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Spatiotemporal profiling of antimicrobial agents during wastewater treatment – a national study

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ABSTRACT

Effective wastewater treatment is critical for public health; however, the persistence of antimicrobial chemicals within these systems presents challenges and potential risks to microbial ecology, treatment efficiencies, the spread of antimicrobial resistance (AMR), and the potential environmental impacts of partially treated effluents. To understand the inputs and removals of antimicrobials in wastewater treatment, we measured, in the most comprehensive study to date, an extended list of 55 antimicrobial agents (AA) and their metabolites at ten wastewater treatment works (WwTWs) across England and Wales over six sampling campaigns in a one-year longitudinal study. A total of 204 wastewater and 135 sludge samples were collected resulting in 79,555 AA data points. Our results revealed significant temporal and spatial variability among the different AA groups, reflecting their variable usage patterns. Most AAs peaked in daily loads during the January or March sampling campaigns (except for lincosamides, quinolones, tuberculosis drugs, azoles, and antiretrovirals), whereas all AA groups experienced a significant reduction in their daily loads during the July sampling campaign. Clear spatial and temporal variabilities in per-capita AA usage were also observed likely due to seasonal usage patterns. Removal of AAs was group and individual AA dependent and highly variable, with >75% removal of lincosamides, some macrolides, nitrofurans, and <75% removal of β-lactams, glycopeptides, with some macrolides and sulfonamides being highly variable. Quinolones had very low removals with mean and median removal rates of –31.7 and –3.7%, respectively. Furthermore, comparison between trickling filter and activated sludge systems, which are the two most common biological treatment processes employed in the UK, suggests that trickling filter systems had comparable or even higher removal rates than the activated sludge systems for most AAs, except for azoles. Furthermore, due to the high variability in AA removal, no significant differences were observed in the overall removal efficiency among the tested sampling sites or across the same technologies. This indicates substantial heterogeneity in AA removal and highlights potential challenges in optimizing wastewater treatment performance for improved AA removal.

1. Introduction

Antimicrobial resistance (AMR) is a global public health threat and is estimated to be responsible for over a million deaths annually(Collaborators 2018). Wastewater is often thought of as an 'AMR hotspot' due to the microbially diverse and chemically rich (e.g., antibiotics, metals, as well as other micropollutants) environment that could facilitate the movement of antimicrobial resistance genes (ARGs) and the selection for resistance. The presence of antimicrobial agents (AAs) in wastewater is mainly driven by their usage in primary and secondary care, and is seasonally- and geographically (by prescribing region) dependent (Elder et al., 2021a; Holton et al., 2023; Holton et al., 2022a; Holton et al., 2022; Sims et al., 2023, 2023). The ever-changing nature of AA composition in wastewater and microbial transformation processes during wastewater treatment is impacted by the physicochemical properties of AAs influencing their metabolic transformation in humans/microorganisms, their physicochemical interactions, and their ability to sorb to solids.

Understanding the presence and fate of AAs is necessary for several reasons. High quantities of AAs can have a detrimental impact on wastewater and natural ecosystem health by disrupting the abundance and diversity of the microbial community(Holton et al., 2022b).

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However, lower antimicrobial concentrations have also been shown to have important ecological effects, particularly in the context of AMR development, whereby selection of resistance can be seen at environmentally relevant and sub-minimum inhibitory concentrations (Gullberg et al., 2011; Murray et al., 2024; Stanton et al., 2020). Indeed, in a UK catchment study, several antibiotics (ciprofloxacin, clarithromycin, azithromycin, and erythromycin) were regularly found exceeding predicted no-effect concentrations (both ecotox and MIC) in wastewater influent and effluent, and occasionally in receiving waters (Elder et al., 2021b).

Several papers have been published on AA presence and fate in the environment (Castrignanò et al., 2018; Diaz-Cruz and Barcelo 2006; Kim et al., 2018; Martinez 2009; Milic et al., 2013; Speltini et al., 2010; Tamtam et al., 2011) and during wastewater treatment(Castiglioni et al., 2006a; Choi et al., 2008; Gros, Petrovic, and Barcelo 2006; Li et al., 2025; Michael et al., 2013; Polesel et al., 2016; Verlicchi, Al Aukidy, and Zambello 2012; Yuan et al., 2019). However, these studies focused on either a small group of AAs, limited wastewater treatment technologies, or ignored the longitudinal dimension encompassing changing AA prescription patterns and seasonality.

Within this study, we performed comprehensive profiling of 55 AAs (Table 1) during the wastewater treatment process at a national scale (England and Wales) over one year. Using this approach, we aimed to account for geographical, AA usage, and seasonality variabilities. A total of 204 wastewater and 135 sludge samples were collected from ten WwTWs, resulting in 79,555 AA data points. This paper is the first in a

Table 1
Antimicrobial Agents (AAs) selected in this study.

S/N	Analyte	Abbreviation	Analyte type	Chemical Group	Chemical sub-group
1	Ciprofloxacin	CIP	Parent	Quinolones	Quinolone
2	Danofloxacin	DAN	Parent	Quinolones	Quinolone
3	Enrofloxacin	ENF	Parent	Quinolones	Quinolone
4	Flumequine	FLUM	Parent	Quinolones	Quinolone
5	Gatifloxacin	GAT	Parent	Quinolones	Quinolone
6	Lomefloxacin	LOM	Parent	Quinolones	Quinolone
7	Moxifloxacin	MOX	Parent	Quinolones	Quinolone
8	Nadifloxacin	NAD	Parent	Quinolones	Quinolone
9	Norfloxacin	NOR	Parent	Quinolones	Quinolone
10	Hydroxy-norfloxacin	NOROH	Metabolite	Quinolones	Quinolone
11	Ofloxacin (Levofloxacin)	OFX	Parent	Quinolones	Quinolone
12	Desmethyl-ofloxacin	OFXDES	Metabolite	Quinolones	Quinolone
13	Ulifloxacin	ULI	Metabolite	Quinolones	Quinolone
14	Sarafloxacin	SAR	Parent	Quinolones	Quinolone
15	Amoxicillin	AMX	Parent	β-lactams	Penicillin
16	Ampicillin	AMP	Parent	β-lactams	Penicillin
17	Penicillin G	PENG	Parent	β-lactams	Penicillin
18	Penicillin V	PENV	Parent	β-lactams	Penicillin
19	Piperacillin	PIP	Parent	β-lactams	Penicillin
20	Tazobactam	TAZ	Parent	β-lactams	Penicillin
21	Cefalexin	CFLX	Parent	β-lactams	Cephalosporins
22	Ceftriaxone	CEFT	Parent	β-lactams	Cephalosporins
23	Aztreonam	AZM	Parent	β-lactams	Monobactam
24	Meropenem	MRP	Parent	β-lactams	Carbapenem
25	Vancomycin	VAN	Parent	Glycopeptides	Glycopeptide
26	Sulfapyridine	SPY	Parent	Sulfonamides	Sulfonamide
27	N-acetyl sulfapyridine	SPYNA	Metabolite	Sulfonamides	Sulfonamide
28	Sulfadiazine	SDZ	Parent	Sulfonamides	Sulfonamide
29	N-acetyl sulfadiazine	SDZNA	Metabolite	Sulfonamides	Sulfonamide
30	Sulfamethoxazole	SMX	Parent	Sulfonamides	Sulfonamide
31	N-acetyl sulfamethoxazole	SMXNA	Metabolite	Sulfonamides	Sulfonamide
32	Trimethoprim	TRM	Parent	Sulfonamides	Trimethoprim
33	4-hydroxy-trimethoprim	TRMOH	Metabolite	Sulfonamides	Trimethoprim
34	Clarithromycin	CLR	Parent	Macrolides	Macrolide
35	N-desmethyl clarithromycin	CLRDES	Metabolite	Macrolides	Macrolide
36	Erythomycin	ERY	Parent	Macrolides	Macrolide
37	N-desmethyl erythromycin	ERYDES	Metabolite	Macrolides	Macrolide
38	Roxithromycin	ROX	Parent	Macrolides	Macrolide
39	Clindamycin	CDMY	Parent	Lincosamides	Lincomycin
40	Thalidomide	THA	Parent	Immunomodulatory	Thalidomide
41	Emtricitabine	EMT	Parent	Antiretrovirals	NRTIs
42	Lamivudine	LAM	Parent	Antiretrovirals	NRTIs
43	Oxytetracycline	OXYTET	Parent	Cyclines	Cycline
44	Tetracycline	TET	Parent	Cyclines	Cycline
45	Chlortetracycline	ClTET	Parent	Cyclines	Cycline
46	Chloramphenicol	CHLAMP	Parent	Amphenicols	Amphenicol
47	Florfenicol	FLOR	Parent	Amphenicols	Amphenicol
48	S,S-ANP	SSANP	Parent	Amphenicols	Amphenicol
49	Metronidazole	MET	Parent	Azoles	Azole
50	Hydroxy-metronidazole	METOH	Metabolite	Azoles	Azole
51	Nitrofurantoin	NIT	Parent	Nitrofurans	Nitrofuran
52	NP-AHD	NPAHD	Metabolite	Nitrofurans	Nitrofuran
53	Isoniazid	IZD	Parent	TB (1st line)	Isoniazid
54	Ethambutol	ETB	Parent	TB (1st line)	Ethambutol
55	Linezolid	LZD	Parent	TB (repurposed)	Oxazolidinone

NRTI – Nucleotide Reverse Transcriptase Inhibitors; S,S-ANP - 2-Amino-1-(4-nitrophenyl)-1,3-propanediol (ANP);.
NP-AHD - 1-(2-nitrobenzylidenamino)-2,4-imidazolidinedione (2NP-AHD).

series of papers aimed at understanding the dynamics and environmental impacts of AAs in wastewater.

This study aimed to answer four key questions as follows:

1. Does AA composition in wastewater influent/sludge change (a proxy for community usage) across different geographies and seasons?
2. What are the drivers of local/regional differences in AA usage?
3. Does treatment process type (i.e., trickling filter, activated sludge) affect AA removals, and does one method outperform the other? Does season or geography affect removal efficiencies?
4. Which AA groups persist throughout the treatment process?

The key novelty of the study lies in its unique spatiotemporal analysis of an extensive dataset, enabling the evaluation of wastewater treatment performance for a large number of antimicrobial agents. The study focuses on ten different technologies over a one-year period, thereby accounting for seasonal variations and changing usage patterns, as well as providing a better understanding of the behaviour of transformation by-products.

2. Materials and methods

2.1. Chemicals

Certified reference standards and deuterated (stable isotope–labelled) standards (mostly employed as internal standards for quantitation purposes) were purchased from Sigma-Aldrich (Gillingham, UK), Toronto Research Chemicals (Toronto, Canada), LGC (Middlesex, UK), or MCE (Cambridge, UK). Further details on these standards can be found in the accompanying electronic supplementary material (ESM Tab. S1). Hypergrade methanol and water for LC-MS were purchased from Merck (Darmstadt, Germany). MilliQ water was of 18.2 MΩ quality (Elga, Marlow, UK). Silanized clear culture tubes employed for elution were purchased from Thermo Fisher Scientific (Miami, OK, USA). Formic acid employed as aqueous mobile phase additive was purchased from Sigma-Aldrich. 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. Sample collection – study sites

Ten sampling sites across England and Wales (as shown in Fig. 1 and Tab. S2), were selected for the screening of antimicrobial agents at different stages of the wastewater treatment processes. Six sampling campaigns were undertaken over 12 months (SR1 – Sept. 2020, SR2 – Nov. 2020, SR3 – Jan. 2021, SR4 – Mar. 2021, SR5 – May. 2021 and SR6 – Jun. 2021). Aqueous samples, including raw influent, secondary and tertiary effluents were collected by Aqua enviro (SUEZ Advanced Solutions UK Ltd) using 24 h composite samplers to account for possible temporal heterogeneity that may be introduced by variations in hydraulic flows and anthropogenic inputs into the wastewater network. A composite sampler was fitted with a sterile internal 12 L stainless steel bucket to collect the sample. Before sampling, a grab sample of 200 mL was used to rinse the tubing and condition the equipment with the sample. The auto-sampler was programmed to collect 220 mL every 30 minutes for 24 hours, resulting in a final volume of ~10.56 L. This sample was then poured several times between two stainless steel buckets to suspend all material and create a homogenised composite of

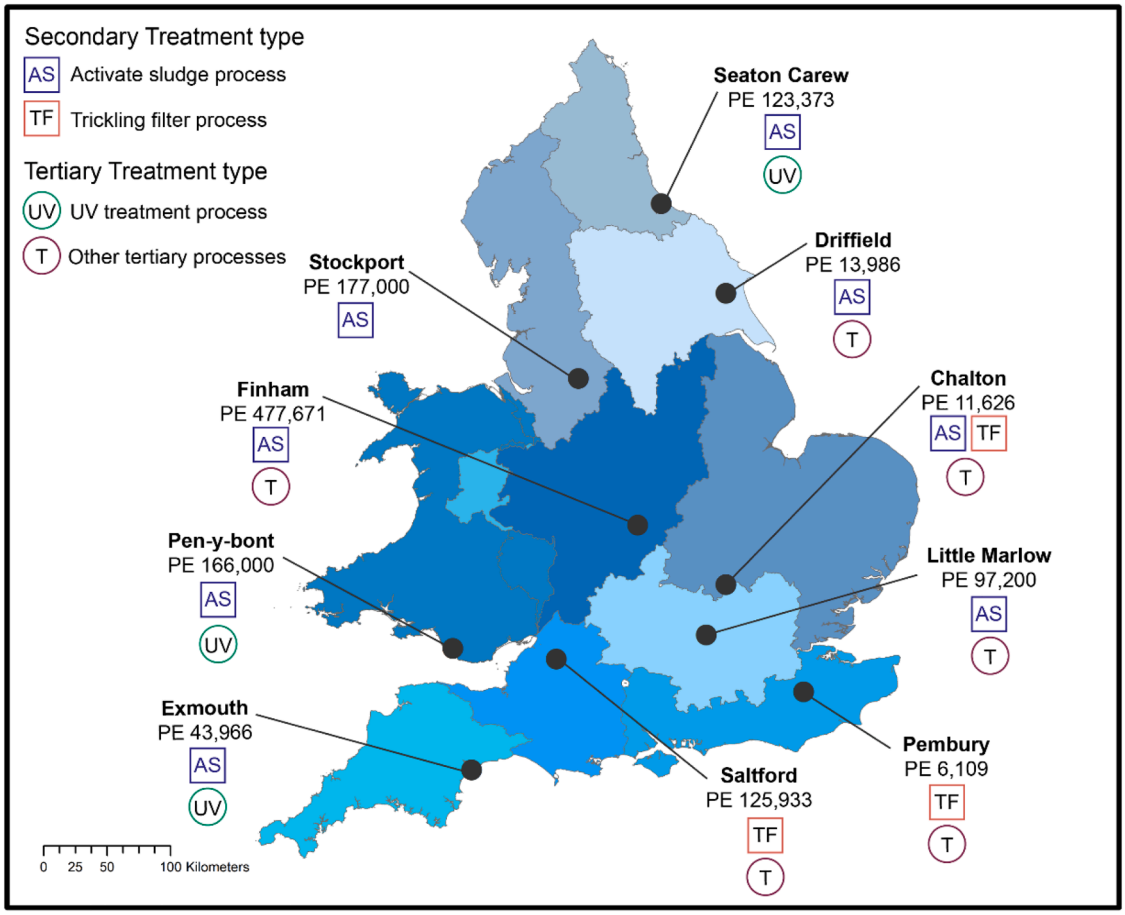

Fig. 1. Map showing the different sampling sites and their respective wastewater treatment processes across England and Wales. Presence of "UV" does not negate the presence of other tertiary treatment processes.

the sample. The homogenised sample was then poured into sterile high-density polyethylene (HDPE) wide neck sample bottles (Nalgene, Thermo Fisher, UK). These included two 500 mL bottles sent to the University of Bath (via UKCEH Wallingford) for organic chemistry analysis.

Sludge samples (both liquid and thickened solids) were collected as single grab samples due to the inability to perform composite sampling on many of these sites (for example, sampling taps on sludge lines). In theory, grab samples are less optimal compared to the 24 h composites, as the influence of temporal variability may be higher. In practice, sludges represent extremely well-homogenised samples, and whilst not tested, we expect the impact of grab versus composite sampling for these sample types to be minimal. Sludge samples were collected either directly into the sample bottles or via a clean, sample-rinsed stainless-steel bucket. All sludge samples were collected in 125 mL HDPE wide neck bottles (Nalgene, Thermo Fisher, UK), and, as for the liquid samples, two were dispatched to the University of Bath (via UKCEH Wallingford) for the screening of antimicrobial agents. Contamination was minimised by using brand new, unopened, HDPE sampling bottles.

To reduce biological degradation during sampling, the 24-h composite samplers were packed with ice to keep the composite sample cool. At the time of collection, samples were kept in insulated boxes packed with dry ice (approximately −78.5°C, which is the temperature of dry ice) during the collection on-site. Samples were shipped using a commercial courier to UKCEH Wallingford on dry ice (overnight), or where logistically possible, delivered by hand by Aqua Enviro staff on the same day as sampling. On arrival at UKCEH Wallingford, samples were stored immediately at −80°C and transferred to the University of Bath on dry ice to minimise sample degradation.

2.3. Sample preparation

2.3.1. Solid phase extraction for liquid samples

The details of the procedures employed for the extraction of selected antimicrobials from wastewater samples in this study had been previously reported(Holton and Kasprzyk-Hordern 2021), although with some minor modifications. Briefly, 50 ml of wastewater samples were measured and transferred into narrow-mouth 125 ml Fisherbrand polypropylene bottles. The samples, which were measured in triplicates, were then spiked with 50 μl of 1 μg/ml of a mixture of internal standards (see: Tab. 1). The samples were kept at 4°C and allowed to equilibrate for 1 h.

Prior to the commencement of the solid-phase extraction (SPE), the samples were filtered with 0.7 μm Whatman GF/F filter papers while the SPE cartridges were preconditioned using 2 ml MeOH followed by 2 ml H_2O under gravity. The resulting filtrates were loaded under vacuum onto the pre-conditioned HLB cartridges at a flow of approximately 2.5 ml/min. Thereafter, the cartridges were dried under vacuum for 1 h, wrapped with aluminium foil and stored at −20°C until the elution phase of the sample preparation. The adsorbed antimicrobials were eluted under gravity with 2 × 2 ml of methanol into silanized clear culture glass tubes (Thermo Fisher Scientific, Miami, USA). The eluates were then dried under a gentle stream of nitrogen gas at 40°C. The dried extracts were subsequently reconstituted with 500 μl of 80:20 v/v (H_2O:MeOH), vortexed and transferred into LC vials for further instrumental analysis.

2.3.2. QuEChERS for solid samples

The solid samples consisting of primary sludge (G1), secondary sludge (G2) and dewatered sludge (G3) samples were freeze-dried (ScanVac, Lynge, Denmark), homogenised and sieved with a 40-micron stainless steel sieve. Depending on the quantity of the dried samples, approximately 0.10 – 0.50 g was weighed and transferred into a 50 ml centrifuge tube (VWR, Radnor, PA, USA). The samples, which were in most cases prepared in duplicates, were then spiked with 50 ng (50 μl of 1 μg/ml in methanol) of a mixture of internal standards. To prevent possible adsorption onto the walls of the sample container, the internal standard solution was spiked directly on the sample and the samples were allowed to stand for 10 min. to allow for solvent evaporation. Each sample was then vortexed for 30 s and stored overnight at 4°C.

To each of the centrifuge tubes containing the samples, 5 ml of ultrapure water and 5 ml of acetonitrile were added. The samples were immediately vortexed for 30 s to disperse the sample in the diphasic solvent system. Then, the QuEChERS extraction kit (Citrate extraction tube (55227-U), Merck, Denmark) was added to each sample and the initial vigorous shaking by hand and vortexing steps were repeated. To dissipate the heat generated during the exothermic reaction taking place within the tubes, each of the tubes was immediately placed in the ultrasonic bath. At this stage, the samples were ultrasonicated with no heating for 5 min. Each of the tubes were then wiped and centrifuged at 4,000 rpm for 5 min resulting in a distinct separation of the solids, water, and acetonitrile.

To minimise possible matrix effects, ~4.5 to 5 ml of the supernatants (organic phase) were collected and transferred into 15 ml centrifuge tubes for an additional clean-up pre-treatment step, which is necessary for eliminating potential interference. Then, the QuEChERS clean-up kit (Supel™ QuE-PSA tube (55227-U), Merck, Germany) was added into each tube containing the supernatant. This step was followed by initial vigorous shaking by hand, vortexing, and ultrasonication as previously described for the extraction step. After the ultrasonication, the tubes were wiped and centrifuged at 4,000 rpm for 5 min. At this point, ~4.5 ml of the supernatant was transferred into a silanized tubes for onward transfer into the TurboVap LV concentration workstation for solvent evaporation. The supernatant was then completely dried under a gentle stream of high purity nitrogen gas at a temperature of 40°C. The dried extract was reconstituted with 500 μl of 80:20 (H_2O:MeOH, v/v), vortexed and transferred into 1.5 ml Eppendorf tube. To minimise the possibility of clogging during chromatographic analysis, the samples were further centrifuged at 6,000 rpm for 10 min on a small benchtop centrifuge. The supernatant was finally transferred into LC vials without disturbing the solid residues that had settled on sides of the Eppendorf tubes.

2.4. Analysis with ultraperformance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC-Xevo TQD)

The chromatographic separation of the targeted antimicrobial compounds and their metabolites were performed on Waters ACQUITY UPLC™ system (Waters, Manchester, UK) employing a reversed phase BEH C18 column (150 mm x 1 mm, 1.7-micron particle size) manufactured and supplied by Waters. The eluents from the UPLC system were fed into Xevo Triple Quadruple (TQD) mass spectrometer (Waters, Manchester, UK), which was equipped with an electrospray ionisation (ESI) source. The mass spectrometer was operated in positive ESI mode since majority of targeted compounds preferentially ionised in this mode producing consistent MS spectra for all the compound classes.

In all cases, 20 μl of standard solutions, blanks, quality control (QC) and real samples were injected into the UPLC system. Upon injection, the gradient elution of analytes was then effected using two mobile phases (A and B) consisting of 0.1% formic acid in 95:5 (v/v, H_2O:MeOH) and 100% MeOH, respectively. The gradient programme started at 100% A and was maintained for 1 min. This was reduced to 40% A over 8.5 min, and was further reduced to 0% A over 4.5 min. and held under this condition for 3 min. The gradient was then ramped to its starting condition over 0.5 min and was maintained under this condition for 3 min for re-equilibration of the column. The mobile phase additive as well as LCMS grade methanol were obtained from Merck (Darmstadt, Germany), while deionised water (18.2 MΩ) for mobile phase preparation was obtained from a Milli-Q system.

Both the UPLC and MS systems were controlled by MassLynx (Waters, Manchester, UK), while post-acquisition data processing was performed using the TargetLynx software, which is part of the MassLynx

(version 4.1) compendium. The intricate capabilities of enhanced sensitivity of the triple quadruple system were explored by quantifying the targeted compounds using a multiple reaction monitoring (MRM) mode of analysis after a series of optimisation studies had been performed. For these purposes, nitrogen was employed as a nebulising gas and was obtained from a high-purity nitrogen generator (Waters, Manchester, UK). High-purity argon (99.998%), which was employed as the collision-induced dissociation (CID) gas was supplied by BOC. The following MS parameters were optimised: capillary voltage, collision energy, source temperature, desolvation temperature, cone gas flow, desolvation gas flow, among others. Under the optimum conditions, two MRM transitions representing parent-daughter ions were selected for each analyte, except for the mass-labelled internal standards, in which case one transition is sufficient for their confirmation. In this case, the transition with the highest abundance was designated as quantification ion while the other transition was designated as a confirmatory ion. The full details of the method performance, optimised conditions for both LC and MS systems as well as the MRM transitions of the targeted compounds investigated have been previously reported(Holton and Kasprzyk-Hordern 2021). They can be also found in Tabs. S3-4.

2.5. Quality control

All samples were enriched with known amounts (50 ng) of appropriate internal standards to compensate for matrix suppression and potential losses of analytes during sample preparation. In this study, all the aqueous samples were analysed in triplicates, including the quality control samples employed for the evaluation of the method performance and validation studies.

To evaluate the sensitivity of the method employed, the limit of detection (LOD) and the limit of quantitation (LOQ), which are defined as the concentration corresponding to the signal-to-noise (S/N) equivalent to 3 and 10 in real samples, respectively, were determined. Similarly, the instrument's LOD and LOQ were also estimated employing the lowest concentration ranges of the calibration samples using the same S/N criteria. For most of the targeted AAs, the linearity was assessed using up to thirteen calibration points typically ranging from 0.005 to 1,500 ng/ml, although some of the AAs had variable concentration ranges with a maximum of 400 ng/ml.

The instrument and method's accuracy and precision were assessed at three different levels, including low (10 ng/L), medium (100 ng/L), and high (500 ng/L). In these instances, the mean recovery of these replicate analyses provides a measure of their accuracy, while the estimated standard deviation of the replicate analyses provides a measure of their precision. In a more practical manner, matrix-spiked samples were employed to evaluate the accuracy of the extraction method employed. Given the high native wastewater concentration values observed for some of the AAs, including azithromycin, clarithromycin and their targeted metabolites, 100% recovery was assumed. This is consistent with the performance of other targeted AAs with similar chemical characteristics. The detailed summary of all the instrument and method performance parameters, which were assessed for the targeted AAs are presented in Tabs. S4-6.

2.6. Calculations and statistical analysis

Daily mass loads (*DLs*) of AAs (mg day^{-1}) were calculated by multiplying total AA concentrations (mg L^{-1}) in 24 h composite raw wastewater samples by daily wastewater flows (L day^{-1}) using the following equation:

$$DL = C\ x\ V$$

where: *C* is the total concentration of *AAs* (mg L^{-1}) in untreated influent wastewater, and *V* is the volume of wastewater received by the WWTP per day (L day^{-1}).

Population (inhabitants; inh) normalised daily mass loads (*PNDLs*) of *AAs* (mg day^{-1} $1000inh^{-1}$) were calculated using the following equation:

$$PNDL = \frac{DL}{PE_{WW}}\ x\ 1000$$

where: *DL* is the daily mass load of *AAs* (mg day^{-1}) in influent wastewater, PE_{WW} is the water utility PE estimate.

Concentrations of individual antimicrobial agent (and their respective antimicrobial groups in influent, effluent, and sludge samples were statistically analysed using one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) post-hoc test. For each sample type, data were grouped by sampling site ($n = 10$) and sampling period ($n = 6$ bimonthly events over 12 months). ANOVA assessed whether antimicrobial levels differed significantly across sampling sites or sampling periods within each sample type. A significant ANOVA result ($p < 0.05$) indicates variability among groups, prompting Tukey's HSD to identify pairwise differences between specific sites and sampling periods. For all sample types, spatial (site-to-site) and temporal (period-to-period) variations were tested due to distinct contamination dynamics, especially for the aqueous samples. In all cases, analytes whose concentration were detected below the limit of detection were assigned a value equivalent to half of their respective LODs prior to undertaking the statistical test. All analyses were conducted using R version 4.3.1 (2023-06-16) with *stats* package.

3. Results and discussion

3.1. Occurrence of AAs in WwTWs: insights into geographical and seasonally driven AA fingerprints

Fifty-five AAs grouped into thirteen different classes were quantified in wastewater samples from ten WwTWs in England and Wales (Fig. 1) in six sampling campaigns over 12 months (giving 79,555 data points in 339 (204 wastewater and 135 sludge) samples). Sulfapyridine and its main metabolite sulfapyridine N-acetyl, trimethoprim, and metronidazole were detected in tested influent and effluent samples with 100% frequency. Similarly, sulfamethoxazole, erythromycin and its main metabolite erythromycin desmethyl, were detected in all the effluent samples collected from each of the sampling sites. Clindamycin, clarithromycin, clarithromycin desmethyl, ofloxacin, vancomycin, emtricitabine, isoniazid, metronidazole hydroxyl-, and sulfadiazine N-acetyl were also consistently detected in at least six of the ten sampling sites in both influent and effluent samples.

As seen in Fig. S1, the most prevalent AA groups are the lincosamides, quinolones, tuberculosis drugs, sulfonamides, and macrolides contributing as much as 63% (Saltford), 60% (Seaton Carew), 22% (Driffield), 17% (Pen-y-bont) and 13% (Pembury), respectively. Cyclines, β-lactams and antiretrovirals generally contributed as much as 10% (Stockport), 8% (Pen-y-bont), and 3% (Stockport) respectively to the total AA concentrations. The contributions of other AA groups were generally below 1% throughout the sampling campaigns. Overall, lincosamides, quinolones and sulfonamides represent the dominant AAs groups in the influent samples contributing a minimum of 18%, 10% and 5%, respectively to the total AA concentrations at each of the sampling sites.

For the effluent samples, a somewhat similar pattern was observed except that relatively high contributions of sulfonamides, quinolones and azoles were seen. In this case, the predominance pattern of the AA groups is as follows: sulfonamides > quinolones > macrolides > β-lactams > lincosamides > cyclines > azoles where their contributions to the total AA concentrations were 15-29%, 11-29%, 9-22%, 8-19%, 3-17%, 4-16%, and 2-12%, respectively. The observed patterns in this study are consistent with the findings from similar studies where the predominance of these AA groups has been reported (Holton et al., 2023; Sims, Kannan, et al., 2023, 2023; Xu et al., 2024).

The spatial profiles of the different AAs concentrations in influent samples, as summarised in Fig. S1, provide some insights on their usage patterns in the respective catchment areas. The observed spatial patterns in the concentrations of the targeted AAs in the influent samples significantly differ ($p = 0.0493$) from one sampling campaign to the other possibly reflecting AA usage preferences throughout the year. The total concentrations of the AA groups at these sites are generally below 200,000 ng/L except for Saltford and Seaton Carew where their measured total concentrations exceed this threshold during the May and July 2021 sampling campaigns, respectively. The elevated levels of the AAs at these sampling sites are largely driven by unusually high levels of lincosamides (up to 1,707 ng/L for CDMY at Saltford) and quinolones (as high as 425 ng/L for ULI at Saltford). Interestingly, their levels were significantly different from those observed at the same sampling sites during the other sampling campaigns. Apart from these two sampling sites, the highest total AA concentrations in influent samples are mostly observed at Pembury where different dominant AA groups contribute to the observed trends and patterns.

For effluent samples, the spatial patterns and temporal trends of the antimicrobial groups in terms of their measured mean and median concentrations showed some degrees of consistency across the sampling sites. The measured total AA concentrations for effluent samples at each

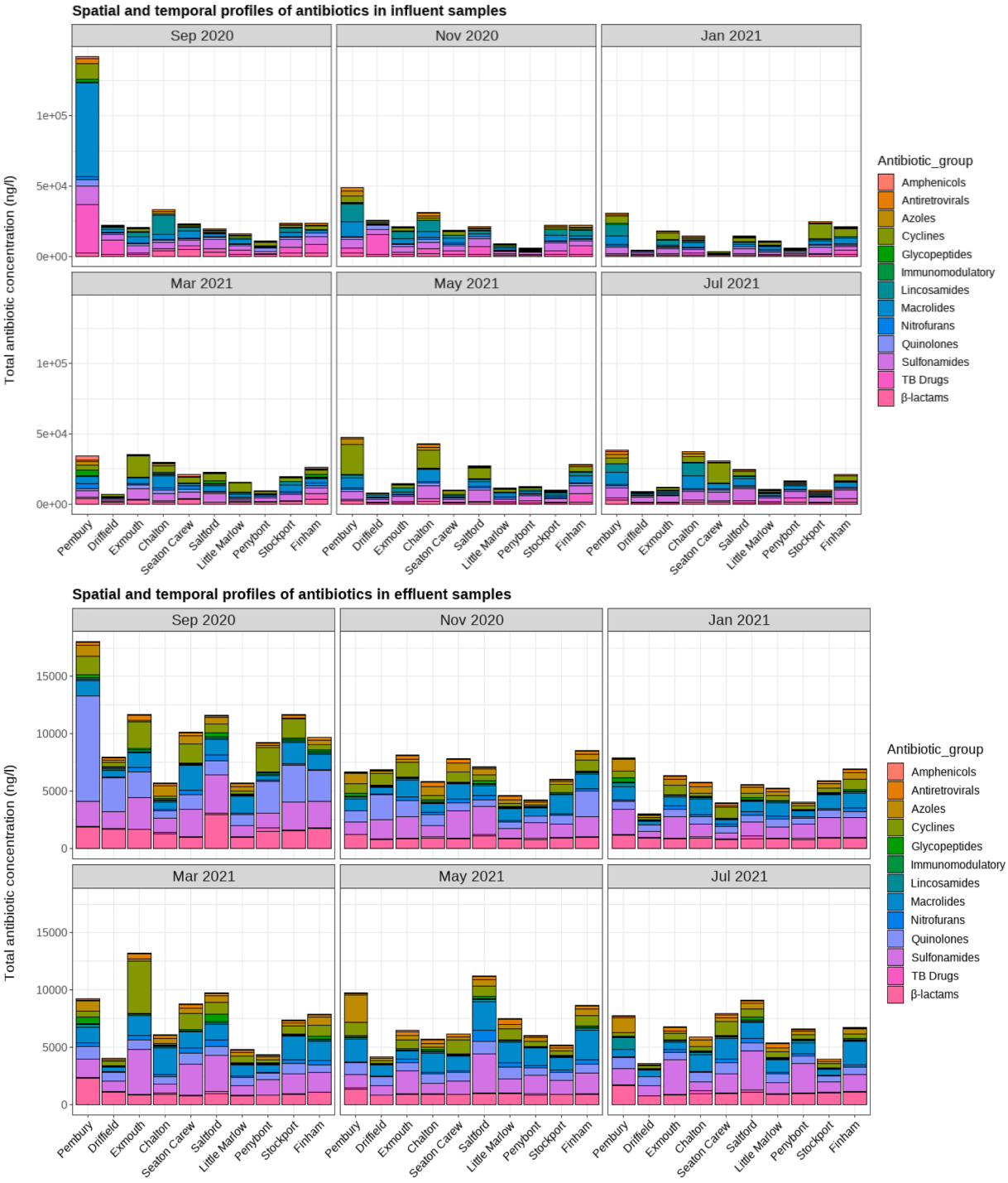

Fig. 2. Spatial and temporal profiles of AA concentrations in influent, effluent wastewater in 6 sampling campaigns across 10 WWTPs in England and Wales.

site are generally below 10,000 ng/L except for some sampling sites where this threshold was exceeded during the September 2020, March, and May 2021 sampling campaigns. As noted for Exmouth during the May 2021 sampling campaign, unusually high levels of the targeted AAs in treated effluent can signify temporal wastewater treatment inefficiency and might indicate system performance issues. The ranges of mean (and median) effluent concentrations of amphenicols, antiretrovirals, azoles, cyclines, glycopeptides, immunomodulatory, lincosamides, macrolides, nitrofurans, quinolones, sulfonamides, TB drugs and β-lactams were as follows: 13.5 −23.9 (6.1-25.7) ng/L, 60.7 – 175.5 (63.3 −141.2) ng/L, 75.3 −622.5 (78.6 – 571.4) ng/L, 85.4 – 571.1 (64.4 – 447.5) ng/L, 41.5 – 322.6 (37.0 – 272.4) ng/L, 61.8 – 102.1 (61.5 – 95.0) ng/L, 76.5 – 1000.2 (79.1 −325.5) ng/L, 114.9 – 341.7 (91.6 – 216.6) ng/L, 3.81 – 13.1 (2.9 – 11.4) ng/L, 66.8 – 177.9 (52.5 – 93.5) ng/L, 117.9 – 351.8 (49.2 – 254.9) ng/L, 11.5 – 53.2 (9.5 – 24.0) ng/L, 84.9 – 158.8 (52.6 – 74.5) ng/L, respectively. Despite the observed patterns in the levels of the targeted AAs in effluents samples, some notable spatial differences among the antimicrobial groups were observed. For instance, the highest concentrations of 6 out of the 13 antimicrobial groups: 7,893.9 ng/L (lincosamides), 6,474.5 ng/L (quinolones), 4,017.1 ng/L (cyclines), 361.3 ng/L (antiretrovirals), 155.0 ng/L (immunomodulatory), and 35.5 ng/L (nitrofurans) were detected at Exmouth while the highest concentrations for the other antimicrobial groups were detected at four other wastewater treatment works with concentrations as high as 1,862.0 ng/L (β-lactams), 1,753.5 ng/L (sulfonamides), 1,460.1 ng/L (macrolides), 268.1 ng/L (TB drugs), 692.4 ng/L (glycopeptides), 1,624.5 ng/L (azoles), 84.5 ng/L (amphenicols) for Saltford, Seaton Carew, Pen-y-bont, Saltford, Pembury and Seaton Carew, respectively.

Temporal profiles of the antibiotic groups in raw and treated wastewater are captured in Fig. 2 for wastewater influent and effluent respectively. The observed temporal differences in concentrations in each AA group for the influent samples were highest in macrolides followed by lincosamides, quinolones, TB Drugs, cyclines and sulfonamides. Both glycopeptides and nitrofurans had moderate temporal concentration differences. The AA groups with the lowest temporal variability include immunomodulatory, β-lactams, amphenicols, antiretrovirals and azoles. This is likely due to their broad spectrum efficacy against bacterial infections making them a preferred choice for prescribers all year long.

3.2. Occurrence and spatiotemporal variability of AAs in sludge

Forty out of the original 55 AAs that were detected in the aqueous wastewater samples were frequently detected in the primary, secondary, and thickened or treated sludge samples that were analysed in this study. Similar detection of fewer AAs in sludge samples has been attributed to their medium to high polarities, thus making them to exhibit higher partitioning in the aqueous phase (Bijlsma et al., 2021; Östman et al., 2017).

In primary sludge samples, SPY was the most detected AA having a detection frequency of 100% at 9 out of 10 sampling sites investigated in this study (Fig. S2). This is followed by SDZ, CLR, CLRDES, and TRM with each having 100% detection frequency at 8 out of 10 sampling sites. Despite their relatively low detection frequencies, METOH (3,869.1 ng/g at Pembury), CDMY (3,316.5 ng/g at Pembury), CDMY (3,106.9 ng/g at Stockport) and PENG (2,554.3 ng/g at Finham) were the AAs with the highest concentrations. AAs in the secondary sludge samples were dominated by SPY, TRM and CLRDES with each AA having 100% detection frequency at 9 out of 10 sampling sites. These were followed by ERY and CLR with each having 100% detection frequency at 8 and 7 out of 10 sampling sites, respectively. AAs with the highest concentrations in the secondary sludge include: ISNA (4,418.5 ng/g at Saltford), TRM (2,947.3 ng/g at Saltford), CDMY (2,820.9 ng/g at Pembury) and CIP (2,728.6 ng/g at Pembury).

Unlike their occurrence in the primary and secondary sludge samples, most of AAs were less frequently detected in the treated sludge samples. When detected, AAs were mostly found at lower concentrations. CLR was the most dominant AA in the treated sludge sample having 100% detection frequency at 3 out of 10 sampling sites investigated. ERYDES, CDMY, ClTET and ISNA had the highest concentrations in the treated sludge sample with concentrations: 3,573.7 (Finham), 1,283.7 (Chalton), 1,212.7 (Pen-y-bont) and 1,050.5 ng/g (Chalton), respectively. In terms of their overall detection frequencies, approximately 48, 49 and 38% of the targeted AAs had detection frequencies of at least 75% in the primary, secondary and treated sludge samples, respectively. The AAs featuring in this group are mainly dominated by AAs representing the macrolides (CLR, CLRDES, ERY, ERYDES, ROX), sulfonamides (SPY, SPYNA, SDZ, SDZNA, SMX, TRM), antiretrovirals (EMT), quinolones (OFX, FLUM), TB drugs (LZD, ISNA) and β-lactams (PENG, PENV). AAs with detection frequencies $< 50\%$ were: the glycopeptides (VAN), amphenicols (FLOR, CHLAMP) and those of lincosamides (CDMY and CDMYDES) feature more prominently in this category. Despite the elevated concentrations of the lincosamides, their detection frequencies were mostly found below 50% in all the sludge types investigated.

The patterns exhibited by AAs representing the dominant AA groups (macrolides, sulfonamides, quinolones and lincosamides) in the different sludge types are somewhat consistent throughout the sampling campaigns. For instance, among the quinolones, CIP had the highest concentration in the primary sludge (1,811.0 ng/g with a median value of 881.1 ng/g (Pembury)), secondary sludge (2,728.6 ng/g with a median value of 474.2 ng/g (Pembury)), and treated sludge (984.3 ng/g with a median value of 18.7 ng/g (Pen-y-Bont)).

The spatiotemporal profiles of these AAs representing their specific chemical groupings are presented in Fig. 3. Although each of the sludge types investigated in this study had varying compositional profiles across the sampling campaigns, the measured levels of each of the targeted AA groups did not vary significantly except at Chalton, Saltford, and Stockport. At these three sites, the levels of the AA groups in the primary sludge were found to be statistically different ($p < 0.05$) from their corresponding secondary sludge (Chalton and Saltford) and thickened or treated sludge samples (Stockport and Chalton).

In terms of the spatiotemporal patterns observed for the different AA groups in the primary sludge samples, not only were notable differences in the total concentrations of these AA groups observed, but there were also marked changes in their compositional profiles throughout the sampling campaign (Tab. 3). For instance, there was increased dominance of quinolones, sulfonamides and, in some cases, β-lactams during sampling campaigns that coincided with the colder months (September 2020 to January 2021). Incidentally, the observed pattern gradually shifted to an increasing dominance of the lincosamides and macrolides during the warmer months, which was particularly notable during the July 2021 sampling campaign. Such a gradual shift may have been driven by changes in antibiotic usage within the catchments of these sampling sites. It could also possibly demonstrate the influence of rising temperature/changes in the composition of the matrix on the adsorption of AAs during wastewater treatment processes, which might warrant further investigations.

3.3. Community AA usage: insights into spatiotemporal usage trends

3.3.1. Daily loads (DLs) of AAs in WWTPs

Flow normalised daily loads of AAs (DLs) are shown in Fig. 4. Normalisation to flows allows for seasonal AA usage to be explored without season driven flow variability. It needs to be noted that sampling took place during the 1st year of COVID pandemics and therefore AA DLs will likely be heavily impacted by COVID prevalence(Xu et al., 2024).

It is interesting to note that sites with highest DLs also have highest PE. We reported in our previous work that population size is the key driver of AA DLs(Elder et al., 2021a; Sims et al., 2023, 2023). This is clearly seen in Fig. 2 where WWTPs with the highest PE (Finham)

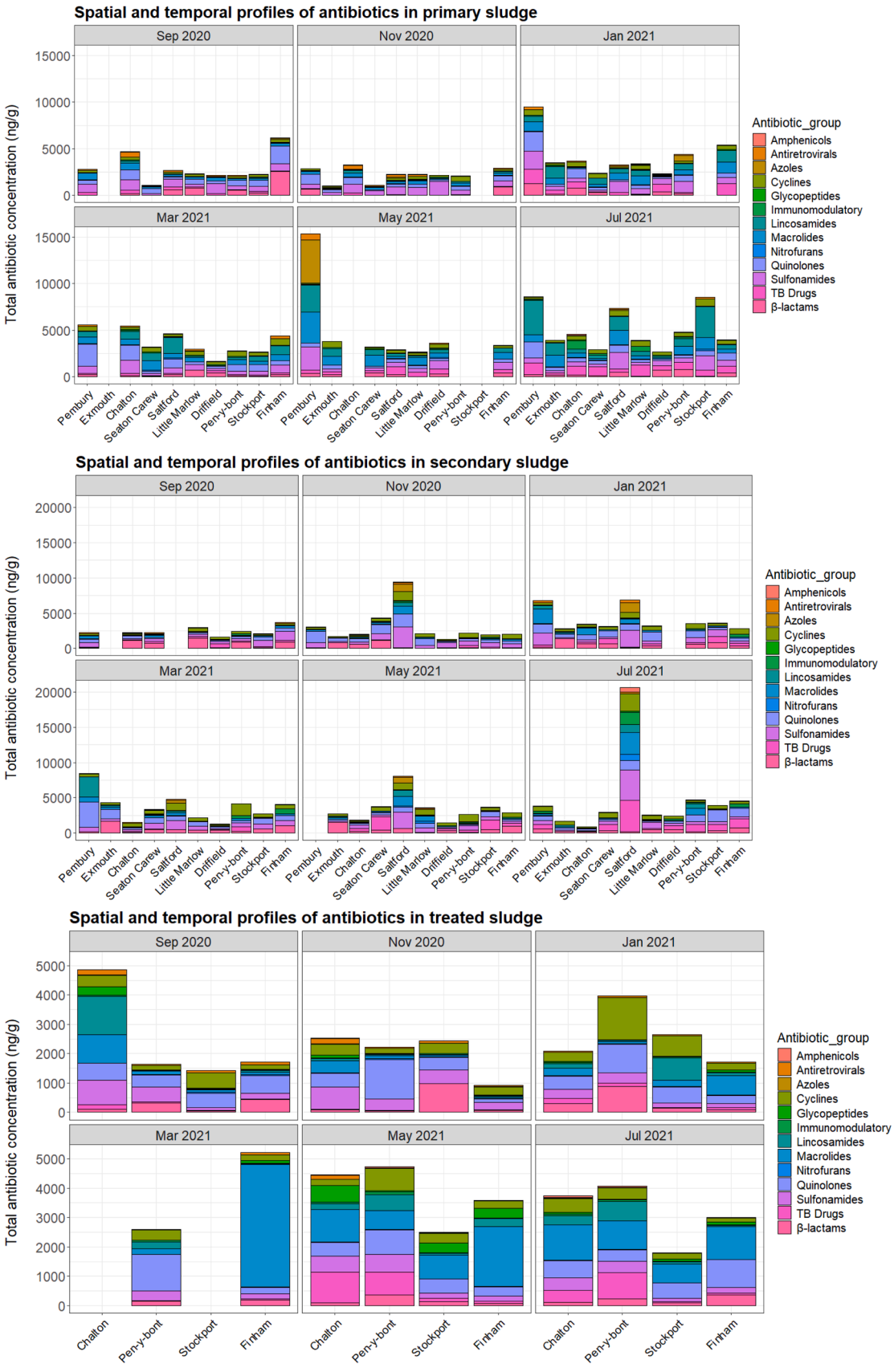

Fig. 3. Spatial and temporal profiles of AA concentrations in primary, secondary and treated sludge samples in 6 sampling campaigns across 10 WWTPs in England and Wales.

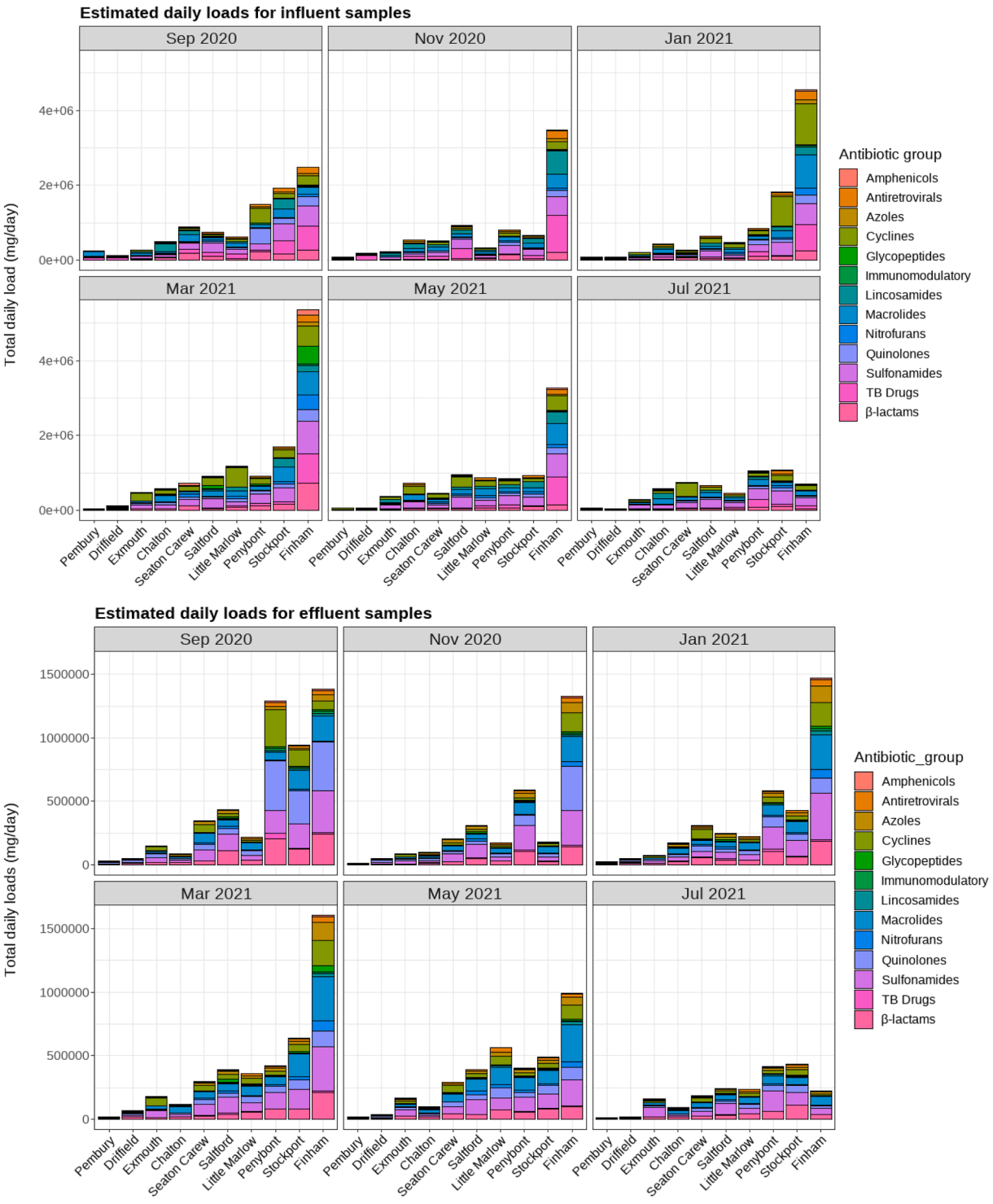

Fig. 4. Spatial and temporal profiles of AA (and their metabolites) daily loads in influent and effluent samples in 6 sampling campaigns across 10 WWTPs in England and Wales.

serving ~478k inh has on average highest AA loads. These generally range from 0.4 g/day for NOROH to as high as 647.4 g/day for IZD. Pembury WWTP with the lowest population of ~6k inh. has the lowest AA loads ranging from 0.004 g/day for NOROH to 20.3 g/day for ERY. Interestingly, however, this relationship does not fit strongly the linear regression model as in the case of other pharmaceuticals (Kasprzyk-Hordern et al., 2023; Kasprzyk-Hordern et al., 2022). This is due to seasonal disease prevalence driven usage of AAs. While pharma used in chronic conditions strongly correlates with population size (Kasprzyk-Hordern et al., 2023), this is not always apparent in the case of AAs.

As seen in Fig. 4 and Tab. S7, there are significant temporal and spatial variabilities in DL among the different AA groups possibly reflecting their overall usage and consumption patterns. These data suggest that there are no clear patterns exhibited by AAs representing each of the AA groups in terms of their collective variabilities. For instance, IZD with an estimated mean daily load of 108 g/day has the highest total daily load and variability while ETB (1.3 g/day) belonging

to the same AA group as IZD is among the AAs exhibiting the least variability. It is therefore pertinent to note that AAs belonging to the same therapeutic grouping may not necessarily exhibit similar daily load patterns, especially if their prescription dosage and specific prescription frequency differ at different stages of the treatment regimes. Other AAs demonstrating high variability include: OYTET, CDMY, SPYNA, CLR, ERY, TRM, SPY, CEFT, EMT, TET, and NIT having mean daily loads of 91, 70, 46, 46, 46, 42, 40, 37, 32, 28, and 21 g/day, respectively. Interestingly, AAs representing the quinolones (NOROH, LOM, ENF, GAT, OFX, MOX, NAD, OFXDES, DAN, SAR) amphenicols (SSANP, CHLAMP) and β-lactams (TAZ, AMP, AMX, AZM, PIP, PENG, PENV, MRP) are among the least variable AAs. The observed patterns suggest that AAs with the least variability in their daily loads are consistently prescribed all year round, and thus do not tend to exhibit any profound seasonal variations.

Considering the observed trends of the total daily loads for each of the AA groups, it is worth mentioning that majority of the AA groups peaked either during the January or March sampling campaigns except for lincosamides, quinolones, TB drugs, azoles and antiretrovirals. Despite these unique seasonal variabilities, all the targeted AA groups had a significant reduction in their total daily loads during the July sampling campaign which coincides with the summer season. These findings agree with those reported in similar studies where a considerable reduction in AA mass fluxes were observed during summer relative to other seasons of the year(Castiglioni et al., 2006b). For instance, (Coutu et al., 2013) reported an increase of up to 3 to 4 times of AA mass

Fig. 5. Spatial and temporal profiles of AA (and their metabolites) population normalised daily loads in influent and effluent samples in 6 sampling campaigns.

loads in influent samples of some Swiss WWTPs during winter than summer. In a typical WWTP in South Africa, (Holton, Archer, et al., 2022b)also observed higher AA mass loads in winter than were observed for summer attributing the disparities in the measured loads to weather-based seasonality that may have influenced the transport and degradation of AA within the catchment area.

In a review by(Harrower et al., 2021), it was noted that seasonal variations in AA loads were observed in different environmental matrices, including wastewater samples, where higher concentrations were reported during the winter season compared to summer possibly due to their enhanced stability during the colder season. The study by (Zhang et al., 2020) in China revealed that AA concentrations in hospital wastewater exhibited distinct seasonal patterns, with higher levels during summer, which further emphasises the impact of seasonal variability on AA loads in different regions. Overall, these cited studies demonstrate the consistent presence of seasonal variations in AA loads in wastewater treatment plants across different regions. These findings underscore the need for improved understanding and management of seasonal influences on AA concentrations to develop effective strategies for mitigating their environmental impacts.

3.3.2. Population normalised daily loads (PNDLs)

While DLs are very useful in understanding AA mass balance and the overall environmental burden, only double normalisation of AA concentrations to flows and population size allows for understanding of spatial variabilities in antibiotic usage. Fig. 5 (and Tab. S8) shows PNDLs for AA groups. It is clearly seen that there are spatial and temporal variabilities in per capita AA usage in different WWTP catchments. Except for Pembury where the highest PNDL for macrolides (ERY – 17,318.4 mg/day/1000inh) and TB drugs (IZD – 10,075.6 mg/day/1000inh) were found, the highest PNDLs estimated for all the other AA groups were found at Chalton. AAs with the highest PNDLs in this particular WWTP representing each of the AA groups include: CDMY (17,503.1 mg/day/1000inh), OXYTET (16,934.2 mg/day/1000inh), SPYNA (4,550.1 mg/day/1000inh), EMT (3,264.1 mg/day/1000inh), CFLX (2,107.9 mg/day/1000inh), MET (2,067 mg/day/1000inh), NIT (1,798.7 mg/day/1000inh), VAN (1,539.5 mg/day/1000inh), CIP (1,231.1 mg/day/1000inh), THA (481.8 mg/day/1000inh) and FLOR (441.9 mg/day/1000inh). On the other hand, Finham, despite its relatively high influent flow and population equivalents, is among the WWTPs with the lowest per capita mass fluxes of AAs with its PNDLs ranging from 0.1 (FLUM) to 2,063 mg/day/1000inh (IZD).

Comparing our findings with some pre-COVID pandemic studies shows higher per capita usage of the AAs included in this study. For instance, the PNDLs obtained for sulfonamides in this study are higher than those reported in a Finnish study where their PNDLs were found to range from 8 to 305 mg/day/1000inh(Kortesmäki et al., 2020). However, they are in line with our pre-pandemic work (albeit often higher at some sites/sampling campaigns)(Sims et al., 2023). Similarly, the PNDLs estimates for quinolones represented by CIP in an Iranian study, which were found to range from 55.3 to 151.3 mg/day/1000inh for influent and 7.4 to 63.2 mg/day/1000inh for effluent (Mirzaei et al., 2019) were significantly lower than those obtained in this study: 9.8 to as much as 1,231 mg/day/1000inh in influent while its estimates in effluent ranged from 3.7 to 267.1 mg/day/1000inh. These levels are closer to PNDLs recorded in our pre-pandemic study where ciprofloxacin regularly exceeded 100mg/day/1000inh in influent wastewater(Sims et al., 2023). These findings further highlight the fact that the onset of COVID-19 might have resulted in increased usage of AAs at different sampling locations covered in this study.

No statistically significant differences ($p > 0.05$) were found in the mass fluxes of AAs exiting the WWTPs during the entire sampling campaign at Chalton, Exmouth, Saltford and Seaton Carew possibly reflecting consistent operational efficiency of these treatment plants. For the other WWTPs, statistically significant differences were usually observed between PNDL estimates for warmer months (May and July 2021) and colder months (September, November 2020 and January 2021).

3.4. AA removal during WwTW treatment: impacts of geography and seasonality

3.4.1. Removal of AAs in the WwTWs: focus on different groups of AAs

Fig. 6 (and Tab. S9) shows the overall removal patterns of the targeted AAs and their metabolites computed as average removal rates, which were estimated using their respective daily loads in the influent and effluent samples. Each boxplot in the Fig. represents data for all the ten WwTWs investigated in this study. Therefore, the differences in the operational design, treatment capacities and processes of each of the WwTWs should be taken into consideration when interpreting the observed removal patterns estimated for each of the AAs.

For instance, the lincosamides represented by clindamycin (CDMY) are positively removed during the treatment process across all the investigated WwTWs with removal rates ranging from 59.9% (Pembury) to as high as 94.3% (Exmouth). For clindamycin, higher removal rates exceeding 75% were observed at 7 out of the 10 sampling sites examined in this study. This pattern was significantly different from what was generally observed for macrolides in this study.

For macrolides, the removal pattern for each of the AA and their respective metabolites representing this antimicrobial group was quite different. For instance, erythromycin (ERY) and its metabolite (ERYDES) with average removal rates ranging from 23.1 (Seaton Carew) to 87.2% (Pembury) and −41.0 (Driffield) to 87.3% (Pembury), respectively, were better removed during the treatment process than the other macrolides. In this AA group, only ERY, ERYDES and CLRDES had average removal rates exceeding 75% at two of the ten sampling sites. Their respective removal rates at these sites are 87.2 ± 14.5 (Pembury), 87.3 ± 8.0 (Pembury), 87.2 ± 14.5 (Pembury), 79.6 ± 19.7 (Chalton), and 76.6 ± 10.6 (Pembury) for ERY, ERYDES, ERYDES, and CLRDES, respectively. Incidentally, CLR and ROX having overall removal rates ranging from −4.1 (Seaton Carew) to 74.0% (Chalton) and −9.0 (Driffield) to 66.1% (Pembury), respectively, were not removed at a rate exceeding 75% at any of the WWTPs investigated in this study. Given their relatively high prescription rates in England and their extremely poor removal rates across most of the sampling sites in this study, it is pertinent to suggest that incessant releases of these AAs into environment via effluent discharges may promote selection pressure within the bacterial communities in the receiving waterbodies.

Quinolones with 14 AAs consisting of 11 parent compounds and 3 active metabolites are the most represented antimicrobial groups in this study. Their removal efficiencies were remarkably different from the rest of the antimicrobial groups with most of the targeted AAs having negative removal rates. Only 29% of the targeted AAs (CIP, FLUM, NOROH, OFX and ULI) had a mean removal rate exceeding 0% with majority of these having an average removal rate of <50% except for NOROH. Among the quinolones, only 2 AAs (CIP and OFX) had an average removal rate exceeding 75% only at Chalton. These observed trends largely suggest that majority of the wastewater treatment works evaluated in this study do lack the capacity to efficiently remove these antimicrobials during the treatment process.

For sulfonamides, all the targeted AAs in this group except for trimethoprim hydroxy- (a metabolite of TRM) were positively removed having average removal rates ranging from −301.9 to 97.5%. Among the targeted sulfonamides, only SMX, SMXNA, SDZ, SDZNA, SPYNA, and TRM had removal rates exceeding 75% at 1, 7, 3, 1, 3 and 1, respectively out of the 10 sampling sites investigated in this study. Despite the positive removal outlook observed for this antimicrobial group, these findings further suggest that majority of the WwTWs investigated in this study are still incapable of removing these AAs. For this AA group, there is a clear distinction between the removal rates of the parent AAs and their respective metabolites. Similar to the removal patterns observed for the nitrofurans (NIT and NPAHD), higher removal rates were

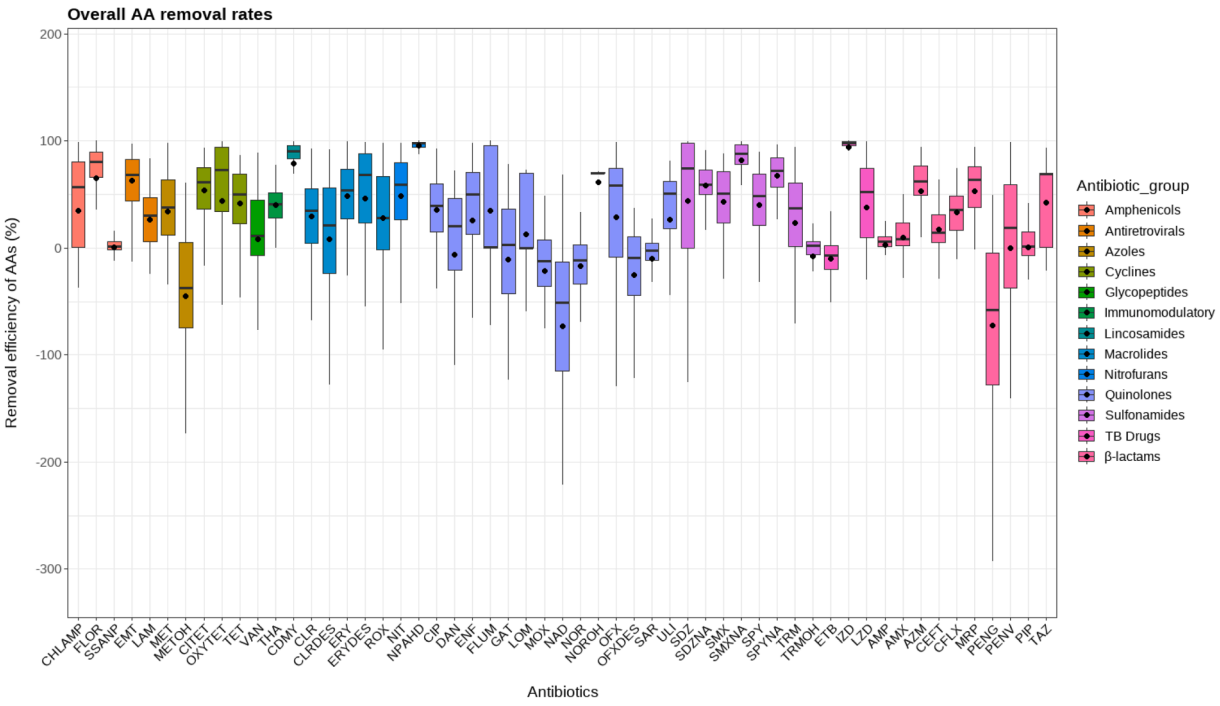

Fig. 6. Percentage removal of AAs and their metabolites: focus on different groups of AAs.

generally observed for the metabolites than their respective parent compounds except for TRM whose metabolite was mostly negatively removed. For instance, SMX whose highest average removal rate was 75% (range: −5.7 (Driffield) to 75.4% (Finham)) was poorly removed compared to its main metabolite (SMXNA) where up to 97% (range: 60.2 (Saltford) to 97.0% (Stockport)) removal rates were observed. Similar patterns of removal rates were observed for the other sulfonamides where their metabolites had correspondingly high removal rates than their respective parent compounds. For these parent-metabolite pairs as with other sulfonamides, no statistically significant difference ($p > 0.05$) was observed in their removal rates except for SPY/SMXNA and TRMOH/SMXNA with p-values of 0.021 and 0.013, respectively.

Similar to the patterns observed for the quinolones, the β-lactams investigated in this study had high variations in their removal rates with negative rates notably observed for PenG and PenV. Interestingly, none of the AAs representing this group was removed at a rate exceeding 75%. Except for AZM and MRP, a vast majority of the β-lactams were generally removed at an average removal rate of <40%, thus suggesting that WwTWs investigated in this study are grossly inefficient in removing these antimicrobials. Within this group, statistically significant differences in the removal rates of PenV and other β-lactams, including AZM ($p = 0.0016$), CEFT ($p = 0.0328$), CFLX ($p = 0.0090$), MRP ($p = 0.0016$), and TAZ ($p = 0.0072$) were observed. Similar trends were also noted for the removal rates of PenG and AZM ($p = 0.0211$) as well as PenG and MRP ($p = 0.0214$).

The removal rates for the 3 AAs representing the TB drugs are highly variable ranging from −32.5 to 3.16%, 73.6 to 99.5% and −98.7 to 83.9% for ETB, IZD and LZD, respectively. Among the AAs in this group, IZD was efficiently removed with removal rates exceeding 75% at 9 out of the 10 WwTWs investigated in this study while LZD was removed at a similar rate, but at only one of the ten sampling sites. This observed pattern clearly suggests that IZD could be efficiently removed regardless of the treatment process employed. In contrast, ETB which is also commonly prescribed for the treatment of tuberculosis was poorly removed across the sampling sites. This difference in the treatment efficiency of WwTWs to remove contaminants belonging to the same chemical groupings highlights the need for enhanced complementary technologies to the existing treatment works and regular testing of wastewater from these facilities.

FLOR, EMT, MET and OXYTET representing amphenicols, antiretrovirals, azoles and cyclines, respectively, were the only AAs which had average removal rates of 75% or more at 4, 5, 1, and 3 out of the 10 sampling sites, respectively. Other AAs, including CHLAMP, SSANP, LAM, METOH, ClTET and TET belonging to these antimicrobial groups were poorly removed with removal rates ranging from −146.3 to 72.0%. Of all the thirteen AA groups investigated in this study, β-lactams, glycopeptides and immunomodulatory were the only groups which had <75% removal rate across all the sampling sites. For the two latter groups, which were only represented by vancomycin (VAN) and thalidomide (THA), their removal rates ranged from −33.8 – 51.8% and 27.6 – 50.3%, respectively. In contrast, NPAHD belonging to the nitrofurans and having removal rates ranging from 86.7 to 99.0% is the only AA group that had 75% or more removal rates across all the sampling sites.

3.4.2. Removal of AAs in the WwTWs: focus on different WwTW technologies used

To evaluate the treatment efficiencies of the different biological treatment processes employed by the wastewater treatment works investigated in this study, the removal rates of WwTWs employing trickling filter (TF), bio bead biological aerated flooded filter (BAFF) and other variants of the activated sludge (AS) treatment process were compared. For this comparison, Chalton was grouped separately as "AS_TF" because the AA daily loads in the final effluent used in the estimation of its removal rates results from a 2-in-1 treatment process where both trickling filter and activated sludge systems were employed. On the other hand, both Saltford and Pembury which employ stone trickling filtering system were grouped as "TF" while Exmouth which employs bio bead biological aerated flooded filters was grouped as "BAFF". Other WwTWs (Driffield, Finham, Little Marlow, Penybont, Seaton Carew and Stockport) were based on either the conventional activated sludge treatment process or on different variants of this treatment process.

As shown in Fig. 7, it is clear that the treatment efficiencies of all the treatment works investigated are highly variable. Except for a few AA groups, the trickling filter system as well as TF-AS system at Chalton

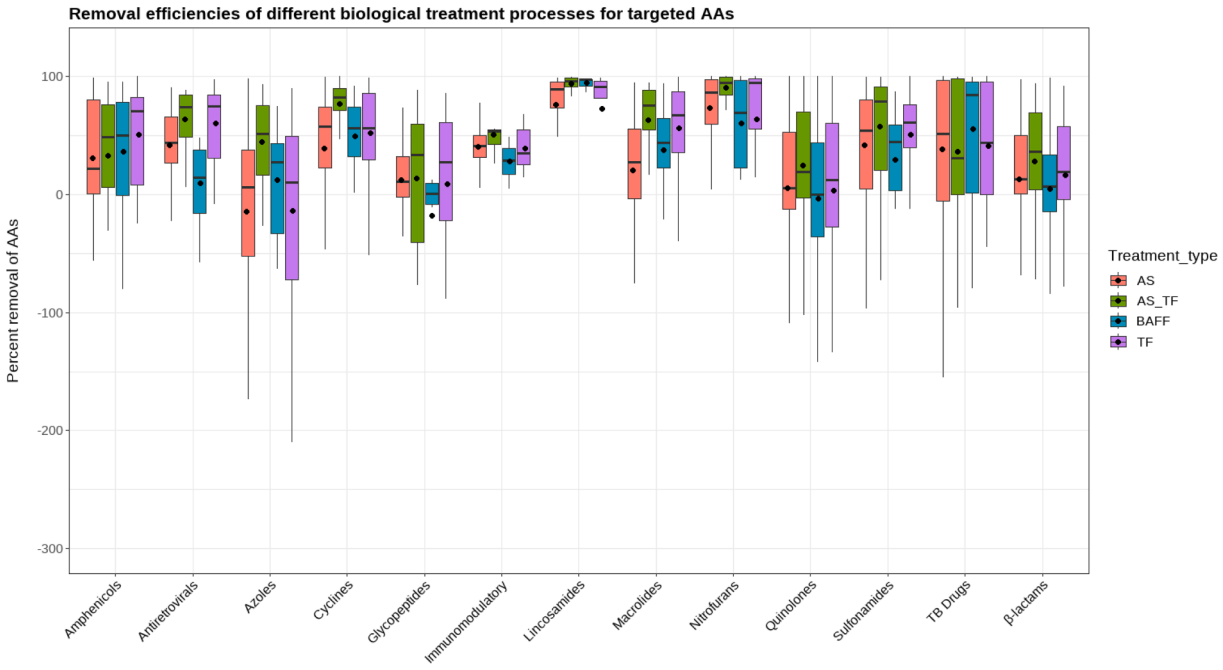

Fig. 7. Percentage removal of AAs and their metabolites: focus on different WwTW technologies used.

generally had relatively high mean and median removal rates than other treatment processes for most of the antimicrobial groups investigated. Furthermore, the comparison between the trickling filter and activated sludge systems, which are the two most common biological treatment processes employed in the UK suggests that the trickling filter systems had comparable or even higher removal rates than the activated sludge systems for most of the antimicrobial groups except for azoles.

To establish whether each of the sampling sites vary significantly in terms of their abilities to remove the targeted AA, the mean removal rates for all the targeted AAs were subjected to Tukey HSD statistical tests. The outcome of the statistical tests revealed that no significant difference exists among the sampling sites, except for Pembury and Chalton. These observations clearly suggest that despite the unique differences in the operational designs and processes of the investigated WwTws included in this study, there seems to be a similarity in their abilities to remove the targeted AAs. Nonetheless, the overriding influence of several factors, including the type and concentrations of AAs being examined, the presence of other co-contaminants such heavy metals and operational conditions such as temperature and pH variations need to be carefully considered when comparing the removal

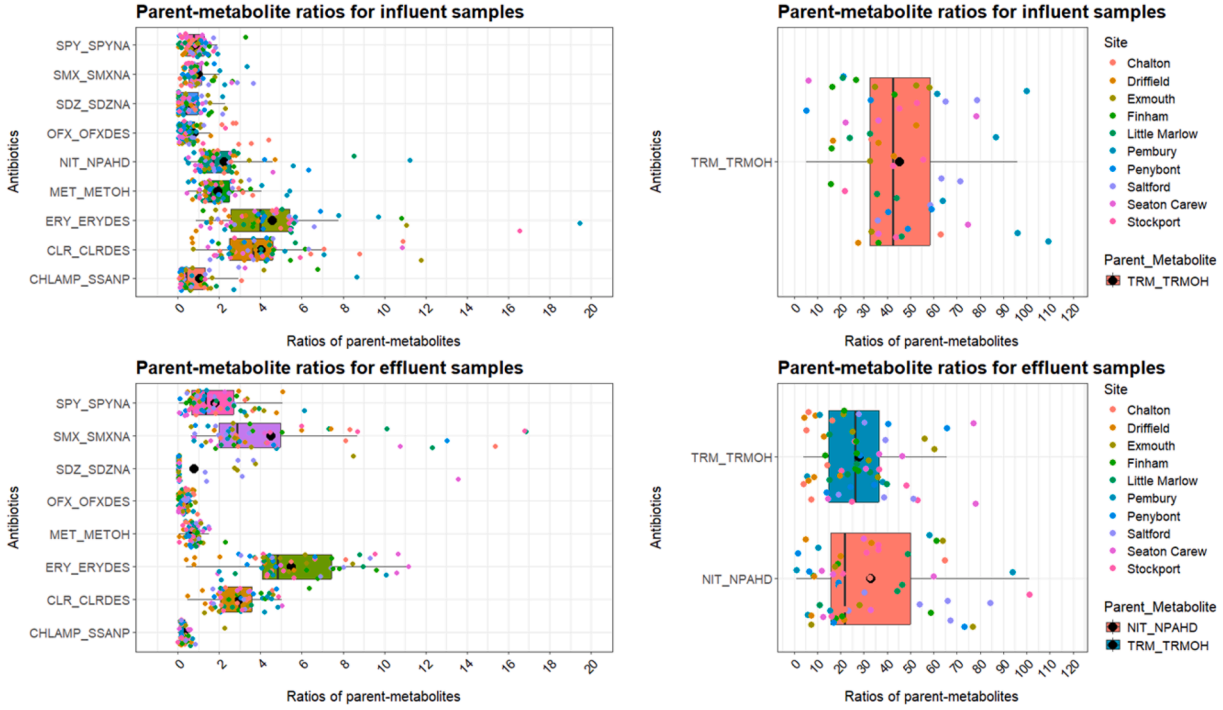

Fig. 8. Box and Whisker plots showing the distribution of the parent-metabolite ratios for selected AAs in influent and effluent sample.

efficiencies of different wastewater treatment works.

Fig. 8, 9 (and Tab. S10 and S11) show parent/metabolite (P/M) ratios for various antibiotic groups. They clearly indicate variable efficiency in parent compound vs metabolite removal with typically lower ratios observed in wastewater influent (e.g. SPY/SPYNA, SMX/SMXNA, ERY/ERYDES) vs effluent due to a relatively better removal of metabolites. Interestingly, and as expected, higher P/M ratios were observed in sludge samples due to the higher sorption potential by more non-polar parent compound vs their more hydrophilic metabolites (e.g. TRM/TRMOH). Further interrogation of data relevealed characteristic general P/M ratio decrease for effluent vs influent for macrolides (CLR_CLRDES, ERY_ERYDES), azoles (MET_METOH) and amphenicols (CHLAMP_SSCLAMP. In contrast, P/M was found to increase for solfonomides and nitrofuranes. Quinololes showed compound depended P/M change with P/M increasing for NOR-NOROH and decreasing for OFX_OFXDES. This shows that chemical structures and physicochemical properties of different AA groups influence their removal potential. It is also apparent that there is a complex interplay between AA removal, metabolites formation followed by their removal, as well as, likely, deconjugation of phase II metabolites leading to the release of parent AA and phase I metabolites. There are also variabilities observed in P/M ratios in TF when compared to AA treatment, but less significant when compared to AA groups P/M patterns.

4. Conclusions

This complex study targeting 55 AA and their metabolites at 10 WWTPs spatially distributed in England and Wales over 6 sampling campaigns in a one-year longitudinal study answered the following questions:

1. Does AA composition in wastewater influent/sludge change (a proxy for community usage) across different geographies and seasons?

There is significant temporal and spatial variability among the different AA groups reflecting their variable usage patterns that are geography and season driven. The majority of the AA groups (measured as DLs) peaked either during the January or March sampling campaigns (except for lincosamides, quinolones, TB drugs, azoles and antiretrovirals) due to their season driven usage. Despite these unique seasonal variabilities, all the targeted AA groups had a significant reduction in their total daily loads during the summer season.

2. What are the drivers of local/regional differences in AA usage?

Clear spatial and temporal variabilities in AA PNDLs were observed in different WWTP catchments across England and Wales. The key driver of this variability is seasonal usage. However, an impact of COVID pandemic should not be underestimated.

3. Does treatment process type (i.e., trickling filter, activated sludge) affect AA removals, and does one method outperform the other? Does season or geography affect removal efficiencies?

Treatment efficiencies of all the treatment works investigated are highly variable. The comparison between the trickling filter and activated sludge systems, which are the two most common biological treatment processes employed in the UK suggests that the trickling filter systems had comparable or even higher removal rates than the activated sludge systems for most of AAs except for azoles. Furthermore, due to high variabilities in AA removal, no significant difference was observed in the overall efficiency of AA removal among the tested sampling sites and the same technologies tested, which indicates high heterogeneity in AA removal and potentiallchallenges in wastewater treatment performance eoptiomisation for better AA removal. .

4. Which AA groups persist throughout the treatment process?

Removal of AAs was group and individual AA dependent and highly variable with lincosamides, some macrolides (ERY, ERYDES and CLRDES), nitrofurans having average removal >75% and β-lactams, glycopeptides having removal <75%, with some macrolides (e.g. CLR and ROX) and sulfonamides being highly variable. Quinolones had very low, no removal observed.

CRediT authorship contribution statement

Adegbenro P. Daso: Conceptualization, Data curation, Formal

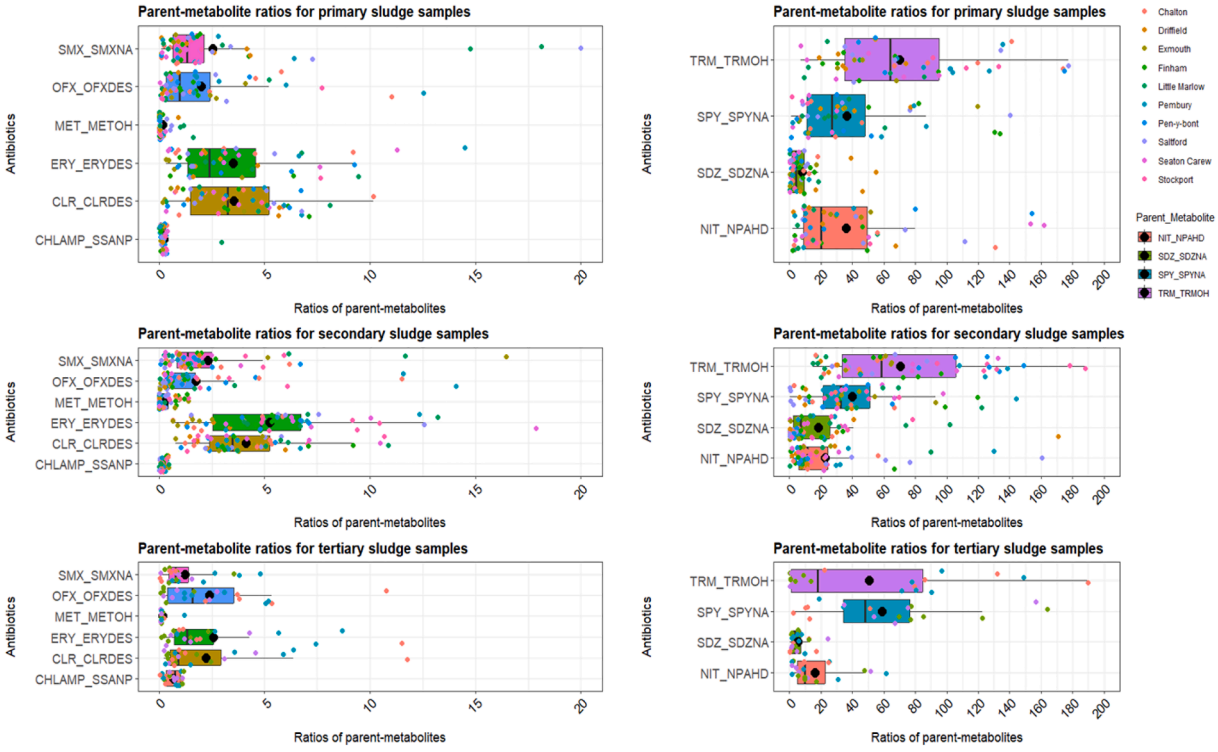

Fig. 9. Box and Whisker plots showing the distribution of the parent-metabolite ratios for selected AAs in primary, secondary and treated sludge samples.

analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Holly J. Tipper:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Daniel S. Read:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing. **Barbara Kasprzyk-Hordern:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, 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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.envadv.2026.100696.

Data availability

Data will be made available on request.

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