10^6 individuals).

Based on studies in other healthcare settings, data privacy and potential misuse of aircraft-derived wastewater may also represent a concern (Wagner et al., 2023; Driver et al., 2024). While our analyses were intended to provide population-level insights, the theoretical possibility of linking results to specific flights or individuals, especially on less frequented routes, could lead to stigmatisation of certain travel patterns or passenger demographics. Furthermore, unintended consequences, such as discriminatory travel restrictions or negative impacts on regional tourism, must be considered (Maal-Bared et al., 2023). A further unintended consequence could be that airports and airlines restrict access to its wastewater infrastructure. Balancing these concerns with public health benefits therefore requires careful consideration (Graham, 2023). As with other healthcare fields, transparency about surveillance programs, robust data protection measures, and clear guidelines on the use and sharing of results are therefore essential (Kalkman et al., 2022; Graham, 2023). Engaging with ethicists, policymakers, and the public in ongoing discussions will help address these complex issues.

4.4. Conclusions

Our study represents the first comprehensive simultaneous investigation into the abundance and diversity of AMR in wastewater from both aircraft and terminals at multiple international airports. Our

findings indicate the role aircraft passenger wastewater may play in rapidly introducing AMR bacteria from diverse global populations into local water cycles. However, further research is required to fully evaluate the source-to-sink dynamics from flight passengers to municipal wastewater systems. We therefore recommend future studies to (i) evaluate the relative contributions of air passengers and the local population to the WWTP influent resistome, (ii) investigate temporal and spatial variations in ARG profiles across different flight routes and seasons, and (iii) assess the risk of cross-contamination in vacuum truck samples. Our results also highlight the advantages and limitations of using aircraft wastewater for global AMR surveillance. To enhance the robustness and comparability of AMR surveillance data, it will also be important to develop standardised sampling protocols, unified methodologies for ARG detection and quantification, consistent data reporting and sharing practices and ethical frameworks addressing privacy concerns and potential misuse of data.

CRediT authorship contribution statement

Margaret E. Knight: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Kata Farkas:** Conceptualization, Funding acquisition, Methodology, Project administration, Writing – review & editing. **Matthew Wade:** Conceptualization, Methodology, Writing – review & editing. **Gordon Webster:** Data curation, Methodology, Software, Validation. **Daniel A. Pass:** Data curation, Formal analysis, Methodology, Software, Validation. **William Perry:** Methodology, Software, Validation. **Peter Kille:** Data curation, Formal analysis, Methodology, Software, Validation, Writing – review & editing. **Andrew Singer:** Conceptualization, Methodology, Writing – review & editing. **Davey L. Jones:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2025.109260.

Data availability

Data will be made available on request.

Host removed microbial sequences are available via NCBI BioProject PRJNA1195052.

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