



Antimicrobials and antimicrobial resistance genes in a one-year city metabolism longitudinal study using wastewater-based epidemiology[☆]

Natalie Sims ^{a,b}, Andrew Kannan ^a, Elizabeth Holton ^a, Kishore Jagadeesan ^a, Leonardos Mageiros ^e, Richard Standerwick ^c, Tim Craft ^d, Ruth Barden ^c, Edward J. Feil ^e, Barbara Kasprzyk-Hordern ^{a,b,*}

^a University of Bath, Department of Chemistry, Bath, BA2 7AY, UK

^b Centre for Sustainable Circular Technologies, Bath, BA2 7AY, UK

^c Wessex Water, Claverton Down Rd, Bath, BA2 7WW, UK

^d Department of R&D, Royal United Hospitals Bath, NHS Foundation Trust, Bath, BA1 3NG, UK

^e Milner Centre for Evolution, Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK



ARTICLE INFO

Keywords:

Wastewater-based epidemiology

Antimicrobials (AA)

Antibiotics

Antimicrobial resistance genes (ARGs)

Hospital effluent

ABSTRACT

This longitudinal study tests correlations between antimicrobial agents (AA) and corresponding antimicrobial resistance genes (ARGs) generated by a community of >100 k people inhabiting one city (Bath) over a 13 month randomised monitoring programme of community wastewater. Several AAs experienced seasonal fluctuations, such as the macrolides erythromycin and clarithromycin that were found in higher loads in winter, whilst other AA levels, including sulfamethoxazole and sulfapyridine, stayed consistent over the study period. Interestingly, and as opposed to AAs, ARGs prevalence was found to be less variable, which indicates that fluctuations in AA usage might either not directly affect ARG levels or this process spans beyond the 13-month monitoring period. However, it is important to note that weekly positive correlations between individual associated AAs and ARGs were observed where seasonal variability in AA use was reported: *ermB* and macrolides CLR-clarithromycin and dmCLR-N-desmethyl clarithromycin, aSPY- N-acetyl sulfapyridine and *sul1*, and OFX-ofloxacin and *qnrS*. Furthermore, ARG loads normalised to 16S rRNA (gene load per microorganism) were positively correlated to the ARG loads normalised to the human population (gene load per capita), which indicates that the abundance of microorganisms is proportional to the size of human population and that the community size, and not AA levels, is a major driver of ARG levels in wastewater. Comparison of hospital and community wastewater showed higher number of AAs and their metabolites, their frequency of occurrence and concentrations in hospital wastewater. Examples include: LZD-linezolid (used only in severe bacterial infections) and AMX-amoxicillin (widely used, also in community but with very low wastewater stability) that were found only in hospital wastewater. CIP-ciprofloxacin, SMX-sulfamethoxazole, TMP-trimethoprim, MTZ-metronidazole and macrolides were found at much higher concentrations in hospital wastewater while TET-tetracycline and OTC-oxytetracycline, as well as antiretrovirals, had an opposite trend. In contrast, comparable concentrations of resistant genes were observed in both community and hospital wastewater. This supports the hypothesis that AMR levels are more of an endemic nature, developing over time in individual communities. Both hospital and community wastewater had AAs that exceeded PNEC values (e.g. CLR-clarithromycin, CIP-ciprofloxacin). In general, though, hospital effluents had a greater number of quantifiable AAs exceeding PNECs (e.g. SMX-sulfamethoxazole, ERY-erythromycin, TMP-trimethoprim). Hospitals are therefore an important consideration in AMR surveillance as could be high risk areas for AMR.

[☆] This paper has been recommended for acceptance by Klaus Kümmeler.

* Corresponding author. University of Bath, Department of Chemistry, Bath, BA2 7AY, UK

E-mail address: [B.Kasprzyk-Hordern](mailto:B.Kasprzyk-Hordern@bath.ac.uk) (B. Kasprzyk-Hordern).

1. Introduction

The evolution and spread of antimicrobial resistance (AMR) limits therapeutic options for a broad range of infectious diseases, and poses a global threat to public health (World Health Organisation, 2020). Whilst genes conferring resistance have evolved naturally prior to the administration of antimicrobial agents (AAs), the inappropriate use of these drugs dramatically accelerated the spread of resistance genes across different species, settings and geographical locations. It has been estimated in 2019 that there were 4.95 million deaths associated with bacterial AMR infections, including 1.27 million deaths directly attributed to resistant bacteria (Murray et al., 2022). These rising global rates of AMR have stressed the importance of effective surveillance systems for understanding the burden of resistance and identifying new or re-emerging threats. Effective surveillance can also feedback into evidence-based policy making and evaluate the effectiveness of public health interventions. As highlighted by WHO's GLASS report (World Health Organisation, 2018), there are clear disparities in AMR surveillance between different countries, due to limited resources and infrastructure. There is also a recognised lack of population-wide surveillance data regarding AMR, highlighting a need to develop both cost-effective and standardised population-wide surveillance AMR systems.

A promising tool for monitoring community-wide AMR surveillance is wastewater-based epidemiology (WBE). Wastewater treatment plants (WWTPs) serve a well-defined community, capturing all the excretion products of this population. These excretion products contain biomarkers of pathogens, pharmaceuticals and other indicators of population health. Estimates of community response resulting from exposure to pathogens, or consumption of pharmaceuticals, can be made by quantifying these biomarkers in influent (untreated) wastewater, whilst accounting for flow rates and population size. The resulting data complement evidence generated through traditional public health approaches.

WBE as a public health tool has clear advantages; it not only provides anonymous population-wide data but is also relatively inexpensive and gives rapid results. In the case of AMR, analysis of AA residues in wastewater can inform on consumption within the community. This can be valuable where prescription data is not easily obtainable, or where AAs are available over-the-counter or online. Complimentary to the analysis of AAs, the analysis of resistance genes or co-factors (constituents in wastewater that can co-select for resistance) can also provide key information on the presence of resistant bacterial communities in wastewater. The ability of WBE to give rapid results provides the potential for rapid responses. In the case of AMR, WBE also holds to the promise of evaluating the effectiveness of antimicrobial policy. However, WBE is not without its challenges. Estimation of population sizes and biomarker suitability pose problems, and remain an active area of research (Been et al., 2014; Chen et al., 2014; Choi et al., 2020; Daughton, 2018).

An overview of WBE studies focusing on the presence of AAs is given in Table S1. WBE has been used to investigate AA usage in flu season (Zhang et al., 2019) and even to demonstrate changing consumption patterns in the wake of the COVID-19 pandemic (Galani et al., 2021). Furthermore, combining analysis of AAs with ARGs can reveal how AA usage impacts on the presence of ARGs. For example, areas with higher quinolone consumption, driven by population size, were found to have a higher prevalence of *qnrS* gene, which encodes resistance to this antibiotic (Castrignanò et al., 2020). Similar results were observed for other AAs and their respective resistance genes (macrolides and *ermB*, sulfamethoxazole and *sul1*, chloramphenicol and *catA*) (Elder et al., 2021).

The development of advanced DNA sequencing techniques offers a promising approach for monitoring the abundance of ARGs in the environment (Guo et al., 2017; Hendriksen et al., 2019; Lanza et al., 2018; Petrovich et al., 2020; Riquelme et al., 2021). High throughput quantitative polymerase chain reaction (qPCR) has also been utilised to

reflect expected clinical resistance trends across Europe (Pärnänen et al., 2019). Complexities of AMR require all proxies (e.g. antimicrobials, genes, co-factors) to be studied simultaneously to give a full picture of AMR prevalence at a community level.

Due to the multifaceted nature of AMR, the One Health approach has proved a valuable framework for tackling this complex issue. One Health aims at holistic understanding and management of public and environmental health and has been successfully adopted in AMR research with considerable global human and animal health, food security, and safety impacts. One Health studies aim to incorporate a dynamic set of biological, chemical, and socioeconomic indicators that are difficult to unravel. Whilst AA and ARG analysis have been undertaken internationally (Hendriksen et al., 2019; Pärnänen et al., 2019; Petrovich et al., 2020) many studies focus on shorter sampling periods with inconclusive results regarding AA-ARG correlations. There is a lack of studies occurring over longer periods, investigating how ARGs (used as proxy for AMR prevalence) correlate with AAs generated by whole communities. Longer term wastewater monitoring of AAs will capture both fluctuating and consistent consumption patterns between seasons and can therefore complement prescription data. Evidence can also be generated on the relationship between the abundance of AAs and associated ARGs in these communities.

This paper is first to undertake an intensive longitudinal study of AAs usage and ARGs prevalence within one city with an aim to test a hypothesis that AA usage correlates (is a driver) of ARG prevalence in a given community.

This paper tests the hypothesis that AA usage drives ARG prevalence in communities. The key objectives of this paper are to:

1. Provide better understanding of AA-ARG associations in the context of a longitudinal 13-month monitoring period of Bath: 120,113 inhabitants (inh) for a suite of AAs and their metabolites (AA/met), and corresponding ARGs.
2. Undertake data triangulation to understand relationships between AA/met levels and corresponding ARGs; as well as water quality indicators (WQIs-see SI) in the context of seasonal AA use.
3. Provide better understanding of AA and ARG contributions in hospital vs community wastewater and their associations in the two types of wastewater.
4. Understand the role of wastewater in the dissemination of AMR, and to explore measured vs predicted no effect concentrations (PNECs) of AAs in both community and hospital wastewater.

2. Materials and methods

2.1. Materials and target compounds

AAs selected for this study cover a broad and diverse range of classes with both parent compounds and metabolites as seen in Table 1, Table S2 and S3. Analytical standards and deuterated (stable isotope-labelled) standards were obtained from Sigma-Aldrich (Gillingham, UK), TRC (Toronto, Canada), LGC (Middlesex, UK) or MCE (Cambridge, UK). The methanol used was HPLC-grade (Sigma-Aldrich), the water was of 18.2 MΩ quality (Elga, Marlow, UK); and the purity of formic acid, used as the mobile-phase additive, was >95% (Sigma-Aldrich). Glassware was deactivated using 5% dimethylchlorosilane in toluene (Sigma-Aldrich) to avoid losses via adsorption. Oasis HLB (60 mg, 3 mL) SPE cartridges, polypropylene LC vials, and Whatman GF/F 0.7-μm filters were purchased from Waters (Manchester, UK).

2.2. Sampling

2.2.1. Wastewater treatment plant sampling

One wastewater treatment plant (WWTP) serving the city of Bath (population: 120,113) was sampled over a sampling period between 2018 and 2019. The site has limited contribution from industrial input

Table 1

AA targets investigated in this study, ordered by class groupings, table adapted from Holton and Kasprzyk-Hordern (2021), <https://rdcu.be/cxqhT>.

Grouping	Chemical	Abbrev
Sulphonamide & Trimethoprim	Sulfadiazine	SDZ
	Sulfapyridine	SPY
	Sulfamethoxazole	SMX
	Sulfasalazine	SLZ
	Trimethoprim	TMP
	N-acetyl sulfadiazine	aSDZ
	N-acetyl sulfapyridine	aSPY
	N-acetyl sulfamethoxazole	aSMX
	4-hydroxy-trimethoprim	hTMP
	Azithromycin	AZM
Macrolide & Lincosamide	Erythromycin	ERY
	Clarithromycin	CLR
	Clindamycin	CLI
	N-desmethyl azithromycin	dmAZM
	N-desmethyl erythromycin A	dmERY
	N-desmethyl clarithromycin	dmCLR
	N-desmethyl clindamycin	dmCLI
	Amoxicillin	AMX
	Ampicillin	AMP
	Flucloxacillin	FLX
β-lactams Penicillin	Penicillin G	PenG
	Penicillin V	PenV
	Amoxicilloic acid	AMXa
	Ampicilloic acid	AMPa
	Penicilloic G acid	PenGa
	Cefalexin	LEX
	Cefixime	CFM
	Ceftiofur	CTF
	Ceftriaxone	CRO
	Aztreonam	ATM
Cephalosporin	Imipenem	IPM
	Meropenem	MEM
	Besifloxacin	BSF
	Ciprofloxacin	CIP
	Danofloxacin	DFX
	Enrofloxacin	ENR
	Flumequine	FLU
	Gatifloxacin	GAT
	Lomefloxacin	LOM
	Moxifloxacin	MXF
Monobactam Carbapenem	Nadifloxacin	NAD
	Nalidixic acid	NAL
	Norfloxacin	NOR
	Oflloxacin (Levofloxacin) *	OFX
	Prulifloxacin	PFLX
	Sarafloxacin	SRF
	Desethylene ciprofloxacin	deCIP
	Hydroxy-norfloxacin	hNOR
	Oflloxacin N-oxide	OFXo
	Desmethyl-ofloxacin	dmOFX
Quinolone	Ulifloxacin	UFX
	Isoniazid	INH
	Pyrazinamide	PZA
	Ethambutol	EMB
	Rifampicin	RMP
	Rifabutin	RFB
	Isonicotinic acid	INA
	Acetyl-isoniazid	aiNH
	5-Hydroxy-pyrazinoic acid	hPZA
	25-desacetyl rifampicin	daRMP
TB (1st line)	25-O-desacetyl rifabutin	darFB
	Capreomycin IA	CAPla
	Capreomycin IB	CAPlb
	Gentamycin C1	GEN1
	Gentamycin C1a	GEN1a
	Gentamycin C2 C2a C2b	GEN2
	Kanamycin A	KAN
	Streptomycin A	STR
	D-cycloserine	DCS
	Delamanid	DMD
TB (other)	Bedaquiline	BDQ
	Linezolid	LZD
	Thalidomide	THAL

Table 1 (continued)

Grouping	Chemical	Abbrev
OTHER Amphenicol	Chloramphenicol	CHL
	Florfenicol	FLO
Cycline	2-Amino-1-(4-nitrophenyl)-1,3-propanediol	ANP
	Doxycycline	DOX
	Oxytetracycline	OTC
	Tetracycline	TET
Nitrofuran	Nitrofurantoin	NIT
	1-(2-nitrobenzylidenamino)-2,4-imidazolidinedione	NPAHD
Azole	Metronidazole	MTZ
	Ketoconazole	KTC
	Hydroxy-metronidazole	hMTZ
	Deacetyl-ketoconazole	daKTC
Antiretroviral	Emtricitabine	FTC
	Lamivudine	3 TC

Multi-drug resistant (MDR), tuberculosis (TB), LC-MS method is not chiral (*).

(<1%) and it has input from a major hospital discharging wastewater into the sewerage catchment area. 24-hour composite samplers, set up for flow proportional sampling every 15 min, were used to sample screened, but untreated, influent wastewater. Samples were transported (immediately after sample collection) on ice to the laboratory for processing.

2.2.2. Hospital effluent sampling

Wastewater samples were collected from a hospital (>700 beds and a catchment of 500,000 people) within the catchment area of Bath during the longitudinal study over five consecutive days (5th–9th August 2019). Hospital effluent samples were collected by 24-h composite samplers set to time-proportional, with 50 mL collected every 15 min. Collected samples were transported on ice to the laboratory to be processed (<1 h).

2.2.3. Water quality indicators and flow measurements

A range of water quality/sanitary indicators (WQIs: biological oxygen demand/BOD, chemical oxygen demand/COD, suspended solids, chloride, ortophosphorus, T phosphorous, suspended organic carbon, ammonia as N, metals: Al, Fe, Mn) were analysed at Wessex Water at certain sampling points over the studied period. Full experimental details may be found in the SI. Influent wastewater flows and rainfall over the studied period in both catchment areas were also reported (Figure S1). Discussion on WQI, AA and ARG associations is available in SI.

2.2.4. Population equivalent estimation

The population equivalent of those served by the WWTP (PE-WW) was estimated by Wessex Water (Table S4). By multiplying the number of properties in the catchment area by occupancy rate (set at district level), the resident population estimate was determined. The resident population also considers the number of multi-occupancy residences that fall within the catchment area, including care homes, residential schools, university halls, and military bases. As a UNESCO world heritage site, Bath has a thriving tourism input. Tourists were considered as non-resident population, and due to challenges estimating input of day-trippers to the catchment area, they were not included. By utilising WQIs, other inputs to the wastewater stream could be calculated. This included commercial waste; determined by considering supply flow to commercial properties and estimates of 60 g BOD per capita per day. Tankered waste imports were determined via the amount of COD present in the known volume of waste and, assuming 120 g COD per capita per day.

2.3. Chemical analysis – AA and metabolite quantification

2.3.1. Sample preparation

Collected influent wastewater samples were transported on ice to the

lab (<1 h). On arrival, samples were portioned into 50 mL and spiked with 50 ng of each internal standard and shaken. Samples were then filtered through GF/F filters (Whatman, UK). Solid phase extraction (SPE) was used to extract target AAs from wastewater, Oasis HLB cartridges were preconditioned using 2 mL of MeOH followed by 2 mL of Milli-Q H₂O at a flowrate of 1 mL min⁻¹. Wastewater filtrates were then loaded onto the preconditioned cartridges at a rate of 5 mL min⁻¹. Cartridges were dried for at least 30 min under vacuum. For the elution step, 4 mL of MeOH were applied at a rate of 1 mL min⁻¹ with the eluate collected in silanised glass vials. Eluates were then dried at 40 °C under N₂ via a TurboVap evaporator. Dried residues were reconstituted with 500 µL of 80:20 H₂O: MeOH and transferred into polypropylene vials and kept at -18 °C until analysis. Further details on the method can be found in Holton and Kasprzyk-Hordern (2021).

2.3.2. Analyte quantification

Full analytical method validation and instrument conditions may be found in Holton and Kasprzyk-Hordern (2021). For the analysis of target AAs, ultra-performance liquid chromatography (UPLC) was coupled with a XEVO triple quadrupole mass spectrometer (TQD-MS). The analytical method is in total 19 min long and the column used was a reverse phase BEH C18 column (50 × 2.1 mm, 1.7 µm). For separation of target AAs, mobile phase A consisted of 95:5 H₂O:MeOH with 0.1% formic acid with mobile phase B as 100% MeOH. Flowrate was set at 0.2 mL min⁻¹ and injection volume was 20 µL. Mobile phase starting conditions were 0% B (held for 1 min), then a gradual gradient to 40% B (8.5 min), gradient up to 100% B (3.5 min), hold of 100% B (3 min) and finally dropping back to 0% B (0.5 min).

Regarding mass spectrometry conditions, briefly the method was achieved in ESI positive mode with the source desolvation temperature at 400 °C. Nitrogen was used as the nebulising and desolvation gas and argon was used as the collision gas. For gas flows, the desolvation gas was at 1000 L h⁻¹ and the cone gas was at 100 L h⁻¹.

2.4. Biological analysis – ARG quantification

To investigate temporal trends of ARGs, four wastewater samples per month from Bath were chosen to investigate selected ARGs using digital PCR (dPCR). Four ARGs were selected according to previous work done in the catchment area for both genes and associated AAs and due to their clinical importance (Elder et al., 2021). The selected genes were *ermB* (macrolide resistance), *sul1* (sulphonamide resistance), *qnrS* (fluoroquinolone resistance), and *intI1* (potential marker of anthropogenic pollution). An additional two genes were investigated in hospital effluent, *tetW* (tetracycline resistance) and *blaTEM* (resistance to β-lactams), to explore the relationships between AAs and ARGs. To quantify these genes, digital PCR (dPCR) was utilised. Two wastewater samples (selected from contrasting seasons: November 2018 and March 2019) were analysed by DNA sequencing to characterise the bacterial communities and genes present in the selected wastewater samples.

2.4.1. DNA extraction

Influent wastewater samples (100 mL) were filtered through NalGene™ Sterile Analytical Filter Units (Thermo Scientific, UK) containing 0.2 µm cellulose filter papers. DNA was then extracted from the membrane directly from the filter paper using FastDNA SPIN Kit for Soil (MP Bio, UK). The amount of extracted DNA was determined using a Qubit 4 Fluorometer (Thermo Scientific, UK). DNA was kept at -20 °C before further analysis.

2.4.2. DNA sequencing and metagenomic analysis

Extracted DNA from the samples was sent to MicrobesNG (Birmingham, UK) where Illumina HiSeq sequencing (rapid run, 2 × 250 bp paired end reads) was conducted with in-house quality control (adapter trimming with Trimmomatic v0.30, with a sliding window quality score cut-off value of Q15). Taxonomical profiling was performed with

Kraken2 2.0.8 and Bracken 2.5 (Lu et al., 2017) using the Genome taxonomy database for improved performance (GTDB) (Méric et al., 2019). ARG relevant to the AAs chosen to select for AA resistant bacteria in this study were identified using the Comprehensive Antibiotic Resistance Database (CARD) and Resistance Gene Identifier (RGI; 5.1.1). An inhouse R script was used to process the metagenomic data. Low abundance taxa were filtered using a pre-processing step by removing Operational Taxonomic Units (OTUs) that had non-zero values in ≤10% of samples.

2.4.3. Digital PCR

Digital PCR (dPCR) analysis was performed using a the QuantStudio® 3D Digital PCR System (Thermo Scientific, UK). The dPCR reaction was made up according to manufacturer instructions of QuantStudio® 3D Digital PCR Master Mix, appropriate TaqMan™ primers with MGB probes, sterile water, and DNA sample. Due to similarities in gene abundance in wastewater samples, *intI1* and *sul1* were duplexed allowing quantification of both genes on one chip with *intI1* having this in FAM™ dye and *sul1* with VIC® dye. The mixture was then portioned onto dPCR chip wells and sealed.

The thermo cycling conditions chosen for PCR involved: a temperature ramp to 95 °C, 10 min hold; a reduction to 60 °C for 2 min; before increasing to 98 °C for 30 s. This cycle between 60 °C and 98 °C was repeated 40 times to allow for efficient gene amplification. The system was then lowered to 60 °C and held for 2 min, before cooling to room temperature. After cooling, each chip was processed using the QuantStudio 3D Digital PCR system chip reader. To analyse chips, the AnalysisSuite™ software was used for quantification of the target gene. Each DNA sample was run in duplicate for each gene investigated.

Negative controls for dPCR were achieved using sterile water (DNA blanks) to confirm non-amplification. For positive process controls, 10 µL of TaqMan™ Universal DNA Spike in Control (Thermo Fisher) was spiked into the lysis step of the DNA extraction kit. Six extracts of the same wastewater sample were spiked to assess recovery efficiency of the extraction kit, giving an average recovery of 41 ± 12% (figure S2).

2.5. Statistical calculations

P values were calculated via paired sample T tests, to investigate any seasonal effects of AAs and ARGs. By combining sample data, the seasons considered were winter (November '18, December '18 and January '19), spring (February '19, March '19 and April '19) and summer (May '19, June '19 and July '19). Statistical significance in all tests was defined as p ≤ 0.05. Pearson correlation coefficients were used to investigate potential relationships between ARG and AA abundance. Positive correlations were considered >0.5 and negative correlations were considered < -0.05. Statistical significance testing was performed to highlight correlation results that were significant.

3. Results and discussion

3.1. AAs and their metabolites in a longitudinal study in the city of Bath

A diverse range of AAs and metabolites were observed and quantified across the 13-month sampling period. In total, 17 parent AAs and 8 metabolites were quantified consistently in wastewater in both sites during the sampling period. AAs and metabolites from the macrolide and sulphonamide classes were well represented, with AAs from the aminoglycoside not detected once during the sampling period. Average wastewater concentrations for the consistently quantified AAs and metabolites were reported at 0.50 ± 0.58 ng/L over all studied months. Whilst the concentration of AAs and respective metabolites in wastewater will be impacted by consumption patterns at the community level, variable wastewater flowrates will also have an impact, highlighting the importance of normalising concentrations with flowrates when monitoring both spatial and temporal trends.

To account for variable flows, daily loads of AAs were calculated. This allowed for further comparisons between different months e.g., AA seasonality (Fig. 1). Full breakdowns of AA loads may be found in the SI for individual points and average monthly loads.

Regarding high abundance in wastewater, SPY- sulfapyridine and aSPY- N-acetyl sulfapyridine was observed in high levels denoting 43.1 ± 16.4 g/day and 32.0 ± 13.0 respectively. As previously mentioned, SPY is no longer prescribed for human use in the UK but is continued to be used as a veterinary medication. Both SPY and aSPY however are major metabolites of SLZ-sulfasalazine, which is prescribed for humans as an anti-inflammatory. In wastewater, SLZ had lower average loads than its respective metabolites, at 7.41 ± 5.10 g/day; previous work has demonstrated SLZ has lower recovery and stability comparatively to its two metabolites (Holton et al., 2022). It is likely that SPY and aSPY residues will be present in influent from a combination of consumption of SLZ on the community level and other sources, including agricultural run-off of SPY into the sewage system.

Regarding temporal variability, total loads of AAs and metabolites in wastewater were generally higher in winter when compared to summer (Fig. 1). Statistical significance was gauged using T-tests to compare the three seasons for which most data was available: winter 2018/19, spring 2019 and summer 2019 (Table S5). SPY had one of the lowest temporal variabilities across the three seasons (average loads, winter: 47.1 ± 17.6 g/day, spring: 39.6 ± 15.8 g/day, summer: 43.5 ± 16.9 g/day, all $p > 0.05$). SMX-sulfamethoxazole also had low temporal variability across the three seasons, with 14.7 ± 5.0 g/day, 14.3 ± 5.7 g/day and 15.5 ± 4.8 g/day in winter, spring, and summer respectively (on average a 5% change between seasons, $p > 0.05$).

AAs that displayed statistically significant higher loads in winter than in summer included MTZ-metronidazole, SLZ-sulfasalazine, LEX-cefalexin, TET-tetracycline, OTC-oxytetracycline, CIP-ciprofloxacin, TMP-trimethoprim, and the metabolites hMTZ-hydroxy-metronidazole and AMXa-amoxicilloic acid (Table S5). The AAs with some of the highest variabilities across the three seasons were the macrolides CLR-clarithromycin and ERY-erythromycin. Statistically significant differences were observed when loads peaked in winter 2018/19 at an average load of 32.8 ± 8.7 g/day, compared to summer loads of 14.5 ± 4.2 g/day ($p \leq 0.05$). Similar observations could be observed for CLR and ERY's major metabolites dmCLR- N-desmethyl clarithromycin and dmERY-N-desmethyl erythromycin. As mentioned previously, CLR and ERY are known to have higher prescribing in winter months, when respiratory illness peaks in the UK. Respiratory infections tend to follow predictable seasonal patterns in temperate climates (Price et al., 2019). In colder months, individuals spend more time in enclosed spaces indoors which can lead to increases in the spread of infectious diseases. As a result, the total prescribed AA mass is often higher in winter-particularly for macrolide, penicillins and cephalosporin classes (Curtis et al., 2019). Furthermore, lower loads of ERY in wastewater, compared to CLR, may be due to the preferences of prescribing CLR over ERY due to better tissue penetration, fewer side effects and greater patient compliance (Amsden, 1996).

Previous work has been undertaken in the same catchment area (Elder et al., 2021), whilst this study in 2015 investigated only a few AAs and a very short (one week) monitoring time, it allowed crossover comparison with this work (Figure S3). Whilst lower daily averages were observed for SMX in Bath in 2015, results were comparable for the other AAs between the two sampling periods. For example, CIP 17.0 ± 8.9 g/day (2018/19) vs 10.2 ± 5.7 g/day (2015) in Bath.

3.2. ARGs in a longitudinal study in the city of Bath

Metagenomic sequencing was performed on community wastewater samples. Taxonomic profiling revealed high bacterial diversity with a total of 509 bacterial species detected in November 2018 and 848 in March 2019. Detailed discussion on taxonomic profiling is available in SI.

In total, 46 ARGs were identified across the two wastewater samples, demonstrating a broad range of resistance across different AA classes (Table S6). Of the 46 ARGs identified, the highest percentage of ARGs was predicted to confer resistance to aminoglycosides and tetracycline classes. With 29% and 17% of the total ARGs detected for November 2018 being associated with aminoglycosides and tetracyclines respectively, with 17% and 23% reported for March 2019 (Fig. 2). Other dominant ARGs were observed for macrolide resistance at 13% and 12% for November 2018 and March 2019 respectively. The resistance mechanisms of identified resistance genes in wastewater samples were also explored. The analysed samples gave very similar compositions, with the dominant resistance mechanisms demonstrated as antimicrobial inactivation at 40% and 46% for November 2018 and March 2019 respectively. This was followed by antimicrobial target protection at 28% and 19% for November 2018 and March 2019.

Selected key, clinically important ARGs (*ermB* (macrolide resistance), *sul1* (sulphonamide resistance), *qnrS* (fluoroquinolone resistance), and *intI1* (potential marker of anthropogenic pollution) were taken forward for quantification (with dPCR) in the longitudinal study (approximately 4/5 samples per month) to explore the relationships between AAs and ARGs (Table S7). *ErmB* had the highest prevalence in extracted samples, with an average concentration of $4.3 \text{ E}+09 \pm 1.7 \text{ E}+09$ copies/L across the sampling period respectively. The genes *intI1* and *sul1* were next prevalent and had similar concentration averages again at $5.8 \text{ E}+07 \pm 2.1 \text{ E}+07$ and $5.5 \text{ E}+07 \pm 1.9 \text{ E}+07$ copies/L. Regarding lowest prevalence, *qnrS* was the lowest detected gene, at an average concentration of $2.0 \text{ E}+06 \pm 1.8 \text{ E}+06$ copies/L. With regards to a previous study done in this catchment area in 2015 on three of the same ARGs studied, similar trends in concentrations were observed (*ermB* > *sul1* > *qnrS*) (Elder et al., 2021). Regarding concentration, higher values were observed during this study in 2019 (*ermB*: $1.8 \text{ E}+08 \pm 1.5 \text{ E}+08$, *sul1*: $5.4 \text{ E}+05 \pm 3.1 \text{ E}+05$ and *qnrS*: $2.3 \text{ E}+05 \pm 10.0 \text{ E}+04$ copies/L). Variations could be due to a number of reasons including changes in flows, changes in population size, and differences in extraction methodology. To account for this, flowrates were also taken into account as a key variable to give gene loads as seen in Table S8.

Relative abundance of *intI1*, the clinical class 1 integron-integrase gene, has been previously suggested as a suitable proxy for anthropogenic pollution (Gaze et al., 2011; Gillings et al., 2015). This has been attributed to its association to a diverse number of genes that confer resistance to AAs, metals and disinfectants, plus it can be found in a number of bacteria (pathogenic and non-pathogenic). The abundance of this gene can also change rapidly due to changes in the environment, as host cells have rapid generation time and adapt to selective pressures of the environment. Recent work has also proposed that clinical *intI1* could be used to indicate the abundance of ARGs and to monitor the removal of ARGs in the wastewater treatment process (Zheng et al., 2020). A common characteristic of *intI1* is its occurrence alongside sulphonamide resistance (M. Gillings et al., 2015). As a result, positive correlations are usually observed between abundances of *intI1* and *sul1* in wastewater (Makowska et al., 2016; Zieliński et al., 2021). Results in this study showed agreement with this, demonstrating strong positive correlations between absolute and relative wastewater loads of *intI1* and *sul1* ($r = 0.90$, $p = < 0.05$ absolute loads, $r = 0.90$, $p = < 0.05$ relative loads). Correlations of *intI1* with other genes investigated were not observed (Table S10 and S11).

3.3. Correlations between AAs and ARGs in the longitudinal study

The overall distribution of ARG loads and the corresponding resistance AA classes were investigated over the studied period (Fig. 3). AAs from the sulphonamide class were the most prevalent, averaging 63 ± 24 g/day, with macrolides and lincosamides following at 26 ± 12 g/day. Quinolones AAs had the lowest prevalence in comparison at 20 ± 11 g/day in wastewater. The inclusion of AA metabolites within AA classes

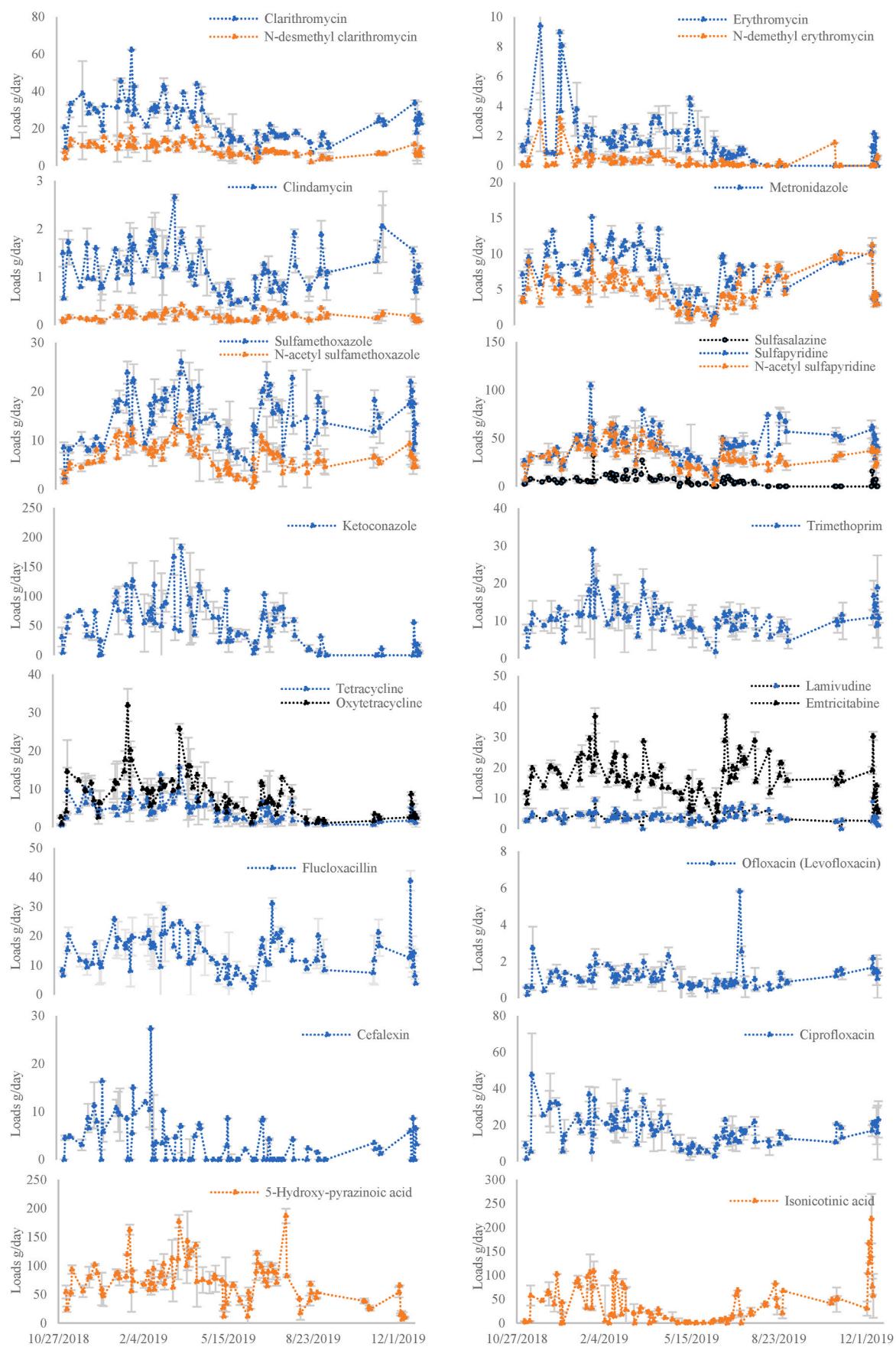
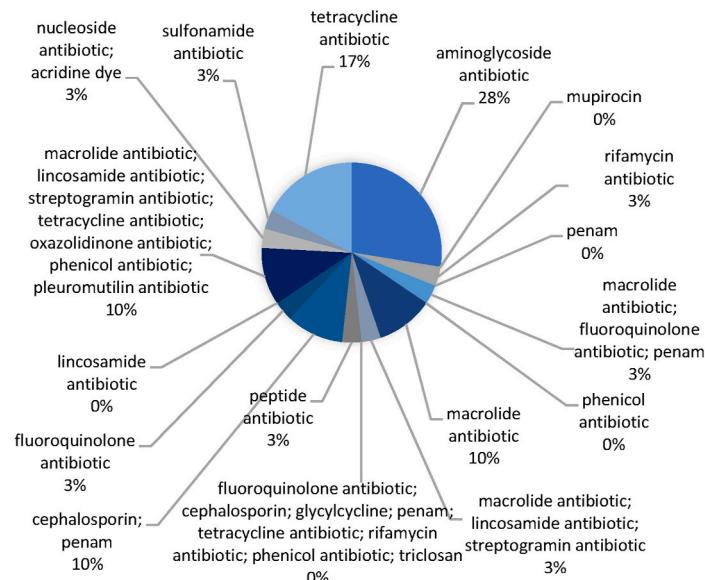
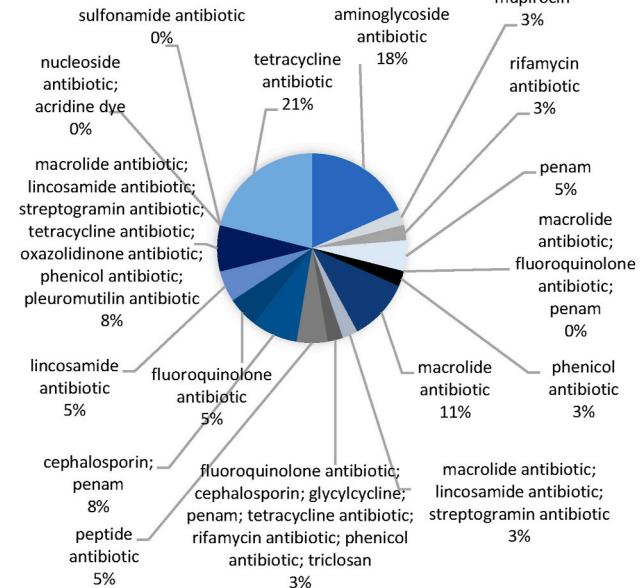


Fig. 1. Scatter plots of average daily loads of AAs and metabolites in influent wastewater of Bath over the sampled period in 2018–2019.

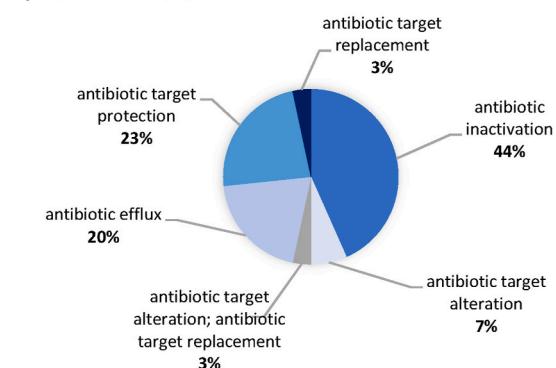
A) NOVEMBER 2018



B) MARCH 2019



A) NOVEMBER 2018



B) MARCH 2019

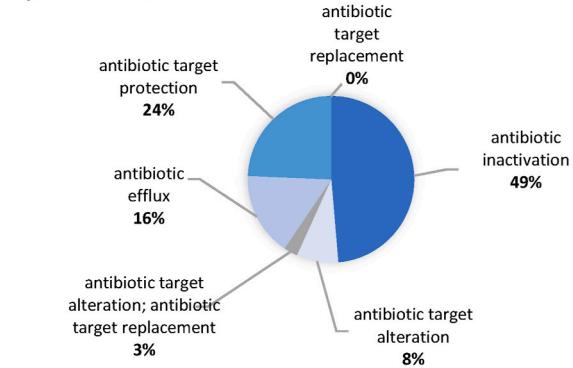


Fig. 2. Composition of resistance gene class types present in influent wastewater November 2018 (A) and March 2019 (B), below composition of resistance gene mechanism types in November 2018 (C) and March 2019 (D).

did not change this overall trend (sulphonamides > macrolides and lincosamides > quinolones), but macrolide and sulphonamides class averages did increase to 100 ± 39 g/day and 36 ± 15 g/day respectively. Quinolone metabolites were rarely detected, which may be because CIP-ciprofloxacin and OFX-ofloxacin are often excreted as parent compounds between 30 and 65% (Bergan et al., 1988) and 73% (Lode et al., 1987) respectively. Another factor could be the limited stability of quinolone metabolites in wastewater with previously reported degradation between 29 and 62%, depending on the metabolite, after 24 h at room temperature (Holton et al., 2022). In comparison, dmCLR- N-desmethyl clarithromycin and the acetyl sulphonamides reported higher stability in the same study. With dmCLR demonstrating little to no degradation in wastewater over a 24 h period at room temperature, and sulphonamide metabolites exhibiting degradation between 10 and 30% over the same time period (Holton et al., 2022).

When investigating seasonal changes of ARGs in wastewater, *ermB*, *sul1* and *intI1* observed no statistically significant different loads in winter 2018/19 when compared to summer 2019 (Table S9). Other studies have observed similar results with regards to ARGs in wastewater. One study investigating seasonal variation of *mecA* gene abundance in wastewater (which confers β -lactam resistance in methicillin resistant *Staphylococcus aureus* (MRSA)), reported variations over a year sampling but no obvious seasonal trend (Börjesson et al., 2009). Another

study investigated 295 ARGs and mobile genetic elements, including resistance associated with tetracyclines, sulphonamides and macrolides, observed no significant seasonal variation of ARGs, except the absolute abundance of genes peaked during the spring (Zheng et al., 2020). In comparison, *qnrS* in this study was present in significantly higher loads in summer than in winter ($p = 0.0018$). Further work is required to understand this phenomenon.

Due to relatively stable ARG levels across 13 months monitoring time, limited correlations were observed between absolute loads of ARGs and total loads of associated AAs by class (Fig. 4). However, correlations between individual associated AAs and ARGs demonstrated some potential correlations where seasonal variability was reported (Table S10). Statistically significant positive correlations were observed for *ermB* and the average monthly loads of the macrolides CLR-clarithromycin and dmCLR- N-desmethyl clarithromycin ($p = 0.45$, 0.48 and 0.58 for CLR, dmCLR $r \leq 0.05$). Other statistically significant correlations again showed weakly positive correlations between ASPY-N-acetyl sulfapyridine and *sul1* ($p = 0.28$, $r \leq 0.05$) and OFX-ofloxacin and *qnrS* ($p = 0.35$, $r \leq 0.05$). Weaker positive correlations were observed between CLR/dmCLR and *ermB* when correlating all the individual sampling points (0.28 and 0.46 for CLR and dmCLR respectively, $r \leq 0.05$) with no statistically significant results observed for ASPY and *sul1* and OFX and *qnrS* (Table S11).

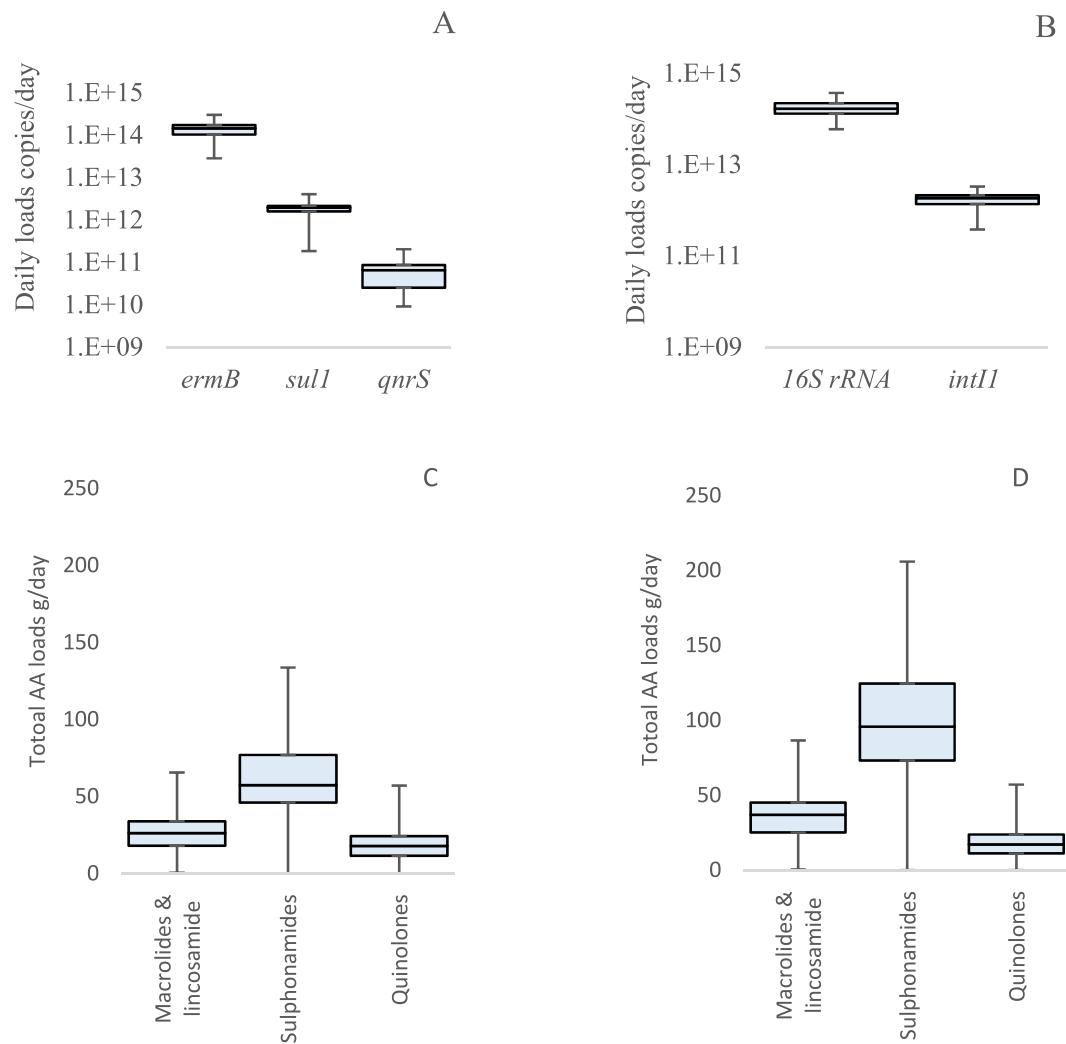


Fig. 3. A) Box plots of absolute loads of ARGs (copies/day) in community wastewater across the sampling period B) Box plots of absolute loads of *16S rRNA* and *intI1* in wastewater C) box plots of total cumulative loads of associated parent AAs (g/day) across the sampling period (metabolites have been removed) D) box plots of total cumulative loads AAs including metabolites. AAs and metabolites included in each class for C) and D) each class are detailed in Table S2.

Several studies have investigated relationships in environmental settings between certain AA classes and respective genes in wastewater (Huerta et al., 2013). For instance, correlations between abundance of TET-tetracycline genes and tetracycline levels (Li et al., 2015; Xu et al., 2015) and *sul* genes and sulphonamides (Gao et al., 2012). Regarding macrolides, Rodriguez-Mozaz et al. reported significant positive correlations between CIP-ciprofloxacin and *qnrS*, OFX-ofloxacin and *qnrS*, CLR and *ermB*, and SMX-sulfamethoxazole and *sul1* in wastewater streams, but no significant correlation between AZM and *ermB* (Rodriguez-Mozaz et al., 2015). Other studies however have observed weak correlations, or not statistically significant relationships, between TET genes and tetracyclines (Gao et al., 2012) and *sul* genes and sulphonamides (Xu et al., 2015).

Lack of strong positive relationship between AAs and their respective ARGs in this one city longitudinal study is an interesting outcome that contrasts with our previous study which focussed on 5 contrasting towns and cities (spanning from 18 k to 867 k inhabitants) (Castrignanò et al., 2020; Elder et al., 2021). Elder et al. observed positive correlations between fluoroquinolones and *qnrS* loads between different locations ($r = 0.997$, $p < 0.004$) (Elder et al., 2021). The study also observed strong positive correlations between macrolide AAs and *ermB* ($r = 0.928$, $p < 0.0002$). However, it was highlighted that strong positive correlations were also observed between both AA and gene loads and population size. It was theorised that correlations between AAs and gene loads are

likely linked to population size as a key driving force. Furthermore in the case for *ermB*, it has been reported that abundance of this gene in influent wastewater could be heavily influenced by the presence of *ermB* in common gut bacteria (Pallares-Vega et al., 2021). Other studies have found that global variation between gene abundance strongly correlates with socio-economic, environmental, and health factors (Hendriksen et al., 2019). In this longitudinal study, $\pm 20\%$ population change might occur at certain times in Bath due to student population and visitors to the city. This change is within method uncertainties, and as such, might not lead to a measurable, statistically significant difference e.g. between seasons. The results presented here support our hypothesis that human population and its size, is a significant driver of AA and ARG levels in the environment. Whilst AA levels in wastewater vary with changing usage patterns, ARGs' prevalence has an endemic, community driven nature.

However, to make fair comparisons between datasets and to aid in accounting for variabilities in the extraction protocol, gene loads were also normalised to *16S rRNA* to measure the estimated abundance of microorganisms present in the sample and to investigate possible selection occurring (Table S8). Relative correlations of ARGs (normalised to *16S rRNA*) with associated AAs were also investigated (Table S10 and S11). In general, normalising each of the gene targets to *16S rRNA* did not change the patterns of gene loads observed across samples. Gene loads were also normalised to the human population of Bath to calculate

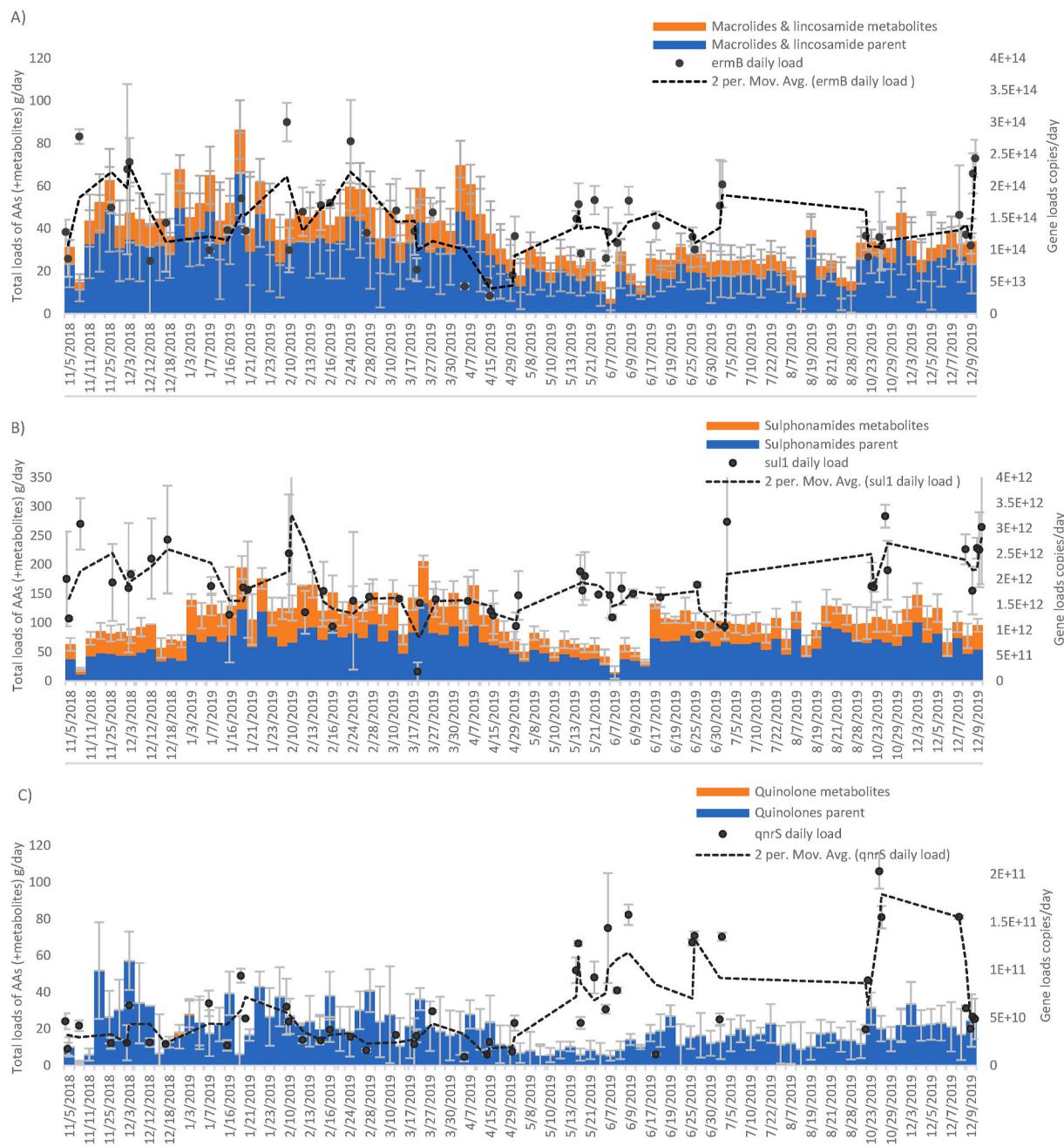


Fig. 4. Daily loads of AAs by class and total loads of associated ARGs in influent wastewater in the studied period. Error bars represent standard deviation, $n = 2$. A) macrolides and lincosamides AAs and *ermB* gene loads B) sulphonamide AAs and *sul1* gene loads C) quinolone AAs and *qnrS* gene loads.

gene loads per capita; these were positively correlated to gene loads normalised by *16S rRNA* (Fig. 5). This observation is an important one, as the results of our study, along with prior research, suggest that the size of the community has a role in determining ARG levels in wastewater. Further research is needed in this area to explore this relationship.

3.4. Hospital vs community wastewater: AA and ARG prevalence

A range of AAs covering different classes were quantifiable in hospital effluent. Regarding ARGs, all seven targets were quantifiable in all samples (S11 and S12). The most prevalent ARG in hospital effluent was *ermB*, at $5.04 \times 10^9 \pm 2.11 \times 10^9$ copies/L; with *qnrS* having the lowest prevalence $2.96 \times 10^6 \pm 2.92 \times 10^6$ copies/L (Fig. 6). The two additional genes investigated in hospital effluent, *TetM* and *bla-TEM*, had

similar abundances in general, at $8.71 \times 10^6 \pm 5.05 \times 10^6$ and $6.38 \times 10^6 \pm 5.00 \times 10^6$ copies/L respectively. Finally, the ARGs, *qnrS* and *tetM* both had low prevalence when compared to other studied genes and observed similar concentrations to each other in hospital effluent.

The overall abundances of AAs linked to the ARGs studied in hospital effluent vs community wastewater were also considered (Fig. 6). Concentrations of total AAs classes in hospital effluent were generally higher than community wastewater. Focusing on hospital effluent, the macrolides and lincosamides were measured at the highest abundance, with five-day averages at 50 ± 43 µg/L and 30 ± 24 µg/L, with and without metabolites respectively. Excluding metabolites, AAs from the beta-lactam class reported the next highest loads with five-day averages at 9 ± 8 µg/L, followed by those in the sulphonamide class at 3 ± 2 µg/L. When including metabolites, due to elevated levels of aSMX N-acetyl sulfamethoxazole on the 06/08/2019 (147 ± 14 µg/L), sulphonamides

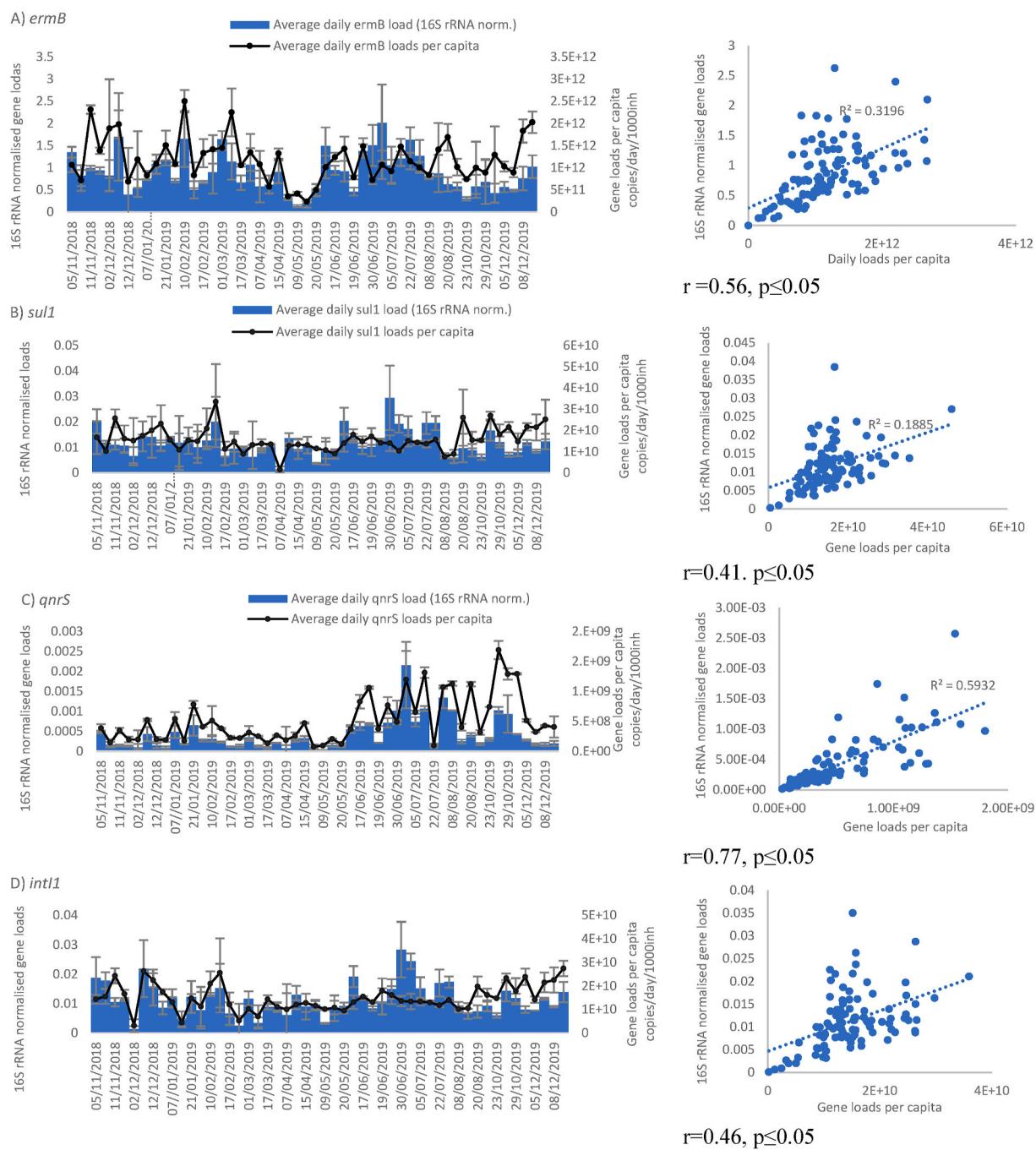


Fig. 5. Average daily loads of ARGs normalised to 16S rRNA and human population size (loads per capita).

observed the highest average after macrolides at $40 \pm 67 \mu\text{g/L}$ with beta-lactams at $11 \pm 7 \mu\text{g/L}$. Quinolone and tetracycline AA were in the lowest abundance in hospital effluent giving five-day averages at $1.0 \pm 1.6 \mu\text{g/L}$ and $0.02 \pm 0.03 \mu\text{g/L}$.

Interestingly, several AAs were present in hospital effluent that were not observed at all or with very low frequency in community wastewater, likely due to dilution of hospital wastewater with communal discharge (Table S14). Many AAs and metabolites were also observed in high concentrations. This is likely due to, with shorter sewage residence time, lower volume of flow and concentration of individuals at the source requiring AAs. LZD-linezolid is an oxazolidinone AA which is used in severe bacterial infections in the UK. It is often prescribed in hospital environments as it requires specialist supervision (National Institute for Health and Care Excellence, n. d.). It can be used to treat

serious respiratory illness (pneumonia) and to treat skin and soft tissue infections caused by methicillin resistant *Staphylococcus aureus* (MRSA). It has also been identified by WHO as a recommended treatment of multi-resistant TB (World Health Organisation, 2019). Whilst LZD was not detected in community wastewater, three of the hospital effluent samples observed levels between 0.03 and 5.2 $\mu\text{g/L}$. AMX-amoxicillin on the other hand is prescribed in both community and hospital settings and is a popular AA due to its effectiveness against both gram-negative and gram-positive infections. AMX was not detected in any samples in community wastewater but was quantifiable in all hospital effluent samples, ranging between 0.3 and 6.0 $\mu\text{g/L}$. The absence of AMX in community wastewater is likely due to lack of stability of the constrained beta-lactam ring (Hirte et al., 2016).

Fluoroquinolones are frequently found at high levels in hospital

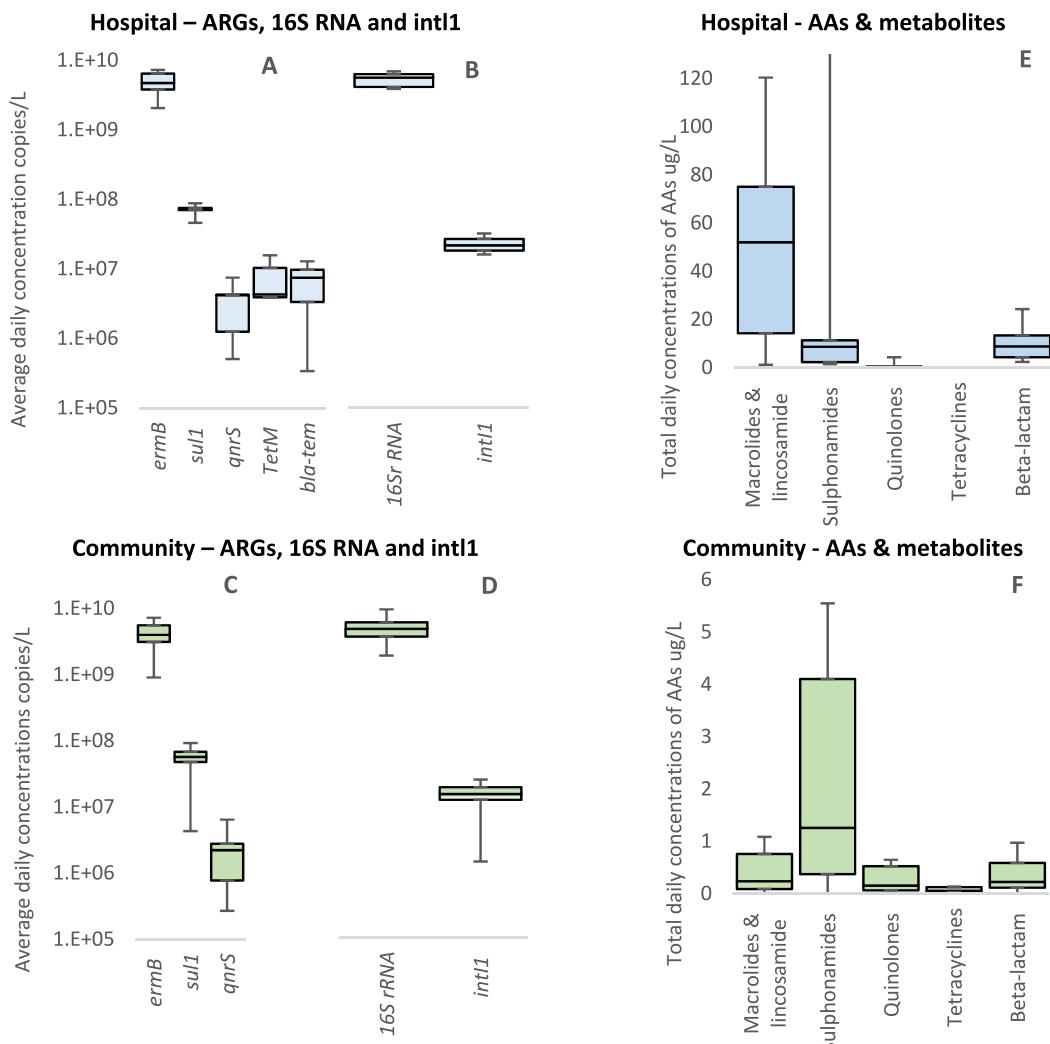


Fig. 6. Box plot of concentrations in A) ARGs in hospital effluent; B) 16S rRNA and *intI1* in hospital effluent; C) ARGs in community wastewater (Bath), August 2019; D) 16S rRNA and *intI1* in community wastewater (Bath), August 2019; E) total AAs grouped by class with respective metabolites included in hospital effluent; and F) total AAs grouped by class with respective metabolites included in community wastewater (Bath) in August.

effluents, particularly in comparison to municipal wastewater (Rodríguez-Mozaz et al., 2015; Varela et al., 2014). Fluroquinolone AAs are a commonly prescribed class of AAs that are used to treat a wide-range of infections, including pneumonia, septicaemia and urinary tract infections. Their high prevalence in environmental matrices is explained by their stability and due to the fact they are often excreted as parent compounds in urine (30–85%) (Novelli and Rosi, 2017). Regarding CIP-ciprofloxacin, the average levels in hospital effluent for the five days were reported at $0.9 \pm 1.6 \mu\text{g/L}$, in comparison to the yearly average of CIP in community wastewater at $0.52 \pm 0.24 \mu\text{g/L}$. The highest concentrations of CIP were reported on the 06/08/2019 in hospital effluent at $3.9 \pm 0.5 \mu\text{g/L}$. Similar variable concentrations of CIP have been reported elsewhere in hospital effluents (Aydin et al., 2019; Varela et al., 2014; Verlicchi et al., 2012), with some studies reporting significantly higher quantities, ranging 101–236 $\mu\text{g/L}$ (Diwan et al., 2010; Lindberg et al., 2004).

Other AAs in higher concentrations in hospital effluent (relative to community wastewater) included SMX-sulfamethoxazole, TMP-trimethoprim, and MTZ-metronidazole. With SMX ranging from 0.22 to 4.23 $\mu\text{g/L}$ and TMP between 0.6 and 7.6 $\mu\text{g/L}$, these corresponding high levels are likely as SMX and TMP are often co-prescribed together. These are in range with other reports of hospital effluents; SMX has been reported between 0.15 and 373 $\mu\text{g/L}$ (Aydin et al., 2019; Lindberg et al.,

2004; Rodriguez-Mozaz et al., 2015; Verlicchi et al., 2012) and TMP at 0.14 and 7.6 $\mu\text{g/L}$ (Lindberg et al., 2004; Rodriguez-Mozaz et al., 2015; Verlicchi et al., 2012). MTZ ranged from 10.2 to 17.1 $\mu\text{g/L}$ in hospital effluent. High quantities of MTZ have also been reported in hospital streams in Sweden (Lindberg et al., 2004), Spain (Gómez et al., 2006) and Vietnam (Lien et al., 2016). High levels in hospital effluent were also noted with respective metabolites aSMX (0.7–146.6 $\mu\text{g/L}$) and hMTZ (6.8–37.6 $\mu\text{g/L}$). Whilst higher concentrations were often observed for hospital wastewater when compared with community wastewater, several AAs had the opposite trend. For example, TET-tetracycline and OTC-oxytetracycline had lower concentrations reported in hospital effluent when compared to community wastewater, with OTC at 0.05 ± 0.03 and $0.24 \pm 0.16 \mu\text{g/L}$, respectively; and TET at 0.03 ± 0.01 and $0.13 \pm 0.08 \mu\text{g/L}$. This could be attributed to the specific AA usage, TET and OTC are not typically associated with hospitals in the UK, being largely used to treat chlamydia and skin conditions, such as acne and rosacea.

Plotting sample composition by AA class (Fig. 7) demonstrated a high percentage of macrolides in hospital effluent (43%) compared to community wastewater influent (12%). Interestingly a higher percentage composition of sulphonamides was observed for community wastewater (37%) versus hospital effluent (15%). A similar trend was noted for antiretrovirals, with AAs of this class making up 7% of

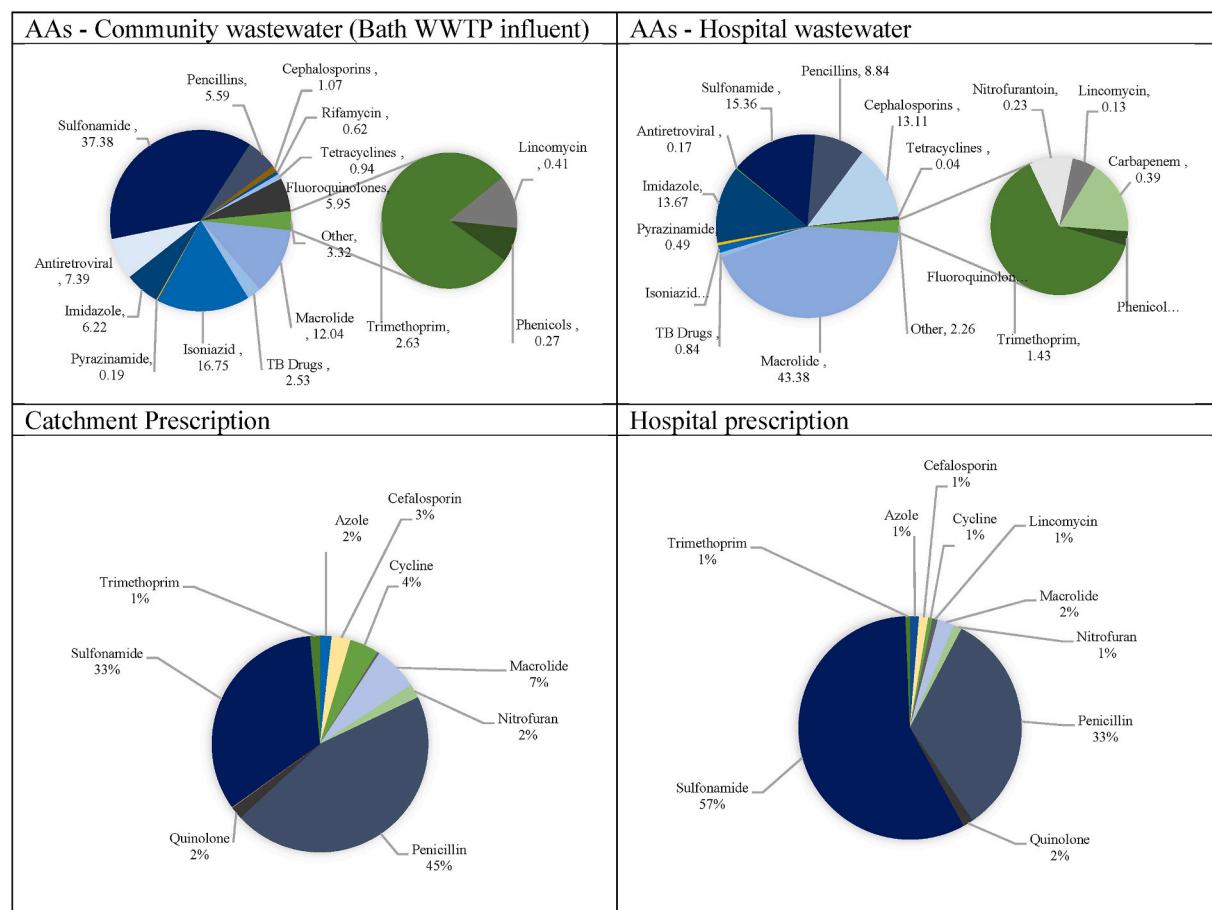


Fig. 7. Comparisons in concentration composition of AA classes in community wastewater ($n = 7$) (A) and hospital wastewater ($n = 5$) in August 2019. The labels under each AA class are percentages. C) Percentage comparison of prescriptions by AA class of hospital versus the rest of the catchment in August 2019.

community wastewater, compared to <1% of hospital effluent. By comparing percentage catchment prescription versus hospital prescriptions (Fig. 7), the percentage of sulphonamides prescribed in hospitals was significantly higher than in the wider catchment (57% versus 33% respectively). Prescriptions of macrolides however were lower in hospital prescribing (2%) in comparison to community prescribing (7%). Why the same composition patterns were not reflected in wastewater is likely due to various reasons, including metabolism and degradation of AAs in wastewater, as well as patient compliance.

Interestingly, in contrast to AAs, comparable concentrations of ARGs were observed in community and hospital wastewater and denoted 5 E+09, 6.7 E+07 and 3 and 4 E+06 for *ermB*, *sul1* and *qnrS* respectively (Figure S4).

A similar observation has been reported with Rodriguez-Mozaz et al., where the absolute concentration of genes *blaTEM*, *qnrS*, *ermB*, *sul1*, and *tetW* had comparable abundances between hospital and urban wastewater (Rodriguez-Mozaz et al., 2015). Furthermore, when investigating relationships between ARGs in hospital effluent, no clear relationship was established (Table S15 and S16). This supports the theory that AMR levels are more of an endemic nature, developing over time in individual communities. This is also reflected in the low variability of AA prescribing.

3.5. Hospital vs community wastewater: environmental impacts

It is considered that the exposure of sublethal concentrations of AAs to bacterial communities present in wastewater streams could lead to selective pressures of ARGs and could influence the microbial ecology (Chow et al., 2021). However, this relationship between AAs and ARGs,

particularly in aquatic environments (wastewater, surface waters etc) is not always clear. Predicted no effect concentrations (PNECs), have been considered to explore whether AA levels are high enough to influence ARG abundance – they predict the concentration that a chemical, if exceeded, will likely cause adverse effects in an ecosystem. This can be used to investigate whether hospital effluent poses a higher risk, compared to community wastewater. PNECs often guide environmental risk assessments and, in the case of AMR, identify the risk of AAs in environmental matrices.

The PNEC table consists of two different values; (1) PNEC-Minimum inhibitory concentration (PNEC-MIC), published by Bengtsson-palme and Larsson (Bengtsson-Palme and Larsson, 2016), based on resistance promotion, and derived from EUCAST breakpoint data for AAs; (2) Environmental PNEC (PNEC-ENV), based on eco-toxicology, and intended to protect ecological systems. The lowest of the two PNEC values are used to regulate environmental AA levels. Hospitals have previously been highlighted as an area of concern. One study measuring particularly high AA burdens estimated 44% of AAs exceeded the PNEC values (Booth et al., 2020). This was in comparison to municipal wastewater, with 99% of AAs exceeding PNECs.

Average concentrations across the sampling period, for both hospital and community wastewater, have been compared with the PNEC values (Table 2). A handful of AAs were reported to have concentrations that fell below PNEC values for both hospital and community wastewater, including TET-tetracycline, OTC-oxytetracycline, and CHL-chloramphenicol. However, several AAs exceeded the PNEC in both communal and hospital wastewater, including CLR-clarithromycin with average concentrations 0.47 ± 0.32 and $0.72 \pm 0.31 \mu\text{g/L}$ in hospital and domestic wastewater respectively, above the PNEC of $0.08 \mu\text{g/L}$.

Table 2

Average concentrations of AAs in hospital effluent (five days sampled) and Bath (community) wastewater over the year along with respective PNEC values.

Bath	Average Wastewater Concentration ug/L		PNEC ug/L		
AA	Hospital	Community	PNEC-MIC	PNEC-ENV	Lowest PNEC
Chloramphenicol	0.18 ± 0.09	0.15 ± 0.17	8	N/A	8
Sulfamethoxazole	1.47 ± 1.58	0.46 ± 0.19	16	0.6	0.6
Azithromycin	39.5 ± 41.56	0.31 ± 0.30	0.25	0.02	0.02
Clarithromycin	0.47 ± 0.32	0.72 ± 0.31	0.25	0.08	0.08
Erythromycin	2.20 ± 2.31	0.06 ± 0.05	1	0.5	0.5
Ciprofloxacin	0.93 ± 1.66	0.52 ± 0.24	0.064	0.57	0.064
Oflloxacin	0.04 ± 0.05	0.04 ± 0.03	0.5 (levofloxacin)	10	0.5 (levofloxacin)
Amoxicillin	11.13 ± 8.92	–	0.25	N/A	0.25
Clindamycin	0.27 ± 0.43	0.04 ± 0.02	1	0.1	0.1
Metronidazole	12.49 ± 3.40	0.23 ± 0.09	0.125	N/A	0.125
Nitrofurantoin	0.52 ± 0.51	–	64	N/A	64
Oxytetracycline	0.05 ± 0.03	0.24 ± 0.16	0.5	18	0.5
Tetracycline	0.03 ± 0.01	0.13 ± 0.08	1	3.2	1
Ethambutol	0.07 ± 0.05	0.11 ± 0.12	2	N/A	2
Sulfadiazine	–	0.008 ± 0.007	N/A	13	13
Trimethoprim	3.25 ± 2.76	0.33 ± 0.12	0.5	100	0.5
Flucloxacillin	5.66 ± 5.97	0.44 ± 0.23			

*Highlighted values exceed the lowest PNEC value.

Similar observations were observed for CIP-ciprofloxacin, with average concentrations 0.93 ± 1.66 and $0.52 \pm 0.24 \mu\text{g/L}$ in hospital and domestic wastewater, again above the PNEC value of $0.064 \mu\text{g/L}$. Both CIP and CLR have been previously reported to exceed the PNEC values, in a range of environmental matrices including hospital, municipal and surface waters (Booth et al., 2020; Hartmann et al., 1998). The levels exceeding PNEC values could have the potential to promote AMR.

Several AAs exceeded PNECs in hospital effluent, but often fell below in community wastewater (Table S17). For example, AA concentrations in hospital and community wastewater respectively: SMX 1.47 ± 1.58 and $0.46 \pm 0.19 \mu\text{g/L}$, PNEC of $0.6 \mu\text{g/L}$; ERY 2.20 ± 2.31 vs $0.06 \pm 0.05 \mu\text{g/L}$, PNEC of $0.5 \mu\text{g/L}$; and TMP 3.25 ± 2.76 and $0.33 \pm 0.12 \mu\text{g/L}$, PNEC at $0.5 \mu\text{g/L}$. Thus for certain AAs, hospital effluents could pose a greater risk of selective pressures of ARGs. The hospital effluent contribution to Bath will likely contribute a small amount to the overall wastewater reaching the WWTP, however the unique environment that hospital effluents constitute (with concentrated AAs exceeding PNECs), highlights the importance of including hospitals in AMR surveillance.

However, previous work by Stanton et al. has observed that environmental concentrations of macrolide AAs (ERY, CLR, and AZM) do not positively select for resistance genes (Stanton et al., 2020). Instead, lowest observable effect concentrations in this study for macrolides were significantly higher than PNECs and the measured environmental concentrations. In this case, it was theorised that PNECs for macrolides could be underestimated when considering combined exposure effects. The same study also demonstrated for CIP, (whilst no significant selection of *qnrS* was observed), positive selection of *intI1* at environmentally relevant concentrations were demonstrated (>7.8 and $< 15.6 \mu\text{g/L}$). Due to this the authors theorised the likelihood that genes conferring resistance to different antimicrobials may also be co-selected by CIP, due to *intI1* association (e.g. *sul1* gene is frequently found on the class 1 integrons backbones). Results such as these could potentially indicate AA levels do not drive ARG levels in wastewater. This study has not only highlighted the need for compound specific assessment for selective potential of genes, but also that further research is essential for more informed AA and ARG regulation, for both environmental and public health purposes.

3.6. Limitations of the study

This study was the first to provide an extensive and comprehensive monitoring programme of AAs and ARGs in a city. AMR is a complex phenomenon. It requires layers of data to unravel. This paper addresses a key knowledge gap by providing AA profiling in a longitudinal, 13

months study. However, as any work of this complexity and scale, this study is not free from limitations. The key limitations are:

1. Selection of both AA and ARG targets. Although as informed as possible (based on literature data and prescription datasets), selection of targets brings limitations, especially when the relationship between AAs and ARGs are being explored. Future work should open opportunities for non-target approaches including high resolution mass spectrometry datasets providing chemical profiles combined with sequencing data providing genetic profiles. These need to be followed by fit for purpose statistical modelling approaches.
2. Future work needs to account for chemical drivers of AMR other than AA. These include biocides, wider pharmaceutical groups as well as metals.
3. A large scale monitoring programme of several urban, and indeed, rural areas across the globe should provide much needed insights into geography driven AMR drivers.
4. Hospital contribution needs further attention. Future work should also account for veterinary usage. The success of future work lies in study design accounting for all drivers of AMR at a city/river catchment level. This direction is critical to enable informed One Health actions.

These limitations need to be accounted for in data interpretation as well and should serve as a starting point for future studies.

4. Conclusions

The manuscript presents results from a 13-month longitudinal study (with randomised sampling of 4 samples per week) aimed at providing insight into AA usage and ARGs prevalence in community and hospital wastewaters in the city of Bath (120 k inh) with an overarching aim to test the hypothesis that AA usage drives ARG prevalence. The key conclusions are as follows:

1. Several AAs in wastewater had higher loads in winter when compared to summer, including macrolide AAs and metabolites, aligning with increases in winter respiratory infections. In contrast, AAs such as SMX-sulfamethoxazole and SPY-sulphapyridine, stayed consistent over the study period.
2. As opposed to AAs, ARGs were found to be less variable, which indicates that fluctuations in AA usage might either not directly affect ARG levels or this process spans beyond the 13-month monitoring period. However, it is important to note that weekly positive

- correlations between individual associated AAs and ARGs were observed where seasonal variability in AA use was reported: *ermB* and macrolides CLR-clarithromycin and dmCLR-N-desmethyl clarithromycin ($p = 0.45, 0.48$ and 0.58 for CLR, dmCLR $r \leq 0.05$), aSPY and *sul1* ($p = 0.28, r \leq 0.05$) and OFX-ofloxacin and *qnrS* ($p = 0.35, r \leq 0.05$).
3. Gene loads normalised to *16S rRNA* (gene load per microbe) were positively correlated to the gene loads normalised to the human population (gene load per capita), which indicates, yet again, that the abundance of microorganisms is proportional to the size of human population and that the community size, and not AA usage, is a major driver of ARG levels in wastewater.
 4. Hospital wastewater showed higher number of AAs and their metabolites, their frequency of occurrence and concentrations when compared to community wastewater. Examples include: LZD-linezolid (used only in severe bacterial infections) and AMX-amoxicillin (widely used, also in community but with very low wastewater stability) that were found only in hospital wastewater. CIP-citalopram, SMX-sulfamethoxazole, TMP-trimethoprim, and MTZ-metronidazole, macrolides were found at much higher concentrations in hospital wastewater while TET-tetracycline and OTC-oxytetracycline, as well as antiretrovirals had an opposite trend as these AAs are used in communities to treat milder conditions. In contrast, comparable concentrations of resistant genes were observed in both community and hospital wastewater. This supports our hypothesis that AMR levels are more of an endemic nature, developing over time in individual communities that are not affected by short term variability in usage patterns of AAs in studied communities.
 5. Both hospital and community wastewater had AAs that exceeded PNEC values (e.g. CLR-clarithromycin, CIP). In general, though, hospital effluents had a greater number of quantifiable AAs exceeding PNECs (e.g. SMX-sulfamethoxazole, ERY-erythromycin, TMP-trimethoprim). Hospitals are therefore an important consideration in AMR surveillance as could be high risk areas for AMR.

Credit author statement

Natalie Sims: Conceptualisation, Methodology (experimental design, sampling, analysis of AAs with LCMS and analysis of ARGs with dPCR), Formal analysis, Data curation, Writing – original draft, Writing – review editing; Andrew Kannan: Methodology (experimental design and sampling); Elizabeth Holton: Writing – original draft, Writing – review editing; Kishore Jagadeesan: Methodology (experimental design and sampling); Writing – review editing; Leonardos Mageiros: Methodology (sequencing), Formal analysis, Data curation, Writing – original draft, Writing – review editing; Richard Standerwick: Methodology (conceptualisation, sampling, WWTP information) Writing – review editing; Project administration, Resources. Ruth Barden: Methodology (experimental design), Funding acquisition, resources, Writing – review editing; Ed Feil: Methodology (sequencing), Writing – review editing; Barbara Kasprzyk-Hordern: Conceptualisation, Methodology (experimental design, sampling and analysis), Writing-original draft, Writing – review editing, Supervision, Project administration, Funding acquisition, Resources.

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.

Data availability

Data is available in SI

Acknowledgements

The support of Engineering and Physical Sciences Research Council (EP/P028403/1), Wessex Water Services Ltd and EPSRC Impact Acceleration Account (Project number: EP/R51164X/1, ENTRUST IAA) is greatly appreciated. The authors would like to thank EPSRC Centre for Sustainable Circular Technologies for their support. The support of Wessex Water is also greatly appreciated.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2023.122020>.

References

- Amsden, G.W., 1996. Erythromycin, clarithromycin, and azithromycin: are the differences real? Clin. Therapeut. [https://doi.org/10.1016/S0149-2918\(96\)80179-2](https://doi.org/10.1016/S0149-2918(96)80179-2).
- Aydin, S., Aydin, M.E., Ulvi, A., Kilic, H., 2019. Antibiotics in hospital effluents: occurrence, contribution to urban wastewater, removal in a wastewater treatment plant, and environmental risk assessment. Environ. Sci. Pollut. Res. 26, 544–558. <https://doi.org/10.1007/s11356-018-3563-0>.
- Been, F., Rossi, L., Ort, C., Rudaz, S., Delémont, O., Esseiva, P., 2014. Population normalization with ammonium in wastewater-based epidemiology: application to illicit drug monitoring. Environ. Sci. Technol. 48, 8162–8169. <https://doi.org/10.1021/es5008388>.
- Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: proposed limits for environmental regulation. Environ. Int. <https://doi.org/10.1016/j.envint.2015.10.015>.
- Bergan, T., Dalhoff, A., Rohwedder, R., 1988. Pharmacokinetics of ciprofloxacin. Infection 16. <https://doi.org/10.1007/BF01650500>.
- Booth, A., Aga, D.S., Wester, A.L., 2020. Retrospective analysis of the global antibiotic residues that exceed the predicted no effect concentration for antimicrobial resistance in various environmental matrices. Environ. Int. <https://doi.org/10.1016/j.envint.2020.105796>.
- Börjesson, S., Melin, S., Matussek, A., Lindgren, P.E., 2009. A seasonal study of the *mecA* gene and *Staphylococcus aureus* including methicillin-resistant *S. aureus* in a municipal wastewater treatment plant. Water Res. 43, 925–932. <https://doi.org/10.1016/j.watres.2008.11.036>.
- Castrignanò, E., Yang, Z., Feil, E.J., Bade, R., Castiglioni, S., Causanilles, A., Gracia-Lor, E., Hernandez, F., Plósz, B.G., Ramin, P., Rousis, N.I., Ryu, Y., Thomas, K.V., de Voogt, P., Zuccato, E., Kasprzyk-Hordern, B., 2020. Enantiomeric profiling of quinolones and quinolones resistance gene *qnrS* in European wastewaters. Water Res. 175, 115653 <https://doi.org/10.1016/j.watres.2020.115653>.
- Chen, C., Kostakis, C., Gerber, J.P., Tscharke, B.J., Irvine, R.J., White, J.M., 2014. Towards finding a population biomarker for wastewater epidemiology studies. Sci. Total Environ. 487, 621–628. <https://doi.org/10.1016/j.scitotenv.2013.11.075>.
- Choi, P.M., Bowes, D.A., O'Brien, J.W., Li, J., Halden, R.U., Jiang, G., Thomas, K.V., Mueller, J.F., 2020. Do food and stress biomarkers work for wastewater-based epidemiology? A critical evaluation. Sci. Total Environ. 736, 139654 <https://doi.org/10.1016/j.scitotenv.2020.139654>.
- Chow, L.K.M., Ghaly, T.M., Gillings, M.R., 2021. A survey of sub-inhibitory concentrations of antibiotics in the environment. J. Environ. Sci. (China). <https://doi.org/10.1016/j.jes.2020.05.030>.
- Curtis, H.J., Walker, A.J., Mahtani, K.R., Goldacre, B., 2019. Time trends and geographical variation in prescribing of antibiotics in England 1998–2017. J. Antimicrob. Chemother. <https://doi.org/10.1093/jac/dky377>.
- Daughton, C.G., 2018. Monitoring wastewater for assessing community health: sewage Chemical-Information Mining (SCIM). Sci. Total Environ. 619–620, 748–764. <https://doi.org/10.1016/j.scitotenv.2017.11.102>.
- Diwan, V., Tamhankar, A.J., Khandal, R.K., Sen, S., Aggarwal, M., Marothi, Y., Iyer, R.V., Sundblad-Tonderski, K., Stålsby- Lundborg, C., 2010. Antibiotics and antibiotic-resistant bacteria in waters associated with a hospital in Ujjain, India. BMC Publ. Health 10, 414. <https://doi.org/10.1186/1471-2458-10-414>.
- Elder, F.C.T., Proctor, K., Barden, R., Gaze, W.H., Snape, J., Feil, E.J., Kasprzyk-Hordern, B., 2021. Spatiotemporal profiling of antibiotics and resistance genes in a river catchment: human population as the main driver of antibiotic and antibiotic resistance gene presence in the environment. Water Res. 203, 117533 <https://doi.org/10.1016/j.watres.2021.117533>.
- Galani, A., Alygizakis, N., Aalizadeh, R., Kastritis, E., Dimopoulos, M.A., Thomaidis, N.S., 2021. Patterns of pharmaceuticals use during the first wave of COVID-19 pandemic in Athens, Greece as revealed by wastewater-based epidemiology. Sci. Total Environ. <https://doi.org/10.1016/j.scitotenv.2021.149014>.
- Gao, P., Munir, M., Xagoraraki, I., 2012. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. Sci. Total Environ. 421–422, 173–183. <https://doi.org/10.1016/j.scitotenv.2012.01.061>.
- Gaze, W.H., Zhang, L., Abdousslam, N.A., Hawkey, P.M., Calvo-Bado, L., Royle, J., Brown, H., Davis, S., Kay, P., Boxall, A.B.A., Wellington, E.M.H., 2011. Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated

- genes in the environment. *ISME J.* 5, 1253–1261. <https://doi.org/10.1038/ismej.2011.15>.
- Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.-G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME J.* 9, 1269–1279. <https://doi.org/10.1038/ismej.2014.226>.
- Gómez, M.J., Petrović, M., Fernández-Alba, A.R., Barceló, D., 2006. Determination of pharmaceuticals of various therapeutic classes by solid-phase extraction and liquid chromatography-tandem mass spectrometry analysis in hospital effluent wastewaters. *J. Chromatogr. A*. <https://doi.org/10.1016/j.chroma.2006.02.038>.
- Guo, J., Li, J., Chen, H., Bond, P.L., Yuan, Z., 2017. Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic resistance genes and mobile genetic elements. *Water Res.* <https://doi.org/10.1016/j.watres.2017.07.002>.
- Hartmann, A., Alder, A.C., Koller, T., Widmer, R.M., 1998. Identification of fluoroquinolone antibiotics as the main source of umuC genotoxicity in native hospital wastewater. *Environ. Toxicol. Chem.* <https://doi.org/10.1002/etc.5620170305>.
- Hendriksen, R.S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., Röder, T., Nieuwenhuijse, D., Pedersen, S.K., Kjeldgaard, J., Kaas, R.S., Clausen, P.T., L.C., Vogt, J.K., Leekithcharoenphon, P., van de Schans, M.G.M., Zuiderma, T., de Roda Husman, A.M., Rasmussen, S., Petersen, B., Bego, A., Rees, C., Cassar, S., Coventry, K., Collignon, P., Allerberger, F., Rahube, T.O., Oliveira, G., Ivanov, I., Vuthy, Y., Sopheak, T., Yost, C.K., Ke, C., Zheng, H., Baisheng, L., Jiao, X., Donado-Godoy, P., Coulibaly, K.J., Jergović, M., Hrenovic, J., Karpíšková, R., Villacis, J.E., Legesse, M., Eguale, T., Heikinheimo, A., Malania, L., Nitsche, A., Brinkmann, A., Saba, C.K.S., Kocsis, B., Solymosi, N., Thorsteinsdóttir, T.R., Hatha, A.M., Alebouyeh, M., Morris, D., Cormican, M., O'Connor, L., Moran-Gilad, J., Alba, P., Battisti, A., Shakenova, Z., Kiuyukia, C., Ng'eno, E., Rakha, L., Avsejenko, J., Bérzinš, A., Bartkevics, V., Penny, C., Rajandas, H., Parimannan, S., Haber, M.V., Pal, P., Jeunen, G.J., Gemmill, N., Fashae, K., Holmstad, R., Hasan, R., Shakoor, S., Rojas, M.L.Z., Wasyl, D., Bosevska, G., Kochubovski, M., Radu, C., Gasama, A., Radosavljevic, V., Wuertz, S., Zuniga-Montanez, R., Tay, M.Y.F., Gavačová, D., Pastuchova, K., Truska, P., Trkova, M., Esterhuyse, K., Keddy, K., Cerdà-Cuéllar, M., Pathirage, S., Norrgren, L., Örn, S., Larsson, D.G.J., Heijden, T., Van der, Kumburu, H., Sanneh, B., Bidjada, P., Njanpop-Lafourcade, B.M., Nikiema-Pessinabá, S.C., Levent, B., Meschke, J.S., Beck, N.K., Van, C.D., Phuc, N., Do, Tran, D.M.N., Kwenda, G., Tabo, D., adjim, Wester, A.L., Cuadros-Orellana, S., Amid, C., Cochrane, G., Sicheritz-Ponten, T., Schmitt, H., Alvarez, J.R.M., Aidara-Kane, A., Pamp, S.J., Lund, O., Hald, T., Woolhouse, M., Koopmans, M.P., Vigre, H., Petersen, T.N., Aarestrup, F.M., 2019. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nat. Commun.* <https://doi.org/10.1038/s41467-019-10885-3>.
- Hirte, K., Seiwert, B., Schüürmann, G., Reemtsma, T., 2016. New hydrolysis products of the beta-lactam antibiotic amoxicillin, their pH-dependent formation and search in municipal wastewater. *Water Res.* <https://doi.org/10.1016/j.watres.2015.11.028>.
- Holton, E., Kasprzyk-Hordern, B., 2021. Multiresidue antibiotic-metabolite quantification method using ultra-performance liquid chromatography coupled with tandem mass spectrometry for environmental and public exposure estimation. *Anal. Bioanal. Chem.* <https://doi.org/10.1007/s00216-021-03573-4>.
- Holton, E., Sims, N., Jagadeesan, K., Standerwick, R., Kasprzyk-Hordern, B., 2022. Quantifying Community-wide Antimicrobials Usage via Wastewater-Based Epidemiology. <https://doi.org/10.26434/CHEMRXIV-2022-PDSF4>.
- Huerta, B., Martí, E., Gros, M., López, P., Pompéo, M., Armengol, J., Barceló, D., Balcázar, J.L., Rodríguez-Mozaz, S., Marcé, R., 2013. Exploring the links between antibiotic occurrence, antibiotic resistance, and bacterial communities in water supply reservoirs. *Sci. Total Environ.* <https://doi.org/10.1016/j.scitotenv.2013.03.071>.
- Lanza, V.F., Baquero, F., Martínez, J.L., Ramos-Ruiz, R., González-Zorn, B., Andremont, A., Sánchez-Valenzuela, A., Ehrlich, S.D., Kennedy, S., Ruppé, E., van Schaik, W., Willems, R.J., de la Cruz, F., Coque, T.M., 2018. In-depth resistome analysis by targeted metagenomics. *Microbiome*. <https://doi.org/10.1186/s40168-017-0387-y>.
- Li, J., Cheng, W., Xu, L., Strong, P.J., Chen, H., 2015. Antibiotic-resistant genes and antibiotic-resistant bacteria in the effluent of urban residential areas, hospitals, and a municipal wastewater treatment plant system. *Environ. Sci. Pollut. Res.* 22, 4587–4596. <https://doi.org/10.1007/s11356-014-3665-2>.
- Lien, L.T.Q., Hoa, N.Q., Chuc, N.T.K., Thoa, N.T.M., Phuc, H.D., Diwan, V., Dat, N.T., Tamhankar, A.J., Lundborg, C.S., 2016. Antibiotics in wastewater of a rural and an urban hospital before and after wastewater treatment, and the relationship with antibiotic use-a one year study from Vietnam. *Int. J. Environ. Res. Publ. Health.* <https://doi.org/10.3390/ijerph13060588>.
- Lindberg, R., Jarnheimer, P.-Å., Olsen, B., Johansson, M., Tysklind, M., 2004. Determination of antibiotic substances in hospital sewage water using solid phase extraction and liquid chromatography/mass spectrometry and group analogue internal standards. *Chemosphere* 57, 1479–1488. <https://doi.org/10.1016/j.chemosphere.2004.09.015>.
- Lode, H., Hoffken, G., Olszewski, P., Sievers, B., Kirch, A., Borner, K., Koeppe, P., 1987. Pharmacokinetics of ofloxacin after parenteral and oral administration. *Antimicrob. Agents Chemother.* <https://doi.org/10.1128/AAC.31.9.1338>.
- Makowska, N., Koczura, R., Mokracka, J., 2016. Class 1 integrase, sulfonamide and tetracycline resistance genes in wastewater treatment plant and surface water. *Chemosphere*. <https://doi.org/10.1016/j.chemosphere.2015.10.044>.
- Murray, C.J., Ikuta, K.S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S.C., Browne, A.J., Chipeta, M.G., Fell, F., Hackett, S., Haines-Woodhouse, G., Kashef Hamadani, B.H., Kumaran, E.A.P., McManigal, B., Agarwal, R., Akech, S., Albertson, S., Amuasi, J., Andrews, J., Aravkin, A., Ashley, E., Bailey, F., Baker, S., Basnyat, B., Bekker, A., Bender, R., Bethou, A., Bielicki, J., Boonkasidecha, S., Bukosia, J., Carvalheiro, C., Castañeda-Orjuela, C., Chansamouth, V., Chaurasia, S., Chirichù, S., Chowdhury, F., Cook, A., Cooper, B., Cressey, T.R., Criollo-Mora, E., Cunningham, M., Darboe, S., Day, N.P., J., De Luca, M., Dokova, K., Dramowski, A., Dunachie, S.J., Eckmanns, T., Eibach, D., Emami, A., Feasey, N., Fisher-Pearson, N., Forrest, K., Garrett, D., Gastmeier, P., Giref, A.Z., Greer, R.C., Gupta, V., Haller, S., Haselbeck, A., Hay, S.I., Holm, M., Hopkins, S., Iregbu, K.C., Jacobs, C., Jarovsky, D., Javanmardi, F., Khorana, M., Kissoon, N., Koebeissi, E., Kostyanev, T., Krapp, F., Krumkamp, R., Kumar, A., Kyu, H., Lim, C., Limmathurotsakul, D., Loftus, M.J., Luu, M., Ma, J., Mturi, N., Munera-Huertas, T., Musicha, P., Mussi-Pinhata, M.M., Nakamura, T., Nanavati, R., Nangia, S., Newton, P., Ngoun, C., Novotney, A., Nwakanma, D., Obiero, C.W., Olivas-Martinez, A., Olliaro, P., Ooko, E., Ortiz-Brizuela, E., Peleg, A.Y., Perrone, C., Plakkal, N., Ponce-de-Leon, A., Raad, M., Ramdin, T., Riddell, A., Roberts, T., Robotham, J.V., Roca, A., Rudd, K.E., Russell, N., Schnall, J., Scott, J.A.G., Shivamallappa, M., Sifuentes-Osorio, J., Steenkiste, N., Stewardson, A.J., Stoeva, T., Tasak, N., Thaiprakong, A., Thwaites, G., Turner, C., Turner, P., van Doorn, H.R., Velaphi, S., Vongpradith, A., Vu, H., Walsh, T., Waner, S., Wangrangsimakul, T., Wozniak, T., Zheng, P., Sartorius, B., Lopez, A.D., Stergachis, A., Moore, C., Dolecek, C., Naghavi, M., 2022. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet*. [https://doi.org/10.1016/S1040-6736\(21\)02724-0](https://doi.org/10.1016/S1040-6736(21)02724-0).
- National Institute for Health and Care Excellence. n.d. Linezolid [WWW Document]. URL: <https://bnf.nice.org.uk/drug/linezolid.html>. (Accessed 2 February 2022).
- Novelli, A., Rosi, E., 2017. Pharmacological properties of oral antibiotics for the treatment of uncomplicated urinary tract infections. *J. Chemother.* <https://doi.org/10.1080/1120009X.2017.1380357>.
- Pallares-Vega, R., Hernandez Leal, L., Fletcher, B.N., Vias-Torres, E., van Loosdrecht, M.C.M., Weissbrodt, D.G., Schmitt, H., 2021. Annual dynamics of antimicrobials and resistance determinants in flocculent and aerobic granular sludge treatment systems. *Water Res.* <https://doi.org/10.1016/j.watres.2020.116752>.
- Pärnänen, K.M.M., Narciso-Da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T., Elpers, C., Fatta-Kassinos, D., Henrikens, I., Jaeger, T., Karkman, A., Martinez, J., L., Michael, S.G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sorum, H., Stedtfeld, R.D., Tiedje, J.M., Giustina, S.V., Delta, Walsh, F., Vaz-Moreira, I., Virta, M., Manaia, C.M., 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. *Sci. Adv.* 5, eaau9124 <https://doi.org/10.1126/sciadv.aau9124>.
- Petrovich, M.L., Zilberman, A., Kaplan, A., Eliraz, G.R., Wang, Y., Langenfeld, K., Duhamel, M., Wigginton, K., Poretzky, R., Avisar, D., Wells, G.F., 2020. Microbial and viral communities and their antibiotic resistance genes throughout a hospital wastewater treatment system. *Front. Microbiol.* <https://doi.org/10.3389/fmicb.2020.00153>.
- Price, R.H.M., Graham, C., Ramalingam, S., 2019. Association between viral seasonality and meteorological factors. *Sci. Rep.* <https://doi.org/10.1038/s41598-018-37481-y>.
- Riquelme, M.V., Garner, E., Gupta, S., Metch, J., Zhu, N., Blair, M.F., Arango-Artgy, G., Maile-Moskowitz, A., Li, A., Flach, C.-F., Aga, D.S., Nambi, I., Larsson, D.G.J., Bürgmann, H., Zhang, T., Pruden, A., Vikesland, P.J., 2021. Wastewater Based Epidemiology Enabled Surveillance of Antibiotic Resistance. <https://doi.org/10.1101/2021.06.01.21251814> medRxiv.
- Rodriguez-Mozaz, S., Chamorro, S., Martí, E., Huerta, B., Gros, M., Sánchez-Melió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Res.* 69, 234–242. <https://doi.org/10.1016/j.watres.2014.11.021>.
- Stanton, I.C., Murray, A.K., Zhang, L., Snape, J., Gaze, W.H., 2020. Evolution of antibiotic resistance at low antibiotic concentrations including selection below the minimal selective concentration. *Commun. Biol.* <https://doi.org/10.1038/s42003-020-01176-w>.
- Varela, A.R., André, S., Nunes, O.C., Manaia, C.M., 2014. Insights into the relationship between antimicrobial residues and bacterial populations in a hospital-urban wastewater treatment plant system. *Water Res.* 54, 327–336. <https://doi.org/10.1016/j.watres.2014.02.003>.
- Verlicchi, P., Al Aukidy, M., Galletti, A., Petrovic, M., Barceló, D., 2012. Hospital effluent: investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. *Sci. Total Environ.* 430, 109–118. <https://doi.org/10.1016/j.scitotenv.2012.04.055>.
- World Health Organisation, 2020. Antimicrobial resistance [WWW Document]. URL: <https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance>. (Accessed 15 September 2021).
- World Health Organisation, 2019. WHO Consolidated Guidelines on Drug-Resistant Tuberculosis Treatment.
- World Health Organisation, 2018. Global Antimicrobial Resistance Surveillance System (GLASS) Report: Early Implementation 2017–2018.
- Xu, J., Xu, Y., Wang, H., Guo, C., Qiu, H., He, Y., Zhang, Y., Li, X., Meng, W., 2015. Occurrence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river. *Chemosphere* 119, 1379–1385. <https://doi.org/10.1016/j.chemosphere.2014.02.040>.
- Zhang, Y., Duan, L., Wang, B., Du, Y., Cagnetta, G., Huang, J., Blaney, L., Yu, G., 2019. Wastewater-based epidemiology in Beijing, China: prevalence of antibiotic use in flu season and association of pharmaceuticals and personal care products with

- socioeconomic characteristics. Environ. Int. 125, 152–160. <https://doi.org/10.1016/j.envint.2019.01.061>.
- Zheng, W., Huyan, J., Tian, Z., Zhang, Y., Wen, X., 2020. Clinical class 1 integron-integrase gene – a promising indicator to monitor the abundance and elimination of antibiotic resistance genes in an urban wastewater treatment plant. Environ. Int. 135, 105372 <https://doi.org/10.1016/j.envint.2019.105372>.
- Zielinski, W., Korzeniewska, E., Harnisz, M., Drzymala, J., Felis, E., Bajkacz, S., 2021. Wastewater treatment plants as a reservoir of integrase and antibiotic resistance genes – an epidemiological threat to workers and environment. Environ. Int. 156, 106641 <https://doi.org/10.1016/j.envint.2021.106641>.