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Antimicrobial resistance in rural rivers: Comparative study of the Coquet (Northumberland) and Eden (Cumbria) River catchments

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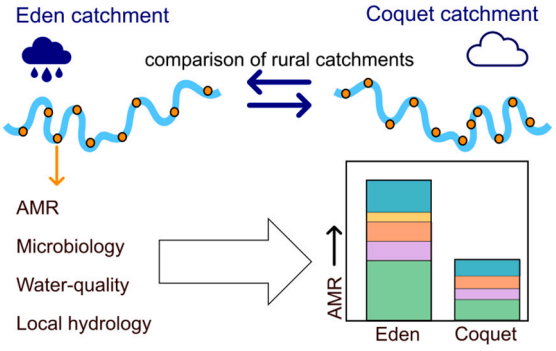
HIGHLIGHTS

- Resistomes and microbiomes were quantified in two rural UK river catchments.
- Differences in resistance were contrasted between the rivers vs local hydrology.
- Microbiomes were assessed through Quantitative Microbial Profiling.
- The wetter Eden River had higher levels of resistance than the drier Coquet River.
- Local hydrology impacts levels of resistance in rivers with diffuse pollution.

GRAPHICAL ABSTRACT



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ABSTRACT

Many studies have characterised resistomes in river microbial communities. However, few have compared resistomes in parallel rural catchments that have few point-source inputs of antimicrobial genes (ARGs) and organisms (i.e., AMR) – catchments where one can contrast more nebulous drivers of AMR in rural rivers. Here, we used quantitative microbial profiling (QMP) to compare resistomes and microbiomes in two rural river catchments in Northern England, the Coquet and Eden in Northumberland and Cumbria, respectively, with different hydrological and geographical conditions. The Eden has higher flow rates, higher annual surface runoff, and longer periods of soil saturation, whereas the Coquet is drier and has lower flowrates. QMP analysis showed the Eden contained significantly more abundant microbes associated with soil sources, animal faeces, and wastewater than the Coquet, which had microbiomes like less polluted rivers (Wilcoxon test, $p < 0.01$). The Eden also had greater ARG abundances and resistome diversity (Kruskal Wallis, $p < 0.05$), and higher levels of potentially clinically relevant ARGs. The Eden catchment had greater and flashier runoff and more extensive agricultural land use in its middle reach, which explains higher levels of AMR in the river. Hydrological and geographic factors drive AMR in rural rivers, which must be considered in environmental monitoring programmes.

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1. Introduction

Antimicrobial resistance (AMR) is recognised as a global health and societal issue. In 2019, it was estimated that 4.95 million deaths could be associated or directly attributed to AMR (Murray et al., 2022) and it has been conservatively projected that up to 10 million additional deaths per year might be expected by 2050 (O'Neil, 2014). As a response to growing concerns, the United Nations, led by the World Health Organisation, is developing an integrated surveillance programme for AMR and antimicrobial use, which was recently highlighted by the G20 Summit Leaders' Declaration aimed at "strengthening global health and implementing a one health approach" (G20 New Delhi Global Leaders (NDGL), 2023). However, AMR prevails across human, animal, and environmental sectors, and surveillance must be inclusive, even in places where the consequences of antimicrobial use are not as evident (UNEP, 2023).

AMR is intrinsic in the environment with antibiotic resistance genes (ARGs) that can encode antibiotic resistance being found in ~30,000 yr-old glacial sediments (Dcosta et al., 2011). However, extensive AMU in healthcare and agriculture has expanded resistomes across the biosphere through a combination of mutations and horizontal gene transfer (HGT) on mobile genetic elements (MGEs), microbial selection, and environmental pollution (Bengtsson-Palme et al., 2018). Human activity, such as agricultural use (Xiang et al., 2018; Neher et al., 2020; Burch et al., 2022) and wastewater discharges (Zhang et al., 2022), has increased resistance in the natural environment, changing the "intrinsic" resistome (i.e., "background" ARGs present in nature before human impact). The question is how to we characterise changes in the intrinsic resistome. Most studies focus on resistance "hot spots", but such locations explain little about changes in intrinsic AMR that require different types of places for study.

As background, hot spots include locations and environments contaminated by wastewater releases (Dhanji et al., 2011; Amos et al., 2014; Devarajan et al., 2015; Tacão et al., 2022), agriculture activity (Seiler and Berendonk, 2012), pharmaceuticals (Šimatović and Udiković-Kolić, 2020; Wilkinson et al., 2022) and heavy metals (Gupta et al., 2022; Zhang et al., 2023a). Further, most studies focus on urban catchments, with less data being available on AMR in rural landscapes, which are critical for baselining the extent of human influence in more impacted locations.

As implied, one way of assessing and explaining changes in intrinsic AMR is to study locations without extensive AMU or overt waste inputs, such as rural river catchments. Catchment studies can contrast subtle effects of different inputs and land uses, with rivers themselves acting as "biomarkers" for native AMR conditions (Vaz-Moreira et al., 2014). Within this context, the United Kingdom (UK) five-year National AMR Action Plan includes environmental AMR surveillance of rivers at the catchment scale to better understand what intrinsic resistance now looks like in the UK.

In broad terms, there is a lack of work that compares rural catchments with similar land-use to reveal underlying drivers of environmental drivers of in situ AMR. Such comparisons are difficult, often due to inconsistency in sampling methods and analytical techniques between different environmental AMR research studies (Hassoun-Kheir et al., 2021). However, comparisons of multiple river catchments with predominantly rural land-use, but different hydrometeorological characteristics, can be used to interpret the potential impact of local hydrology, catchment characteristics, and climate as AMR drivers. Differences in rainfall and runoff likely impact microbiomes and resistomes in receiving rivers, due to different carriage of ARGs and microbes from the land (Almakki et al., 2019). However, relationships between temporal distribution and intensity of precipitation and land use has not been reported in most studies (Hamilton et al., 2020).

In addition to characterising the distribution and abundance of ARGs in a catchment, analysis of microbial taxa is important in understanding land-water interactions. Analysis of next-generation sequencing (NGS) data using quantitative microbial profiling (QMP) has been shown to be an effective approach for such analysis, which overcomes weaknesses in traditional normalisation techniques for NGS data, such as rarefaction (McMurdie and Holmes, 2014). QMP also can improve quantitative abundance estimates of resident microbial communities (Ott et al., 2021b). This approach, first introduced by Vandeputte et al. (2017), rarefies NGS reads to the lowest sampling depth (i.e. sequencing depth divided by cell counts), instead of the traditional minimum read depth (Gloor et al., 2017) that can lead to data from samples being omitted (McMurdie and Holmes, 2014) and false discovery rates from subsequent statistical testing (Mandal et al., 2015; Weiss et al., 2017).

Here we compared hydrology, land use, microbiomes, and resistomes in two rural river catchments in the UK, using the Coquet and Eden Rivers as case studies (Fig. 1). Differences in microbial communities and ARGs between catchments then were used to identify drivers of AMR within rural catchments. Overall, we show the benefit of parallel case-studies in assessing environmental AMR and how they can help inform priorities in AMR surveillance plans.

2. Materials and methods

2.1. Site description and catchment sampling

The Coquet River catchment is in NE England (Northumberland) and spans from the Cheviot Hills to the seaside town of Amble (Fig. 1B). It has numerous small towns, including Shillmoor, Sharperton, Thropton, Rothbury, Warkworth and Amble. The total Coquet catchment area is 606 km^2 and the length of the river is 60 km. In contrast, the Eden River catchment is in NW England (Cumbria) and is larger than Coquet catchment, being 2324 km^2 in area (Fig. 1A). The Eden is split into six sub-catchments, of which the Upper Eden (670 km^2) and Lower Eden (461 km^2) were chosen for work here due to their similar size as the Coquet. In the Eden sub-catchments, there are several small towns, such as Kirkby Stephen, Appleby in Westmorland, and Temple Sowerby as well as the city of Carlisle near the Irish Sea. For practical purposes, these catchments were chosen due to their close geographic proximity and to compare catchments spanning the west-east precipitation gradient in the Northern UK (due to eastern tracking weather systems and orographic effects), which significantly impacts western vs. eastern hydrology.

The Coquet and Eden catchment areas were extracted using ArcMap (Attal, 2017; ESRI, 2018), and land-use was classified using Land Cover Map (LCM) 2015 (Rowland et al., 2017) (Fig. 1). Land-use types were grouped in 'urban', 'rural' and 'pristine' as shown in Table S1. The percentage of land-use within a 2 km buffer around each sampling site was calculated as per Amos et al. (2015) (Fig. 1C). Sample sites were selected to capture a variety of land-uses along the river, whilst also allowing sampling to be safe and logistically suitable. For example, most samples were collected at mid-stream from bridges.

Exact locations of sample sites are shown in Table S2. Twelve and ten sampling sites were chosen for the Coquet and Eden, respectively, where Site A was sampled farthest upstream, and Sites L and J were sampled farthest downstream. Sampling was always performed from upstream to downstream, over three separate days in each catchment on the specified dates in 2020 and 2021 (Table S3). Sampling had been planned to take place over a shorter time, but sampling trips needed to be rearranged due to numerous disruptive lockdowns during the COVID-19 pandemic.

When onsite, river water quality was assessed for temperature, dissolved oxygen (DO) and conductivity using an HQ40 portable multimeter (HACH) and pH using a 500 series portable pH meter (Jenway). River water samples were collected in triplicate, using a bucket cleaned with 70 % ethanol solution between uses and rinsed with the sample before collection. Five litres were collected in total, with three litres being used for DNA extraction and two litres used for all other analyses.

River volumetric flow rate was estimated in situ during two of the

three sampling trips for each catchment (September and October/November), where river velocity was estimated using the float method (Jowett, 1997; Michaud and Wierenga 2005). The cross-sectional area of the river at each site was calculated using the measured river width and depth, which was multiplied by the surface velocity. A correction factor of 0.85 was applied to surface velocity data (Michaud and Wierenga, 2005; Ott et al., 2021a). Flow rate in the March sampling trip was not performed due to including microbial plate colony culturing (Section 2.6), which required extra field- and lab-processing time.

2.2. DNA extraction and quantification of 16SrRNA, ARGs, MGEs and Microbial Source Tracking (MST) probes

For each site visit, three litres of river water (3 × 1.0 L composite samples) were filtered through 0.22 μm cellulose filter paper (Merck Millipore), before subsequent processing. DNA was extracted from microbial cells trapped on the filter paper using the FastDNA Spin kit for soil (MP Biomedicals, UK). Following extraction, samples were assessed for purity using a NanoDrop 1000 Spectrometer (Thermoscientific, UK) and DNA concentration was measured using the Qubit® dsDNA High Sensitivity (HS) Assay Kits (Invitrogen, UK). The extracted DNA was diluted to 5 ng/μL for quantitative polymerase chain reaction (qPCR) analysis to minimise inhibition.

Human and ruminant *Bacteroidetes* MST quantification (HuBac and RuBac respectively) was performed using primers and probes supplied by the Environment Agency (EA) (Porter and Great Britain. Environment Agency., 2008). For the MST probes, and 16S rRNA (total bacteria), Taqman qPCR reactions were conducted using SSoAdvanced™ Universal Probes Supermix (BioRad) (Table S4). Faecal coliforms (Table S4) were quantified using the qPCR SYBR green-based method assay. SYBR-green reactions were conducted using SSoAdvanced Universal SYBR® Green Supermix (Bio-Rad). Assays were completed in duplicate using the Bio-Rad CFX c1000 System (Bio-Rad), with a negative control.

2.3. Next-generation sequencing

Amplification of the 16S rRNA gene was confirmed through PCR and qPCR. The Illumina MiSeq platform at NU-OMICS, Northumbria University (UK) was used to sequence the hypervariable V4 region 515F-806R of the 16S rRNA gene with V2 500 cycle chemistry. Sample

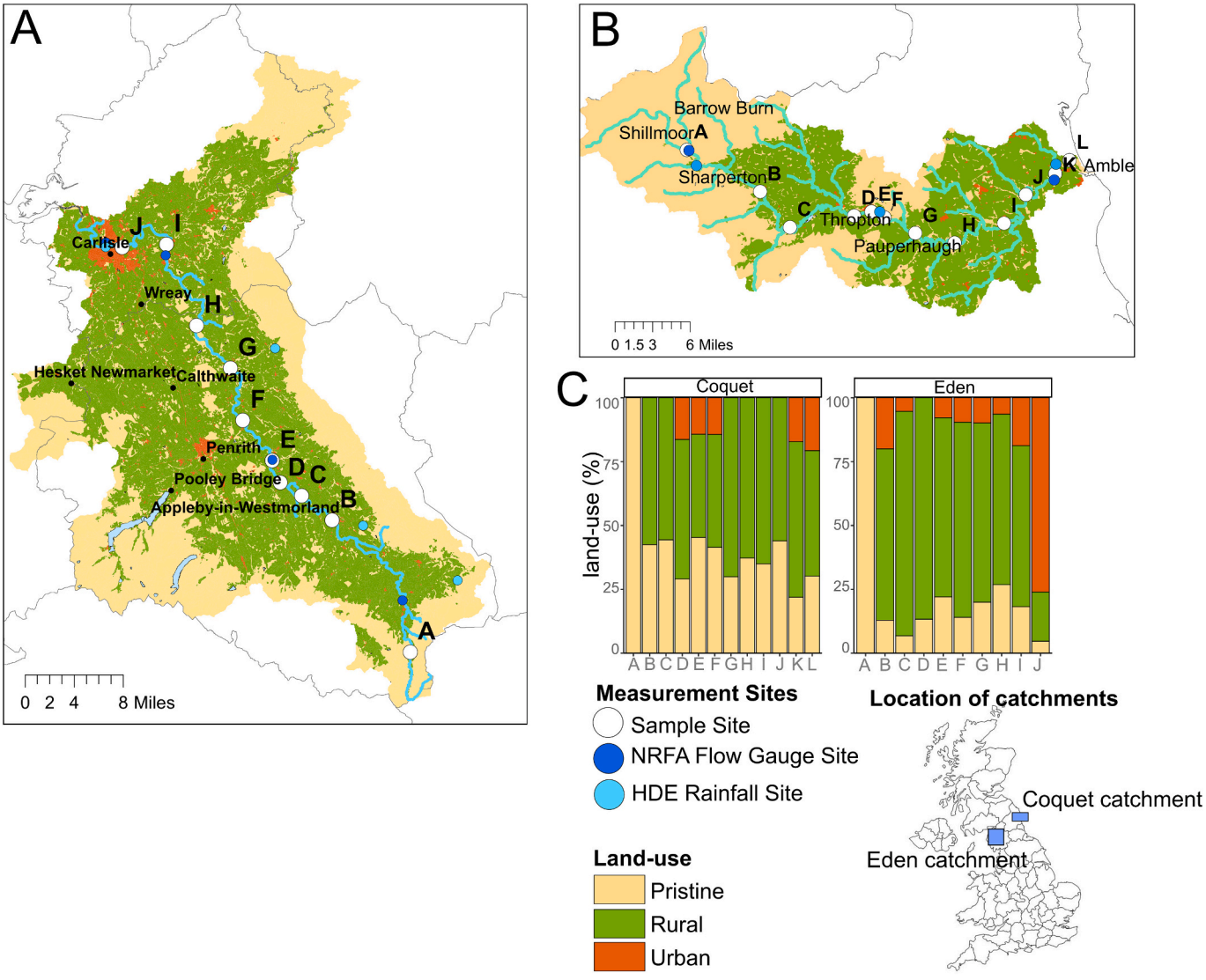


Fig. 1. A) the Eden catchment map with sample locations and land-use B) The Coquet catchment map with sample locations and land-use C) Percentage of Pristine, Rural and Urban land-use within a 2 km buffer in sampling locations. Measurement sites include sample sites for this study, National River Flow Archive (NRFA) Gauge Site (see Section 2.7) and DEFRA Hydrology Database Explorer (HDE) Rainfall measurement sites (see Section 2.7). Land-use classifications according to Land Cover Map 2015 (Rowland et al., 2017) were grouped into Pristine, Rural and Urban according to Table S1.

preparation and sequencing was conducted using the Schloss MiSeq Wet Lab SOP (Kozich et al., 2013), including a positive control (mock community, ZymoBIOMICS Microbial Community DNA Standard, Zymo Research) and negative control (H_2O).

Raw sequences were available as FASTQ files and were processed using QIIME2 (v. 2021.4) (Estaki et al., 2020). Reads were denoised into Amplicon Sequence Variants (ASVs) with DADA2 (Callahan et al., 2016). Then Naïve Bayes classifiers pre-trained on SILVA 138 99 % OTUs full-length sequences were used for taxonomic assigned to genus level. ASVs of <0.1 % of the mean sample depth were removed to account for MiSeq bleed (Comeau et al., 2017). The taxonomy and ASV table biom file were produced for downstream analysis in R Studio (v.4.2.2), with the *phyloseq* (v.1.42.0) and *vegan* (v.2.6-4) packages. ASVs not classified to phylum level, mean value <1 and maximum value <10 was removed. This resulted in a total of 2991 taxa for 66 samples (compared to 4197 taxa pre-quality filtering), with a minimum of 4280, median of 26,212 and maximum of 119,457 reads.

2.4. Quantitative Microbial Profiling and Hill Diversity Analysis

Quantitative Microbial Profiling (QMP) was used to rarefy ASVs as described previously (Vandeputte et al., 2017; Ott et al., 2021b), using R programming from http://www.raeslab.org/software/QMP (Vandeputte et al., 2017). Samples were rarefied to an equal sampling depth (i.e., sequencing depth divided by cell counts), using the R function *rarefy_even_sampling_depth* (seed 711). Following rarefaction, abundances were multiplied with an estimated cell concentration/mL of river water, which was calculated by dividing the 16S rRNA concentration/mL by 4.1, which is the estimated 16S rRNA gene copy numbers per bacterial cell (Klappenbach, 2001).

Hill numbers were used to measure the species diversity. These have been used more frequently in macroecology as an improved method of determining species diversity and address the problem of rare taxa in diversity influences (Boeken and Shachak, 2006; Chao et al., 2014; Alberdi and Gilbert, 2019a). The influence of rare and abundant taxa are assessed through changing the order of diversity (q), where at $q = 1$ relative abundances of ASVs are assessed according to their original values, at $q < 1$ rare ASVs are overweighed and at $q > 1$, highly abundant ASVs are overweighed (Alberdi and Gilbert, 2019b). Hill numbers for QMP abundances were calculated using the *hilldiv* R package (v.1.5.1) (Alberdi and Gilbert, 2019b) and diversity profiles were visualised using ggplot2 (v.3.4.2). As described in Ott et al. (2021b), the Sørenson-type over-lap dissimilarity measure at $q = 1$ was plotted on a Non-metric Multi-dimensional Scaling (NMDS) plot (Alberdi and Gilbert, 2019b), to visualise the proportion of nonshared ASVs between sample sites and between different sampling months.

2.5. Relative abundance of ARGs and MGEs using high-throughput qPCR

Resistomes were characterised through high-throughput qPCR (HT-qPCR), using the Resistomap Oy (Helsinki, Finland) SmartChip Real-time PCR system. DNA from 58 unique samples were analysed. Thirty-two representative samples were analysed for the full array of 384 ARG and MGEs offered by Resistomap (Table S6). Based on these analyses, 96 genes were selected for analysis of the remaining samples (Table S7). DNA samples were first diluted to 10 ng/μL based on concentrations measured with the NanoDrop 1000 Spectrometer (Thermoscientific, UK). DNA samples, qPCR reagents and primer sets were mixed in 100 nL reaction SmartChip™ wells, using the SmartChip™ Multisample Nanodispenser (TakaraBio). It should be noted that there were originally 66 collected samples, but eight had insufficient DNA for HT-qPCR analysis (Table S5), including all the samples from Coquet Site A.

Data processing and statistical analysis was performed through R programming. The threshold cycle (CT) of 28 was used as previously suggested (Stedtfeld et al., 2018). DNA were analysed in three qPCR reactions. Genes which were only present in one technical replicate (i.e., one out of three qPCR reactions), were excluded as false positives. The abundance of each gene was calculated as the relative abundance in proportion to the 16S rRNA gene as previously described (Muurinen et al., 2017).

ARGs and MGEs were transformed into absolute copy numbers by multiplying with 16S rRNA concentration for each sample.

2.6. Physiochemical analysis and microbial colony counts

Water samples were filtered through 0.22 μm polyethersulfone (PES) syringe filters within 24 h of collection and then analysed for soluble chemistry within 48 h. Assessment of water chemistry was conducted using HACH LANGE kits of Chemical Oxygen Demand (COD) (LCK 314: 15–150 mg/L), ammonium-nitrogen (NH_4-N) (LCK 304: 0.015–2 mg/L), orthophosphate as phosphorous (PO_4-P) (LCK 349: 0.05–1.5 mg/L) and Total Nitrogen (TN) (LCK 138: 1–16 mg/L) analysis.

Microbial plating and colony counts were conducted for the third sampling trip for both catchments (March 2021), focusing on non-resistant and resistant *Escherichia coli* (*E. coli)* isolation. River water was filtered onto 0.45 μm cellulose membrane filters (Sartorius™), which were on ChromoSelect agar for *E. coli* (Sigma-Aldrich), and incubated for 38 h at 37 °C. This process was repeated, except filters were placed on Chromoselect Agar with an added supplement for the detection of Extended Beta-Lactamase (ESBL) producing organisms (Sigma-Aldrich) (i.e., 1.5 mg of Ceftazidime and Cefotaxime, 1 mg of Ceftriazone and Aztreoname, and 5 mg of Fluconazole) to quantify *E. coli* resistance to ESBL antibiotics. The amount of water filtered was 2–20 mL for non-ESBL plates and 200–500 mL for ESBL plates. Each site was plated in triplicate.

2.7. Catchment hydrology and land use

Three river gauging sites within the Coquet catchment and four sites within the Eden were identified (Table S8) from the National River Flow Archive (NRFA; https://nrfa.ceh.ac.uk). For each site, the flowrate on each sampling day, various river flow indices, and catchment descriptors were obtained from the NRFA (Table 1). The flow indices were normalized by the upstream contributing area to allow comparisons between sites.

Daily rainfall data was downloaded for the seven gauging sites from the DEFRA Hydrology Data Explorer (https://environment.data.gov.uk/hydrology/explore) (Table S8). The Antecedent Precipitation Index over the previous five days (API_5) was calculated according to the UK Flood Estimation Handbook (FEH; CEH, 2023). This provides a measure of catchment wetness.

Although both catchments are predominantly rural or “pristine”, the

Table 1
Definitions of hydrological parameters (from NRFA, 2023).

Parameter	Definition
Catchment area (km^2)	Area of the catchment upstream contributing to the site
PROPWET	The fraction of the year that soils can be expected to be quite wet, where saturated soils are more likely to contribute to flooding
BFI	Baseflow Index: Proportion of the total flow that comes from groundwater
SAAR (mm)	Average annual rainfall in the standard period (1961–1990) in millimetres
Mean Flow (m^3/s)	Record mean-gauged flow at gauging stations
Mean annual runoff (mm/year)	Long-term mean annual flow as measured at the gauging station normalized by catchment area
95 % Exceedance (Q95) (mm/day)	Low flow parameter: flow which was equaled or exceeded 95 % of the flow record
5 % Exceedance (Q5) (mm/day)	High flow parameter: flow which equaled or exceeded for 5 % of the flow record
Flow rate on the day of sampling (m^3/s)	Gauged flow rate on the day of sampling (From NRFA)

Coquet has more pristine landscapes (48.5 %) compared with the Eden (24.8 %), especially in its headwaters (Sites A-B; see Fig. 1C), whereas the Eden has more urban land use (8.5 % vs 5.0 %). Rural landscapes, frequently used for animal grazing and other agricultural use, cover 66.7 % and 46.0 % of the Eden and Coquet landscapes, respectively, with the middle reach of the Eden (Sites C-G) being 71.0 % rural, predominated by agriculture.

2.8. Statistical analysis

Statistical analysis and data manipulation was performed in the R environment (R Core Team, 2018). Graphics were developed with *ggplot2* and finalised with Inkscape (v.09.4). The Kruskal-Wallis test was performed to analyse differences between the Coquet and Eden catchments and sampling months for MST probe, colony count, and ARG abundance data. The Wilcoxon test was used to find significantly different abundances of taxa at Phylum level between the Coquet and Eden catchments ($p < 0.01$). Otherwise, significance was defined as $p < 0.05$.

The $\log_2$fold change between ARG and MGE concentrations between the rivers Coquet and Eden was computed using the *DESeq2* package, which utilised the Wald test, and p value were adjusted according to the Benjamini Hocherg method (Benjamini and Hochberg, 1995). The $\log_2$fold change was then plotted against statistical significance with a volcano plot using *ggplot2*.

Quantile-Quantile plots (Q-Q plots) were used for the microbiome and resistome data to identify outlier samples. Two datapoints were excluded from the analysis which is discussed further in Sections 3.2 and 3.3. Co-occurrence analysis was also conducted with the ARG and MGE genes, and abundance of ASVs by Order level, to determine possible hosts for ARGs. This was conducted through an initial Spearman correlation, where significance values were adjusted according to Benjamini Hochberg (Benjamini and Hochberg, 1995). Strongly positive correlations ($r_s > 0.8$, $p < 0.01$) were further visualised using network analysis based on the *igraph* R package and *Gephi* software (Bastian et al., 2009). Spearman correlations, with significance values adjusted according to the Benjamini Hochberg method (Benjamini and Hochberg, 1995), were used to assess relationships between water quality, microbial, AMR, and hydrological indicators which were visualised using the *corrplot* (v. 0.92) package.

3. Results

3.1. Differences in hydrological conditions in the Coquet and Eden

Flow data and catchment descriptors, derived using the FEH methodology, were extracted from the National River Flow Archive to describe precipitation patterns and hydrology of both catchments (Table 2). As background, the Standard Average Annual Rainfall (SAAR) reflects altitude and the influence of the Atlantic Ocean, with the highest values in the Eden catchment, particularly in the Eamount at Udford and Kirkby Stephen sites.

High SAAR is associated with long periods of soil saturation (i.e., PROPWET), enhancing the generation of saturation excess surface runoff and higher annual runoff. The ratio of Q5 and Q95 is a measure of flashiness, with high values indicating a flashier flow regime. In each catchment, SAAR, mean annual runoff, and flashiness decrease with decreasing elevation towards the catchment outfall. BFI values, the ratio of baseflow to total streamflow, are similar in the two catchments, except for Kirkby Stephen. This may be due to the hydrological influence of the extensive peat soils, which enhance the flashiness of that subcatchment. The Eamont flow regime is affected by the Haweswater and Wet Sleddale Reservoirs and Ullswater, which may impact on low and high flow values (characterised by Q5 and Q95) and BFI.

The calculated API_5 for both catchments at different rainfall gauges on the day of sampling is summarised in Table S9, i.e., 1.52 ± 0.321 mm in the Coquet and 1.94 ± 1.26 mm in the Eden. Although the API_5 was similar for both catchments, there is higher variability in the Eden, primarily due to high API_5 during the October sampling trip (Table S9).

3.2. Coquet and Eden catchment microbial communities - quantification and diversity

Estimated bacterial cell concentrations in the Coquet (based on 16SrRNA data) varied from $6.37 \times 10^3 \pm 1.37 \times 10^3$ to $1.87 \times 10^5 \pm 7.97 \times 10^4$ cells/mL and in the Eden from $1.6 \times 10^3 \pm 7.48 \times 10^3$ to $8.79 \times 10^4 \pm 3.17 \times 10^4$ cells/mL (Table S10). The lowest cell abundances were recorded in the upstream sites (Site A) in both the Coquet and the Eden, whilst highest abundances were recorded at Site J in the Coquet downstream of Felton and Site J in the Eden within Carlisle city (see Fig. 1). These observations are consistent with greater areas of pristine land at upstream sites versus downstream sites in both rivers.

However, when broadly comparing the sampled sites in the catchments, there were no significant differences in bacterial cell count numbers between the Coquet and Eden based on 16S rRNA data (Kruskal Wallis test $p = 0.55$). There were also no significance differences in cell

Table 2
Catchment descriptors in the Eden and Coquet at relevant sites from the National Flow Archive (NRFA). Definitions of Hydrological Parameters are in Table 1.

		Eden (upstream-downstream)				Coquet (upstream-downstream)		
		Eden at Kirkby Stephen	Eden at Temple Sowerby	Eamount at Udford	Eden at Sheepmount	Usway Burn at Shillmoor	Coquet at Rothbury	Coquet at Morwick
Catchment Area (km^2)		69.4	616.4	396.2	2286.5	21.4	346	569.8
SAAR (mm)		1492	1142	1768	1182	1056	905	850
PROPWET		0.65	0.66	0.66	0.64	0.45	0.45	0.44
BFI		0.25	0.37	0.51	0.48	0.38	0.47	0.44
Mean Annual Runoff (mm/year)		1195	786	1258	752	824	534	486
Mean Flow (mm/day)		3.27	2.15	3.45	2.06	2.26	1.46	1.33
95 % Exceedance (Q95) (mm/day)		0.21	0.28	0.53	0.38	0.32	0.22	0.20
5 % (Exceedance (Q5) (mm/day)		13.07	7.68	10.60	6.45	7.91	4.45	4.52
Flashiness Q5:95 (−)		62.24	27.43	20.00	16.97	24.72	20.23	22.60
NRFA Flow Rate (m^3/s) on day of sampling	Sep	1.497	10.79	NA	55.7	0.621	2.47	2.72
	Oct/ Nov	5.168	40.89	NA	96.94	3.145	4.457	6.28
	Mar	1.221	13.31	NA	65.86	1.486	6.066	9.32

counts when comparing sampling months (Kruskal Wallis Coquet: $p = 0.85$, Eden: $p = 0.95$) (Table S11), however lower cell counts were generally observed in colder months (March/November).

Microbiomes were assessed by 16S rRNA sequencing with Illumina MiSeq. Following QMP normalisation, there were 2975 taxa amongst samples. Sampling depth was highest in the Eden samples, especially in colder months such as November and March (Fig. S1). Conversely, lower sampling depth is seen in the October and September Eden samples, as well as March/November samples at downstream sampling points in the catchment. Lower sampling depth in general was observed in the Coquet samples.

The microbial abundance was plotted for each site in both catchments (Fig. 2), showing the top 25 ASVs in each. Both catchments showed low levels of abundance in upstream sites and an increase in the midstream. The Coquet shows a particularly high abundance at site J, such as a high abundance of *Flavobacteriaceae* in September. Outlier analysis revealed the levels were substantially higher than the typical ASV abundance across all sites and these data were removed from further analysis (Fig. S2).

Analysis of microbial abundances using the QMP approach permits the analysis of significantly more abundant taxa between catchments to enable comparisons. Taxa that had significantly different abundance across all samples in both catchments at Phylum level are presented in Fig. 3. Overall, eight taxa were significantly different between catchments (Wilcoxon test, $p < 0.01$). In the Coquet, *Bdellovirbionota* and *Pastescibacteria* are significantly more abundant compared to the Eden (Wilcox test, $p < 0.01$). In the Eden, *Gemmatimonadota* and *Synergistota* are more significantly abundant compared to the Coquet (Wilcox test $p < 0.01$).

The order of diversity (q) were plotted against microbial diversity represented through Hill numbers (Fig. S3). Decreasing richness of species was evident from up to downstream in the Coquet and decreasing evenness of species. The same pattern was seen in the Eden, except in September when communities were less rich and more uneven along the river.

Beta diversities in each catchment were visualised using a Sørenson-type overlap dissimilarity measure at q = 1 (Fig. S4). There were differences in microbial community structure in the upstream and downstream sites in both catchments, except for some upstream sites in the Eden, which sometimes clustered with downstream sites, possibly due to greater agricultural activity near the river channel at upstream sites, such as Site A (Fig. 1A). Microbial community diversities in both rivers varied across sampling months, which was particular apparent in the Eden catchment.

3.3. Comparing the resistomes and mobilomes in the Coquet and Eden catchments

Relative and absolute abundance of ARGs sorted by antibiotic group are shown in Fig. 4 and Table S15. Outlier analysis indicated that Coquet Site D in September had particularly high relative and absolute abundance of ARGs and MGEs compared to sites in the same catchment, and in the Eden, Site A had particularly high relative abundance of ARGs and MGEs (Fig. S5) consistent with land use (Fig. 1A). As Site D in the Coquet was based on a single sample and it was a statistical outlier, it was removed from further analysis, although data from the site are included in Table S15. For Eden Site A in March, outlier analysis indicated the sample data were in the normal range for the catchment once data were converted to absolute abundances (i.e., ARG and MGE copies/mL). Therefore, Site A in the Eden in March was retained in further analysis.

The Coquet had an average total relative abundance of 0.09 ± 0.13 ARG copies/16S rRNA (mean ± standard deviation) and absolute abundance of $1.6 \times 10^4 \pm 1.7 \times 10^4$ ARG copies/mL. The average relative abundance in the Eden sites was 0.2 ± 0.31 copies/16S rRNA and the absolute abundance was $5.2 \times 10^4 \pm 7.83 \times 10^4$ copies/mL. The Eden had on average higher relative and absolute abundance of ARGs compared to the Coquet, but this was not significant (Kruskal Wallis, $p > 0.05$). However, the Eden catchment had significantly higher ARG diversity, based on the 96 gene assay (Kruskal Wallis, $p = 0.0207$), with 45.6 ± 20 genes detected out of 85 potential ARGs, compared to the Coquet's average of 32.5 ± 16.3 genes (Table S16).

In both catchments, MGEs (including integrons; see Table S16) were detected at all sites (Fig. 5), with an average relative abundance of 0.21 ± 0.22 MGE copies/16S rRNA in the Coquet and 0.34 ± 0.69 MGE copies/16S rRNA in the Eden catchment. In the Coquet, the average absolute abundance of MGEs was $3.98 \times 10^4 \pm 3.6 \times 10^4$ MGE copies/mL and $5.8 \times 10^4 \pm 6.4 \times 10^4$ MGE copies/mL in the Eden. The MGE abundance was not significantly different between the catchments (Kruskal Wallis, $p = 0.46$) (Table S16).

The extent of shared ARGs and MGEs in the Coquet and Eden catchments is provided in Fig. S6. Overall, within the 96 ARG/MGE gene assay, the catchments had similar resistomes, where the Coquet had no

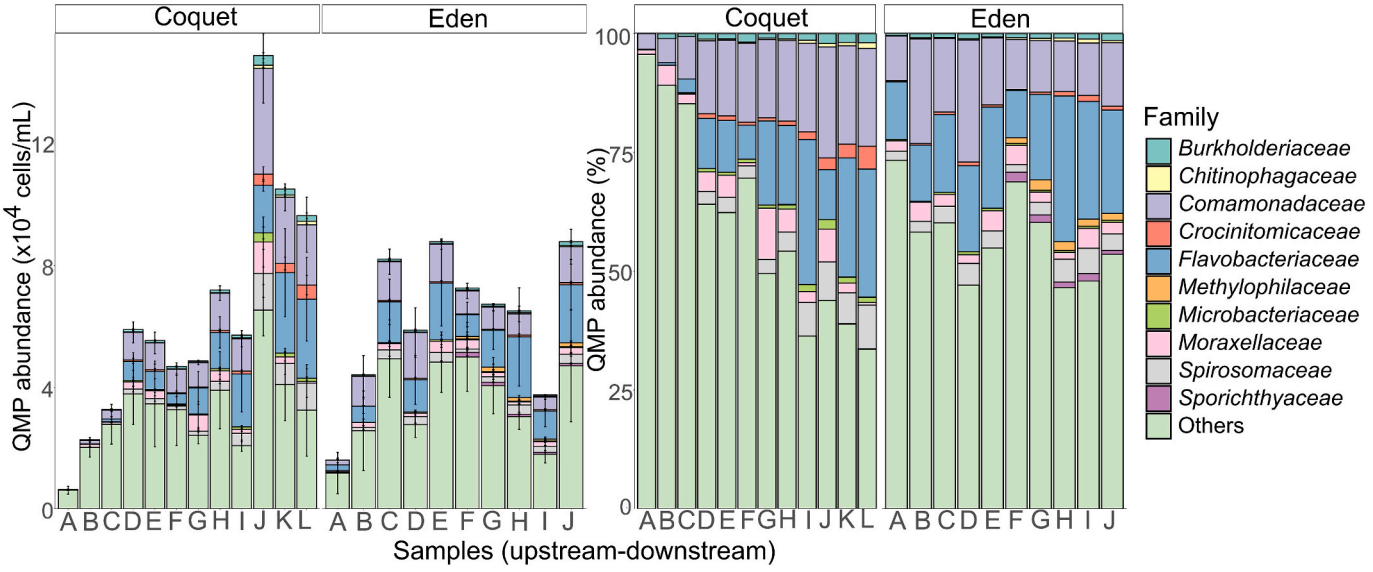


Fig. 2. Bar plots showing Quantitative Microbial Profiling (QMP) abundance of the 25 most abundant amplicon sequencing variants (ASVs) grouped into Families, with the remaining grouped into 'Others'. The error bars show the deviation between sampling months.

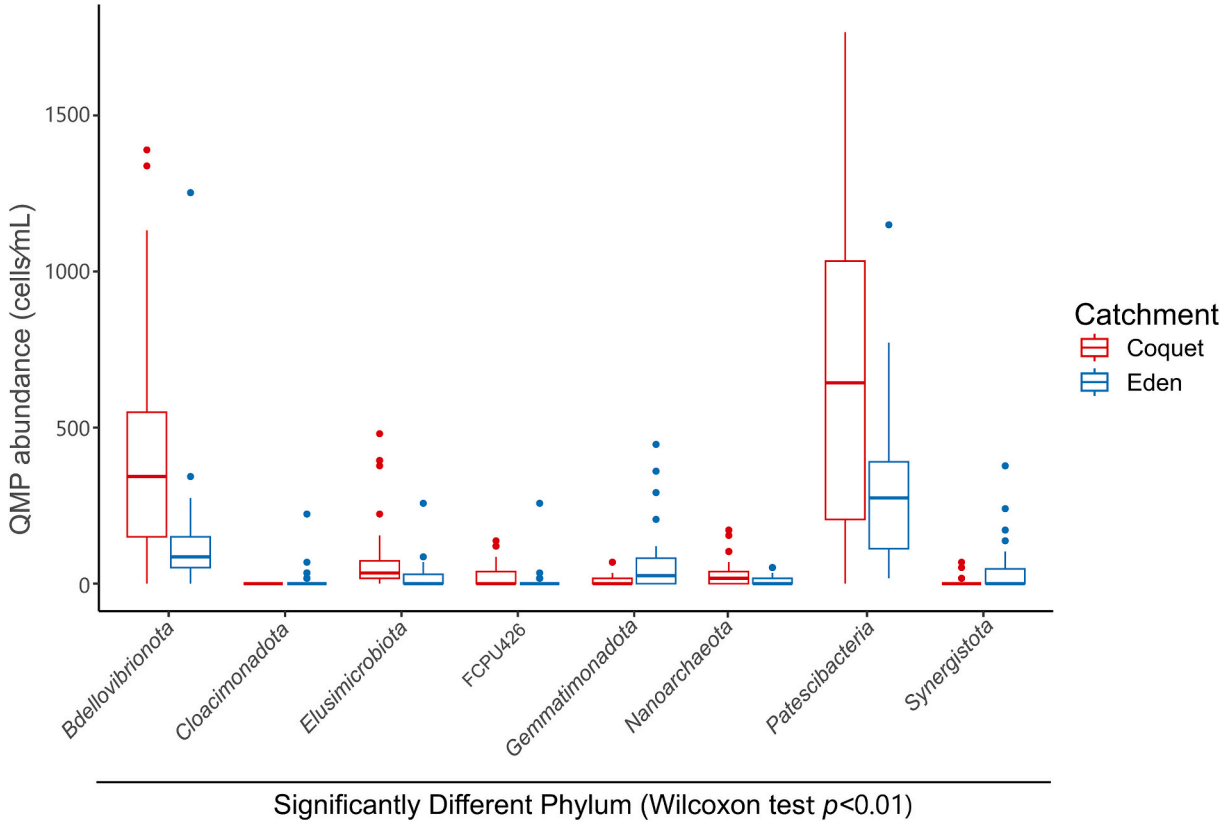


Fig. 3. Significantly different abundant Phylum in the Coquet and Eden catchments. Abundance was assessed through quantitative microbial profiling (QMP) and significant differences were determined through a Wilcoxon test, where the significance threshold was set at $p < 0.01$.

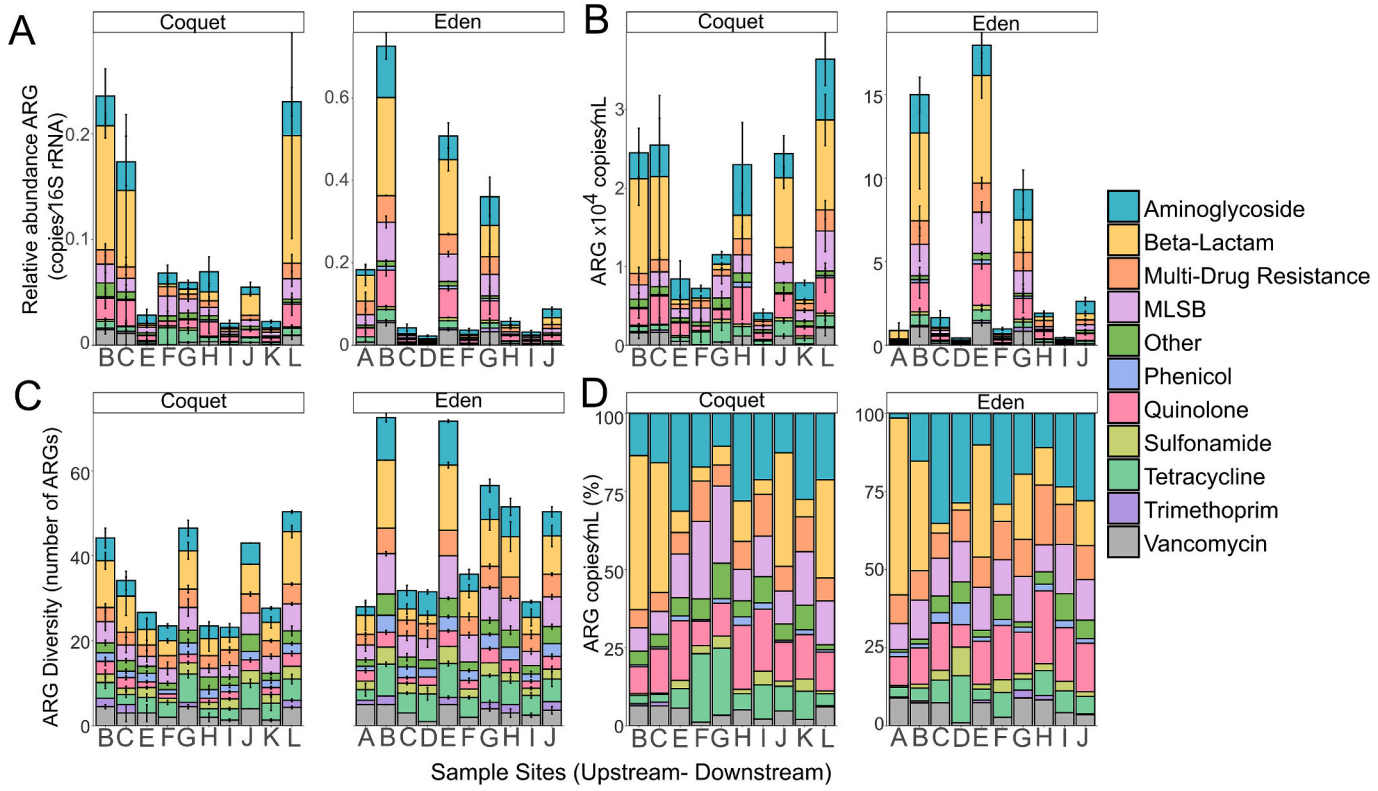


Fig. 4. A) Relative abundance of ARGs/16S rRNA grouped by antibiotic class in the Coquet and Eden B) ARG copies/mL for the Coquet and Eden, c) Diversity of ARGs, D) percentage of absolute abundance (ARG copies/mL) in each catchment.

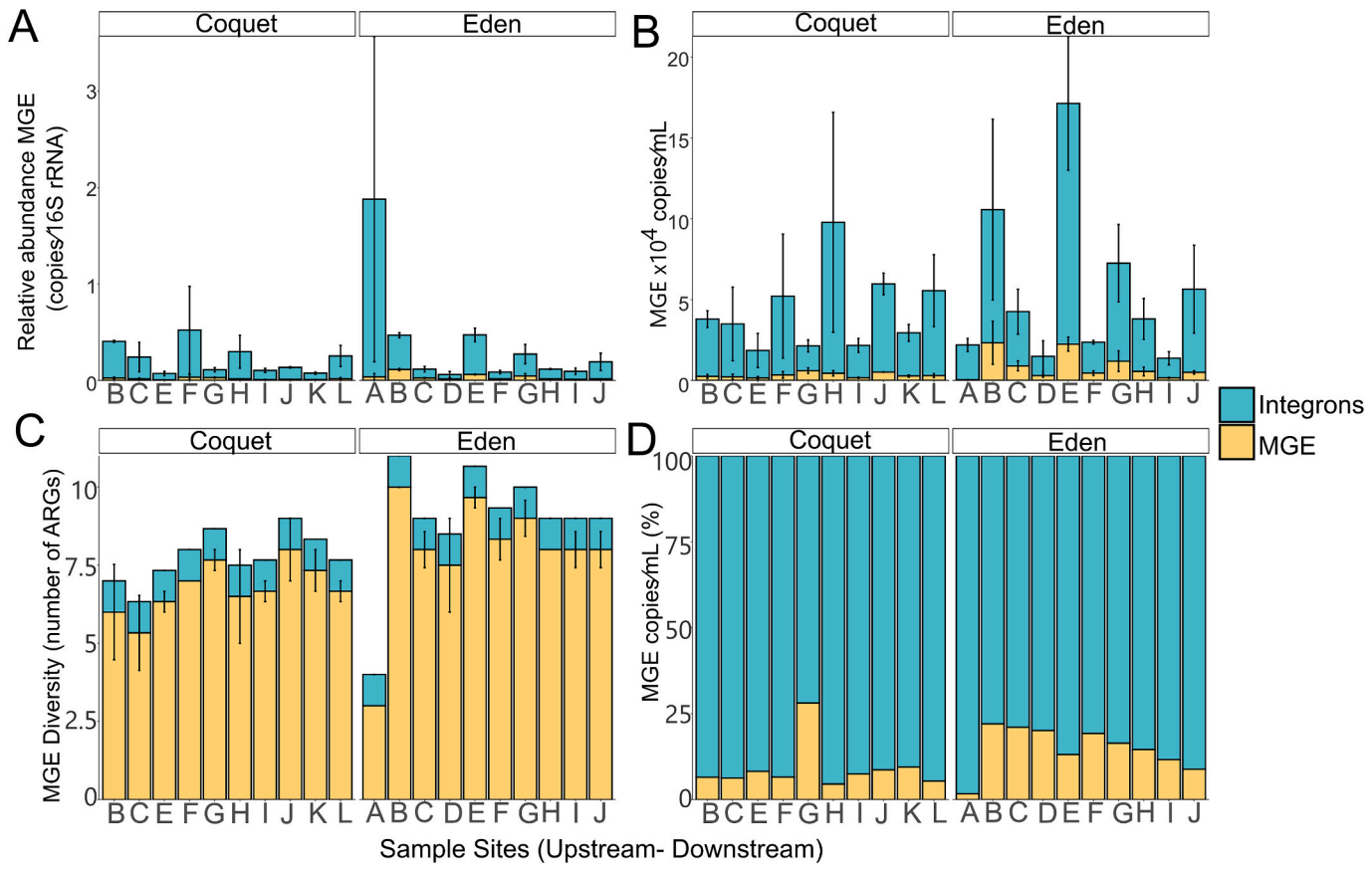


Fig. 5. A) Relative abundance of MGEs/16S rRNA B) MGE/copies/mL C) Diversity of MGEs, D) Percentage of Absolute abundance (MGE copies/mL) in each catchment.

unique genes and the Eden only had four unique genes, two conferring resistances to Beta-Lactams (bla_{KPC} and bla_{GES}), one tetracycline gene (*tetPB_1*), and one MLSB gene (*IsaC*). In terms of abundance, the bla_{KPC} gene and *tetM* gene were found to be significantly more abundant in the Eden catchment (Fig. S7).

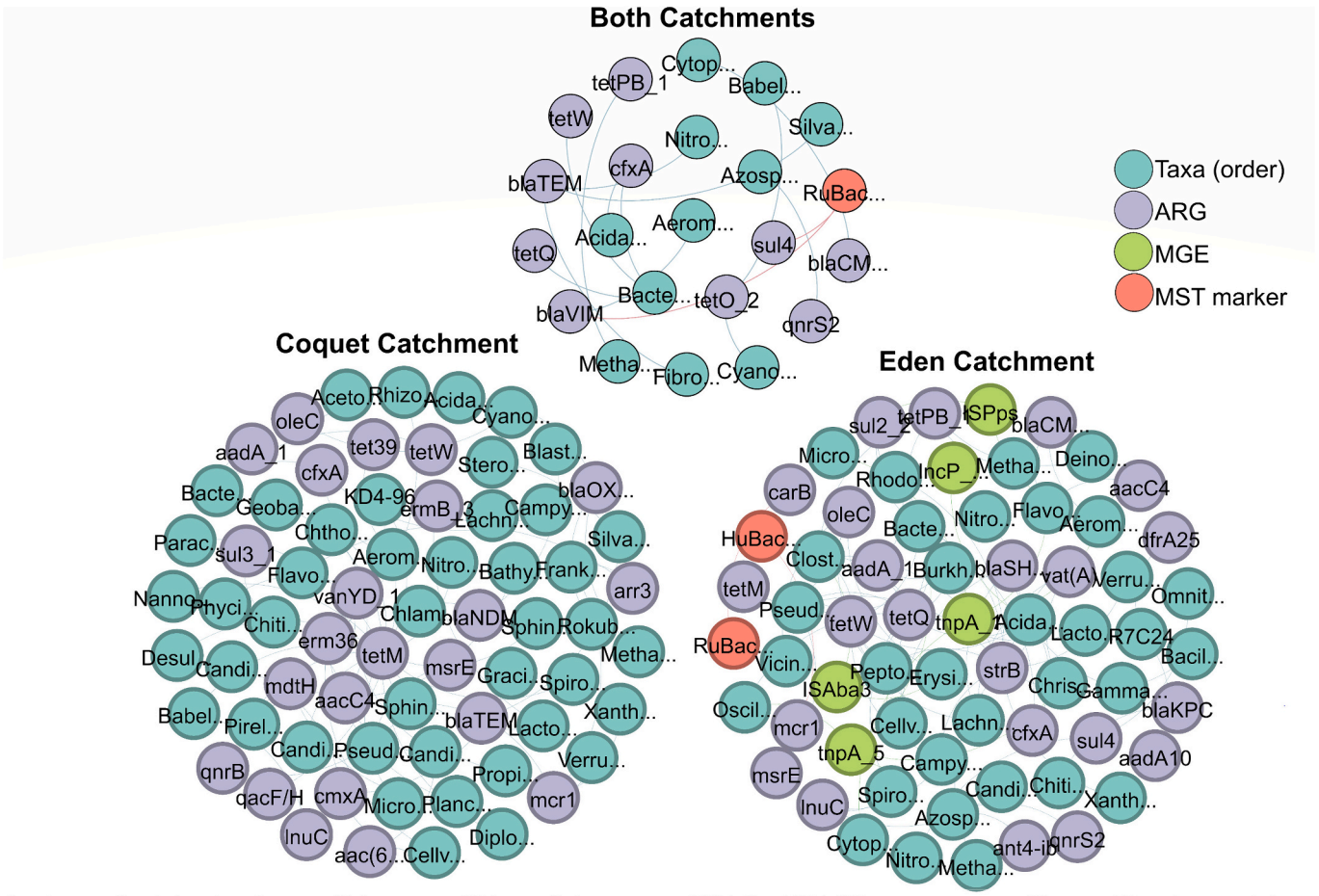
3.4. Possible drivers for microbial community and resistomes in catchments

Microbial source tracking probes (MST probes) for human and ruminant faecal derived *Bacteroidetes* (HuBac and RuBac, respectively) were used to determine potential contributing faecal sources in both catchments. Concentrations of HuBac and RuBac derived *Bacteriodetes* were significantly higher in the Eden compared to the Coquet (HuBac: Kruskal Wallis, $p = 6.83 \times 10^{-5,}$ RuBac: Kruskal Wallis, $p = 0.00257$) (Table S11). HuBac and RuBac concentrations in both catchments increased as one moved downstream, maximising at mid-catchment, but then decreasing downstream (Fig. S8). This pattern was especially evident in the Eden, where upstream RuBac concentrations steadily increase from upstream sites to the Site F (consistent with greater agricultural land use mid-reach in the Eden; Fig. 1A), before decreasing towards the bottom of the catchment. On average, RuBac was about 10-fold more abundant than HuBac concentrations, likely due to the greater farm animal waste inputs associated with agricultural activity in both catchments (see Fig. 1).

Microbiomes and resistomes in both catchments were assessed using network analysis (Fig. 6; Table S17). When both catchments were analysed together, there were 22 nodes, where the highest correlating taxa was *Bacteroidales* (3 degrees). The highest correlating ARGs were bla_{TEM} (three degrees) and *cfXA*, bla_{VIM} and *tetO_2* (two degrees). The MST marker RuBac, also correlated significantly with bla_{VIM} and *sul4* (Spearman, $r > 0.8$, $p < 0.01$). Analysed separately, the Eden and Coquet had a similar number of nodes in their networks, with 64 and 67 nodes respectively. In the Eden catchment, the highest correlated ARGs included *strB* (12 degrees), *tetQ* (10 degrees), *tetW* (8 degrees), and the highest correlating MGEs were *tnpA_5_* (5 degrees), *tnpA_1* (4 degrees) and *ISAba3* (3 degrees). The highest correlating taxa were *Bacteroidales* (7 degrees), *Acideaminococcales, Campylobacterales* and *Peptostreptococcales-Tissierellales* (6 degrees). Both HuBac and RuBac also significantly correlated with noteworthy clinically relevant ARGs in the Eden catchment (e.g., *mcr1* and *carB*, respectively) (Spearman, $r > 0.8$, $p < 0.01$).

In the Coquet, the highest correlating ARGs were *tetM* (16 degrees), *aacC4* (5 degrees), *eerm36*, and *mcr1* (4 degrees). The highest correlating taxa were *Aeromonadales, Bacteriodales, Candidatus Azambacteria and Pirellulales* (all 3 degrees). Interestingly, no MGEs were strongly correlated with taxa in the Coquet catchment, although this may be because the limited number of MGEs quantified (only 10 MGEs). Conversely, more MGEs correlated with taxa in Eden waters.

The data from the two catchments were pooled to identify more general factors driving AMR within the two rural rivers. A correlation matrix was developed which compared parameters measured in this study, grouped into categories called physiochemical parameters, microbial indicators, and AMR indicators (see Fig. S9 for specific indicators). All AMR indicators, whilst not significantly correlated in most cases, are generally positively correlated with water quality (e.g., conductivity and pH) and the microbial indicators. For example, total MGE abundance is significantly positively correlated with 16S rRNA (Spearman, $p < 0.05$). Interestingly, whilst microbial indicators are negatively correlated with DO (i.e., as DO declines, microbial indicators increase), AMR indicators had only a weak, but positive correlation with DO.



Aceto	- Acetobacterales	Chlam	- Chlamydiales	KD4-9	- KD4-96	Planc	- Planctomycetales
Acida	- Acidaminococcales	Chris	- Christensenellales	Lachn	- Lachnospirales	Propi	- Propionibacteriales
Aerom	- Aeromonadales	Chtho	- Chthoniobacterales	Lacto	- Lactobacillales	Pseud	- Pseudomonadales
Azosp	- Azospirillales	Clost	- Clostridiales	Metha	- Methanobacteriales	R7C24	- R7C24
Babel	- Babeliales	Cyano	- Cyanobacteriales	Metha	- Methanomassiliicoccales	Rhizo	- Rhizobiales
Bacte	- Bacteroidales	Cytop	- Cytophagales	Micro	- Micrococcales	Rhodo	- Rhodobacterales
Bathy	- Bathyarchaeia	Deino	- Deinococcales	Nanno	- Nannocystales	Rokub	- Rokubacteriales
Blast	- Blastocatellales	Desul	- Desulfitobacteriales	Nitro	- Nitrosopumilales	Silva	- Silvanigrellales
Burkh	- Burkholderiales	Diplo	- Diplorickettsiales	Nitro	- Nitrososphaerales	Sphin	- Sphingobacteriales
Campy	- Campylobacterales	Erysi	- Erysipelotrichales	Omnit	- Omnitrophales	Spiro	- Spirochaetales
Candi	- Candidatus_ Azambacteria	Flavo	- Flavobacteriales	Oscil	- Oscillospirales	Stero	- Steroidobacterales
	- Candidatus_ Nomurabacteria	Frank	- Frankiales	Parac	- Paracaedibacterales	Verru	- Verrucomicrobiales
	- Candidatus_ Zambryskibacteria	Gamma	- Gammaproteobacteria_Incertae_Sedis	Pepto	- Peptostreptococcales-Tissierellales	Vicin	- Vicinamibacterales
Cellv	- Cellvibrionales	Geoba	- Geobacterales	Phyci	- Phycisphaerales	Xanth	- Xanthomonadales
Chiti	- Chitinophagales	Graci	- Gracilibacteria	Pirel	- Pirellulales		

Fig. 6. Network Analysis for both catchments, and individual catchments revealing co-occurrence patterns amongst taxa at order level (assessed through quantitative microbial profiling (QMP), ARGs/mL, MGEs/mL and MST markers (Ruminant and Human Bacteroidetes (RuBac and HuBac). A connection indicates a strong spearman correlation (rs $>$ 0.8) and significant ($p < 0.01$), which is adjusted with a Benjamini Hochberg correction.

Although the hydrology indicators are not significantly correlated with water quality, microbial or AMR indicators, they do positively correlate with HuBac and RuBac, and negatively correlate with COD. Further, although statistical analysis was not possible based on the available data, water quality qualitatively trends with land use (pristine vs rural vs urban). The Antecedent Precipitation Index over the previous 5 days (API_5) negatively correlated with temperature, pH, DO, conductivity, COD and NH_4. There is also a negative correlation with the flow rate and ESBL coliforms.

4. Discussion

4.1. Impact of hydrological factors on microbial communities in the Coquet and Eden catchments

The hydrology of both catchments was characterised, and the Eden generally had more extreme differences in flow rates, rainfall and run-off compared to the Coquet catchment, especially in the middle of the Eden catchment (NRFA, 2023). More extreme flows will clearly influence flow rates but will also 'flush' upstream and on-land contaminants downstream, impacting river water quality lower in the catchment as seen previously (Chung et al., 2008; Zhang et al., 2014). This effect is most evident in the Eden through the human and ruminant *Bacteroidetes* levels, which increase towards the middle of the catchment before decreasing in concentration farther downstream. Decreases farther downstream are probably due to greater land-management in those reaches, where there are interventions such as fencing or riparian buffers that can protect stream water quality from farm animal-related runoff and pollution (Grudzinski et al., 2020).

In general, the Coquet has higher numbers of the *Bdellovibrionota* and *Patescibacteria* phylum, which tend to be prevalent in less polluted surface water or groundwater environments (Brown et al., 2015; Herrmann et al., 2019; Im et al., 2019; Chaudhari et al., 2021; Li et al., 2021). In particular, the *Patescibacteria* phylum is often present in nutrient-limited conditions (Tian et al., 2020). *Bdellovibrionota*, is a phylum that preys on other bacteria (Sockett and Lambert, 2004). This has been previously associated with low abundance of microalgae (Yang et al., 2023), and maintenance of a healthy and diverse ecosystem through removing dominant bacterial groups (Zhang et al., 2023b). Higher numbers of such strains are consistent with nutritional conditions and evidence of low pollutant inputs in the Coquet, where the Coquet River microbial communities reflect less impacted conditions than the Eden, which is consistent in the other genetic and microbial data for the catchment.

Conversely, the Eden catchment had broadly greater abundances of microbes associated with soils, limnic environments and sediments, such as the phylum *Gemmatimonadiota* (Mujakić et al., 2022) and *Synergistota*, which is often present under conditions impacted by animal faeces, surface soils, and wastewater releases (Bhandari and Gupta, 2012). Longer periods of high soil wetness (PROPWET) and greater soil saturation in the Eden catchment appears to result in greater run-off that contains phylum commonly present in the soils entering the river. This speculation is supported by significantly higher ruminant *Bacteroidetes* abundances in the Eden compared to the Coquet catchment and greater levels of agricultural activity in the Eden, especially near the river mid-reach (Fig. 1).

4.2. Drivers of AMR in the Eden and Coquet catchments

The river water resistomes in the two catchments were similar, although the diversity, as well as absolute and relative abundances of ARGs and MGEs were slightly higher in the Eden compared to the Coquet catchment, and the diversity of ARGs in the Eden was significantly higher. Comparison of the catchments revealed that from the 96 gene assay used in the study, the Eden catchment had four unique genes compared to the Coquet. This included the Beta-lactamase genes, bla_{KPC} and bla_{GES} that encode resistance to carbapenem antimicrobials, which were significantly more abundant in the Eden catchment compared to the Coquet. Both genes are often plasmid-mediated and genetically mobile, and can be shared through HGT (Queenan and Bush, 2007; Bennett, 2008; Mengistu et al., 2022). The high prevalence of the *tetM* ARG was previously found to be a consequence of environmental pollution caused by livestock (Munck et al., 2015).

Carbapenemase genes have been more frequently studied in the context of agricultural and wastewater related contamination of natural waterbodies (Mills and Lee, 2019). In particular, bla_{KPC} represents carbapenem resistance in *Enterobacterales*, which is a problem in hospital settings, especially in the North West of England (Stoesser et al., 2020). In this study, the bla_{KPC} gene was detected once in Site G, and twice in two sites, Site B and E. These sites all have a high percentage of agricultural land use that includes large-animal rearing, and have various small settlements nearby. Therefore, the presence of bla_{KPC} could be a result of community wastewater, septic tanks, or agricultural contamination. Interestingly, bla_{KPC} was not found in the Coquet. More sampling is needed to determine the source of this gene, especially what it might suggest relative to the spread of such ARGs in the environment due to waste releases.

Our network analysis revealed that the ARGs in the Coquet and Eden have multiple potential hosts. In both catchments *Bacteroidales* was highly correlated with ARGs, consistent with previous studies finding that *Bacteroidales* often carry abundant ARGs including tetracycline and Beta-lactam genes (Li et al., 2022). *Aeromonadales*, known to harbour clinically relevant drug resistance (Kneis et al., 2022), also significantly correlated with ARGs. The network analysis further highlighted differences in resistome and mobilome interactions between the catchments. There were multiple MGEs strongly correlating with ARGs in the Eden, whereas no strong correlations were seen between ARGs and MGEs in the Coquet. This further indicates greater anthropogenic impact in the Eden, where the presence of MGEs is indicative of acquired resistance (Datta and Hughes, 1983) and greater human and animal waste inputs to the river.

In addition, human and ruminant *Bacteroidetes* had strong and significant correlations with ARGs in the Eden, whilst there were no correlations with these markers in the Coquet, indicating the increased likelihood of human and/or agricultural related resistance in the Eden catchment compared to the Coquet. However, due to the complexity of the microbial interactions in the environment, this is speculation at best, although it is broadly consistent will qualitative differences between the two catchments and might be useful for considering the effects of differences between river catchments in general terms (Carr et al., 2019).

To understand ecological drivers, correlation matrices were developed to investigate the links between water quality, microbial indicators, resistance indicators and hydrology. Interestingly, unlike previous studies in more contaminated catchments (Ho et al., 2021; Ott et al., 2021a), there were few strong statistically significant positive correlations, although positive correlations were observed between selected AMR indicators, such as ESBL *E. coli* abundance and conductivity (a good indicator for dissolved solids in a river; Abdulsattar et al., 2020). Conductivity may thus be linked to inorganic and organic pollution from fertilisers, or run-off from roads (particularly as most sites were sampled close to roads), which may contain heavy metals that can increase resistance through co-selection (Knapp et al., 2017; Robins et al., 2022). However, this cannot be verified with the available data.

Unlike previous studies in heavily contaminated catchments, DO was only a weak indicator for AMR, whereas previous studies have found a strong, significant negative correlation (Ho et al., 2021; Ott et al., 2021a). This could be due to the difference in AMR sources, where resistance may be primarily derived from diffuse agricultural sources (here) as opposed to point source contamination from wastewater (previous studies). This observation highlights the importance of studying less contaminated sites to understand the drivers for AMR in "average" river environments. In a heavily contaminated site with more discrete waste inputs, DO may be an effective indicator for resistance and be used as a marker for AMR (Ott et al., 2021a), which is analogous to the use of oxygen sag curves for releases of biodegradable carbon. However, in less contaminated sites, which are primarily influenced by more diffuse rural phenomena, DO may not be as good a proxy because local organic waste releases are not as acute.

4.3. The need for further environmental surveillance

The comparison of the Coquet and the Eden catchments illustrates the importance of increased integrated AMR surveillance and insights it

can bring relative to difference catchment dynamics and AMR. The quantitative approach used here lends further support to recent recommendations by the UN Environment Programme (UNEP, 2023). Moreover, this study has indicated that hydrology in conjunction with land use in the Eden catchment, elevated river flows, rainfall, and runoff lead to greater agricultural contamination in the river and increased resistance, a connection that is less in the Coquet catchment.

Our study exemplifies the importance of sampling, analysis, and methods standardisation, which allowed us to better compare and understand subtle drivers of resistance in two "average" UK rivers. With higher flow rates apparently increasing rural in situ resistance, the expected more dynamic rainfall events due to climate change (Watts et al., 2015) may increase resistance in rivers due to surface run-off. Whilst rivers in contaminated environments have been studied more, understanding regional catchments like the work here will provide new insights into the temporal and spatial variation of AMR.

Increased spatial surveillance could further inform environmental risk assessments through understanding of how catchments differ in terms of AMR relative to each other (Burch et al., 2022). Furthermore, this study shows the need for monitoring in different environments, where ultimately the data can be used to inform large scale routine monitoring for AMR in environments (Bengtsson-Palme et al., 2023; Hart et al., 2023). However, we want to emphasise the need for metadata as well as AMR data, such as water quality, hydrology, catchment characteristics, and nutrient conditions, to explain drivers of AMR within environmental studies.

5. Conclusions

This study aimed to compare the microbiome and resistome of two rural catchments in the United Kingdom. By comparing the Coquet and Eden catchments, we show that hydrological and geographic differences between catchments best explain differences in in situ resistance patterns, which would have been less clear from isolated catchment studies. Further, consistent methods for microbiome and resistome characterisation also are important to allow comparisons between studies and catchments. In addition, increased monitoring of AMR in locations that are not overtly contaminated is useful in explaining background AMR. Here, we did not truly determine changes in intrinsic AMR in the catchments, but we show that there are noteworthy levels of resistance potential, although drivers are more nebulous, being dominated by non-point source runoff that is impacted by local hydrology and catchment characteristics. Further work is needed on similar rural catchments to generalise the results herein.

CRediT authorship contribution statement

Katie Robins: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Greg O'Donnell:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis. **Anke Neumann:** Writing – review & editing, Supervision. **Wiebke Schmidt:** Writing – review & editing, Supervision, Resources. **Alwyn Hart:** Supervision, Resources, Writing – review & editing. **David W. Graham:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.172348.

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