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Shotgun metagenomics reveals the microbiome and resistome of water harvesting ponds used by Kenyan rural smallholders

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Abstract

Water harvesting ponds are essential to smallholder farming across sub-Saharan Africa, yet their role in antimicrobial resistance (AMR) transmission remains unclear. Using shotgun metagenomics, we characterised the microbiome and resistome of 16 rural Kenyan ponds, detecting 582 antibiotic resistance gene (ARG) subtypes across 27 classes. Five ARG types (bacitracin, multidrug, polymyxin, beta-lactam and rifamycin) accounted for most ARGs (90.6%). Genome-resolved analyses recovered 1,542 metagenome-assembled genomes, including non-tuberculous *Mycolicibacterium* carrying rifamycin resistance (*rbpA*) and virulence factors including type VII secretion systems, dormancy regulators, and antigen 85 complex. ARG–mobile genetic element co-localisation was rare, and resistome risk scores were moderate (~22.4), indicating limited horizontal transfer potential compared to agricultural or hospital effluents. These ponds act as moderate, persistent environmental AMR reservoirs linked to farming practices. Strengthened antimicrobial stewardship, improved manure management and vegetative buffer zones could help mitigate AMR dissemination and support safer rural water systems under a One Health framework.

Background

Water harvesting ponds, fed by rainfall and surface runoff, are an important water source for many rural smallholder farmers across sub-Saharan Africa (SSA), supporting irrigation, livestock and domestic needs in areas lacking piped infrastructure [1,2]. Across the region,

tens of thousands of such ponds are in use, with many countries hosting several thousand individually [3]. In Kenya, national and county development programmes have constructed 25,091 household ponds, providing 28 million m^3 of water storage to irrigate 14,980 acres of farmland [4]. However, their open, untreated nature makes them highly susceptible to contamination from bioaerosols, human activity, livestock and wildlife, particularly during rainfall events when agricultural runoff and faecal material are washed into the ponds [5,6]. Microbiological surveys have reported levels of faecal indicator bacteria and pathogens in harvested rainwater exceeding World Health Organisation (WHO) guidelines, posing risks of diarrhoeal and other waterborne diseases [5,7]. Increasing evidence also suggests that water harvesting environments and agricultural water sources can act as reservoirs for antimicrobial resistance (AMR) [8–10]. Additionally, chemical contaminants such as pesticides may promote AMR through co-selection mechanisms [11,12]. Given the close association between livestock, agriculture and humans, and water resources, these ponds may facilitate the transmission of pathogens and AMR. However, their role as reservoirs of pathogenic and antibiotic-resistant bacteria remains poorly characterised.

In our previous 16S rRNA amplicon sequencing study of 16 water harvesting ponds in Kenya [13] we found that obligately or facultatively anaerobic bacteria (e.g. *Spirochaeta*, *Opitutus*) dominated the bacterial community. The study also detected the presence of potentially pathogenic bacterial genera, including *Mycobacterium*, *Pseudomonas* and *Neisseria* were detected, and comprised over 1% of the bacterial community, highlighting potential human health risks. While the amplicon sequencing provided a broad overview of community composition, it was limited to genus-level resolution and could not link functional genes, such as antibiotic resistance determinants, to specific microbial taxa [14]. To address these limitations, we extended this work using shotgun metagenomic sequencing to water from the same ponds, enabling strain-level identification [15,16]. Furthermore, this genome-resolved approach allows recovery of metagenome-assembled genomes (MAGs), meaning that taxa carrying antibiotic resistance genes (ARGs), virulence factors and mobile genetic elements (MGEs) can be determined [17].

Material and methods

Sample collection

Surface water was sampled from 16 water harvesting ponds in Laikipia County, Kenya, with three independent replicate samples per pond (n = 48 samples total). We conducted field surveys at 40 smallholder farms in the County of Laikipia, in Kenya to determine if they had working water harvesting ponds [13]. Of these 40 farms, working ponds were found at 14 sites (**Figure 1**). Two farms had two harvesting ponds at the same site (Pond 6/7 and Pond 10/12), meaning 16 ponds in total were sampled. Triplicate surface water (50 ml) was collected from each pond on the same day. All water samples were filtered through a sterile 0.2 µm pore-size syringe filter (Minisar NML, Sartorius). Replicates from each site were filtered using different filters. The three replicates collected from each pond represent independently collected and independently filtered water samples and are therefore treated as biological (field) replicates rather than technical replicates. Replicates were used independently (n = 48) in analyses of alpha diversity, beta diversity, structural correspondence between community and resistome composition, and ARG/taxonomic quantification. Pond identity was included as the grouping factor and, where relevant, permutations were restricted within pond to account for non-independence among replicates from the same pond. For analyses relating pond-level environmental or risk variables to the resistome, replicate values were averaged to a single pond-level mean (n = 16) prior to analysis. Given the modest number of ponds, analyses relating pond-level variables to one another are powered to reliably detect only comparatively strong associations (ρ or $R^2 \geq 0.5$-0.6). The volume of water filtered ranged from 6.54 to 15.3 ml, consistent with rapid clogging of the 0.2 µm filters, likely reflecting a high load of suspended solids or organic matter typical of these shallow, unlined agricultural ponds. Filters were stored in their plastic casing at -80°C prior to DNA extraction.

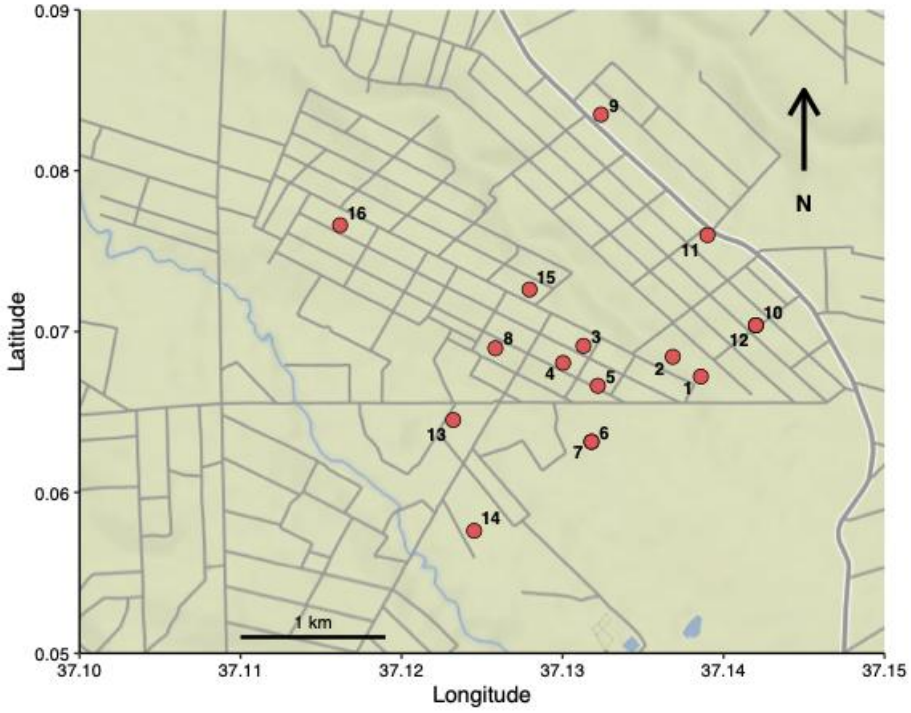


Figure 1 – Map showing the location of rural smallholder subsistence farms in the County of Laikipia, Kenya, where surface water was sampled from water harvesting ponds.

DNA extraction

Filters were removed from their plastic casing using a sterile scalpel and tweezers, cut into smaller pieces using sterile scissors and added to PowerBead Tubes (Qiagen) containing 750 µl of PowerBead Solution and 0.7 mm garnet beads. Filters were subsequently homogenised using a Precellys Evolution Homogenizer (Bertin Technologies) fitted with a 2 ml tube holder and white blocking plate (two 30 s cycles at 10,000 rpm). DNA was extracted from homogenised filters using a DNeasy PowerSoil Kit (Qiagen) according to the manufacturer's instructions and stored at -80°C until use.

Shotgun metagenomic sequencing

Metagenome libraries were prepared using the NEBNext DNA Ultra II FS Kit (New England BioLabs), indexed with custom oligos from Integrated DNA Technologies, and run on an Illumina NovaSeq 6000 using S4 chemistry with paired-end (2 x 150 bp) sequencing at the NERC Environmental Omics Facility (NEOF) at the University of Liverpool. Sequences have been submitted to the NCBI SRA archive under the accession number PRJNA1371399.

Preprocessing of metagenomic reads

The Read_qc module of MetaWRAP [18] was used to pre-process raw Illumina sequencing reads in preparation for assembly and alignment. Raw reads had already been quality-trimmed by the sequencing provider prior to delivery; the Read_qc module was therefore run with the –skip-trimming flag, and any human-derived reads (contamination) are removed with bmtagger (ftp://ftp.ncbi.nlm.nih.gov/pub/agarwala/bmtagger/), removing a mean of 0.13% per sample (range 0.04-0.45%). Read pairs with a single suspected human read are also removed. To make co-assembly of the full pooled read set computationally tractable, the combined reads from all samples were subsequently normalised to 40x covering using BBNorm in the BBTools package (https://sourceforge.net/projects/bbmap/) prior to co-

assembly only. Normalisation was not applied to the reads used for ARG quantification or taxonomic profiling, which were performed on the trimmed, human-read-depleted reads.

Read-based taxonomic profiling and diversity analysis

To provide taxonomic context for the read-based resistome, cleaned, non-normalised reads from each sample were classified using Kraken 2 (v2.0.9) [19] against the standard Kraken 2 database, with abundance re-estimated at species level using Bracken (v2.5.3) [20]. Per-sample profiles were merged using kraken-biom (https://github.com/smdabdoub/kraken-biom) and imported into phyloseq [21] for downstream analysis. Counts were normalised using cumulative-sum scaling (CSS) using the 'metagenomeseq' package [22] prior to diversity analysis. Genus-level relative abundances were visualised as a bubble plot. Alpha diversity (observed richness and Shannon index) was compared among ponds using one-way ANOVA with Tukey's HSD or Kruskal-Wallis with Dunn's post hoc test. Beta diversity was assessed by non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarities, tested by permutational multivariate analysis of variance (PERMANOVA; 999 permutations) with pond as the grouping factor. Homogeneity of multivariate dispersion was confirmed using the 'betadisper' function in 'vegan'.

Identification and quantification of antibiotic-resistance genes (ARGs)

Read-based annotation of ARGs was performed using ARG-OAP v3.0 [23]. The potential ARG sequences were aligned against the structured antibiotic resistance genes (SARG) database using BLASTX with cut-off values of 10^{-7} E-value, 80% sequence identity and 25 amino acids in alignment length. ARG abundance was calculated using the following equation:

$$\text{Abundance} = \sum_{i=1}^{n} \left(k \times \frac{Ni_{\text{ARG-likesequence}} \times Li_{\text{read}} / Li_{\text{ARGsreferencesequence}}}{N_{\text{cellnumber}}} \right)$$

where $Ni_{ARG\text{-}likesequence}$ is the number of ARG-like reads annotated to a given reference sequence; Li_{read} is the read length; $Li_{ARGSreferencesequence}$ is the nucleotide sequence length of the corresponding ARG reference sequence; and *n* is the number of mapped ARG reference sequences belonging to that ARG type or subtype. $N_{cellnumber}$ is the estimated cell number, obtained by mapping reads against a database of essential single-copy marker genes under default settings. The parameter *k* adjusts for reference sequences requiring co-occurrence of multiple gene products for functional resistance (k = 0.5 for two-component systems, k = 0.33 for three-component systems, k = 1.0 otherwise), shown as ARG copies per cell (copies/cell).

To assess whether variation in the read-based resistome corresponded to variation in bacterial community composition, correspondence between Bray-Curtis dissimilarity of genus-level community composition and ARG profile (type and subtype level) was tested using Procrustes analysis (PROTEST, symmetric rotation, 9,999 permutations and Mantel tests (Spearman correlation, 9,999 permutations) in vegan v2.6-10 and permute v0.9-7. Genera and ARG subtypes were retained if present in ≥ 10% of samples with mean relative abundance ≥ 0.01% (671 genera, 277 ARG subtypes and 21 ARG types retained). Tests were performed at both the sample level (n = 48; permutations restricted within pond to account for non-independence of replicates) and pond level (n = 16, pond mean profiles). To identify specific genus-ARG subtype associations, the 30 most abundance bacterial genera (plus four genera of a priori interest, *Brevundimonas*, *Mycobacterium*, *Pseudomonas*, *Sphingomonas*) and ARG subtypes were centred-log-ratio (CLR) transformed and tested pairwise by Spearman's rank correlation (2,370 pond-level comparisons), with Benjamini-Hochberg correction applied across all tests.

Metagenomic assembly and binning

Co-assembly of the cleaned reads (1,823,007,355 reads) from all 48 samples (16 ponds, each sampled in triplicate) was performed with MEGAHIT (v1.2.9) [24] generating 42,491,158 contigs. Co-assembly was chosen over per-sample assembly to improve recovery of genomes shared across ponds but present at low individual-sample coverage. We note this approach

can favour recovery of taxa that are abundant across multiple ponds and may reduce sensitivity to strain-level variation within a single pond. The assembly was formatted to include scaffold length and *k*-mer depth, sorted by length and contigs shorter than 1000bp were removed (6,815,575 contigs remaining). Assembled contigs were binned into three different bin sets within metaWRAP's 'binning' module [18] using MetaBAT (v0.32.5) [25], MetaBAT2 (v2.12.1) [26] and CONCOCT (v1.0.0) [27]. This clusters together contigs based on sequence properties such as tetranucleotide frequency, differential abundance and similar read coverages across samples. The resulting bins were refined (completeness >50%, contamination <10%) using the 'bin_refinement' module within metaWRAP.

Taxonomy and quantification of metagenome assembled genomes (MAGs)

Taxonomy was assigned to the recovered metagenome-assembled genomes (MAGs) using GTDB-Tk (v2.4.1) [28] with GTDB release R226. MAG abundances were calculated using Salmon [29], as part of the 'quant_bins' module in metaWRAP, with default parameters. To further refine the taxonomy assigned by GTDB-Tk, 5S and 16S sequences were extracted from MAGs using Barnnap (v.0.9) (https://github.com/tseemann/barrnap). Extracted sequences were compared to those present in the NCBI nr database using the Basic Local Alignment Search Tool (BLAST) [30].

Identification of antibiotic resistance genes (ARGs), virulence factors (VFs) and mobile genetic elements (MGEs) on MAGs

Antibiotic resistance genes (ARGs) within MAGs were identified using two complementary tools DeepARG v2.0 [31] and the Resistance Gene Identifier (RGI) v5.2.1 from the Comprehensive Antibiotic Resistance Database (CARD) [32]. DeepARG internally aligns sequences using DIAMOND (2.0.14) [33] and applies a deep neural network to classify and score potential ARGs based on similarity to known resistance genes. Analyses were performed in gene prediction mode using the long-sequence model, optimised for assembled contigs. In parallel, RGI was applied to protein sequences predicted from each MAG using Prodigal (v2.6.3) in metagenomic mode [34]. Proteins were aligned against the CARD database (v.3.3.0) using DIAMOND [33] and ARGs were classified based on CARD's curated homology, SNP and rRNA models. To standardise the outputs from each tool we used hAMRonization [35] which converts ARG prediction outputs into a common schema. Predictions from both DeepARG and RGI were filtered to retain only hits with ≥80% sequence identity, generating a unified table of ARG hits including gene identifiers, resistance mechanism, drug classes, alignment scores and associated MAGs.

To identify putative virulence factors (VFs) within each MAG, Prodigal predicted protein sequences were compared against the Virulence Factor Database (VFDB) [36] using BLASTp [37]. BLASTp was run with an e-value cutoff of $1e^{-5}$ and results were filtered to retain only hit with ≥70% sequence identity. Functional annotations for VFDB protein entries were parsed from the FASTA headers and merged with BLAST results using custom Python scripts. Output files included virulence gene IDs, gene names, functional descriptions, alignment statistics and the corresponding MAGs. To assign virulence categories, a metadata table linking VFDB gene identifiers to virulence categories was joined to the annotated results. A final summary file was compiled containing all virulence factor hits across MAGs, including their functional roles and category assignments.

Mobile genetic elements (MGEs) were identified in each MAG by performing BLASTn (v2.6.0) searches against curated reference databases for plasmids, phages, transposons, insertion sequences, and integrons [37]. Custom nucleotide BLAST databases for plasmids and phages were generated using sequences from the NCBI RefSeq plasmid and viral releases, respectively [38]. Integron sequences were obtained from the INTEGRALL databases [39], insertion sequences from ISfinder [40] and transposons from the TnCentral database [41]. Each MAG was queried independently against these databases using BLASTn, with a minimum identity threshold of 90%, a minimum query coverage of 70%, and an e-value cutoff of $1e^{-10}$.

Resistome risk score

Resistome risk, defined as the potential for ARGs to be mobilised via MGEs and transferred to human pathogens, was assessed using MetaCompare [42], which requires contigs and predicted gene sets generates using its documented pipeline and does not accept the MEGAHIT assembled contigs used elsewhere in this study for MAG recovery. Metagenomic reads from each sample were therefore assembled independently into contigs using the IDBA-UD *de novo* assembler [43] and ORFs were predicted with Prodigal [34]. Assembled contigs with predicted ORFs were annotated against reference databases: CARD for ARGs [44]; ACLAME for MGEs [45]; and PATRIC for pathogen associated sequences [46]. Each contig was then categorised based on whether it contained only ARGs, ARGs with MGEs, or ARGs with both MGEs and pathogen signatures, representing increasing levels of potential public health risk. For each sample, the relative abundance of contigs in each risk category is calculated and used as coordinates in a three-dimensional "hazard space". A Euclidian distance-based scoring system is then applied to determine the sample's proximity to the maximum risk vertex (ARG+MGE+Pathogen) and the zero-risk origin, producing a normalized composite resistome risk score, ranging from 0 (low risk) to 100 (high risk). Differences in resistome risk score among ponds, and between ponds compared to previously published valued for other aquatic and engineered environments [42], were tested using one-way ANOVA with Tukey's HSD.

Association between resistome and pond physicochemistry

Physicochemical characterisation of the same 16 ponds, including major cation and anion concentrations, was reported previously [13]. To test whether resistome patterns were associated with pond chemistry, we selected *a priori* four ions indexing agricultural and faecal input (ammonium, nitrate, phosphate, sulphate) and related their pond-level concentrations (mean of triplicate measurements) to total ARG abundance (copies/cell) using Spearman's rank correlation. Associations between these ions and the five dominant ARG types were also examined as an exploratory secondary analysis. P-values were corrected for multiple comparisons using the Benjamini-Hochberg procedure within each analysis. Associations between ion concentrations and resistome composition were further tested by fitting ion vectors onto a principal coordinates ordination of Bray-Curtis dissimilarities among ARG-type profiles and by permutational multivariate analysis of variance (marginal and omnibus models).

Results

Reads-based taxonomic and resistome profiling in water harvesting ponds

Reads-based taxonomic profiling of the 48 samples showed the bacterial community was dominated by *Polynucleobacter*, present above 2.5% relative abundance in 15 of 16 ponds and reaching up to 26.2% (Pond 12) (**Figure 2**). Other recurrent genera included *Bradyrhizobium* (2.7-7.6%)*, Hydrogenophaga* (2.5-6.4%)*, Lacunisphaera* (2.7-26.2%)*,* and *Variovorax* (2.5%-4.7%), together forming a core assemblage present across the majority of ponds. Genera containing recognised pathogens were detected at low relative abundance in a subset of ponds, including *Sphingomonas* (7 ponds), *Brevundimonas* (5 ponds), *Pseudomonas* (2 ponds) and *Mycobacterium* (1 pond).

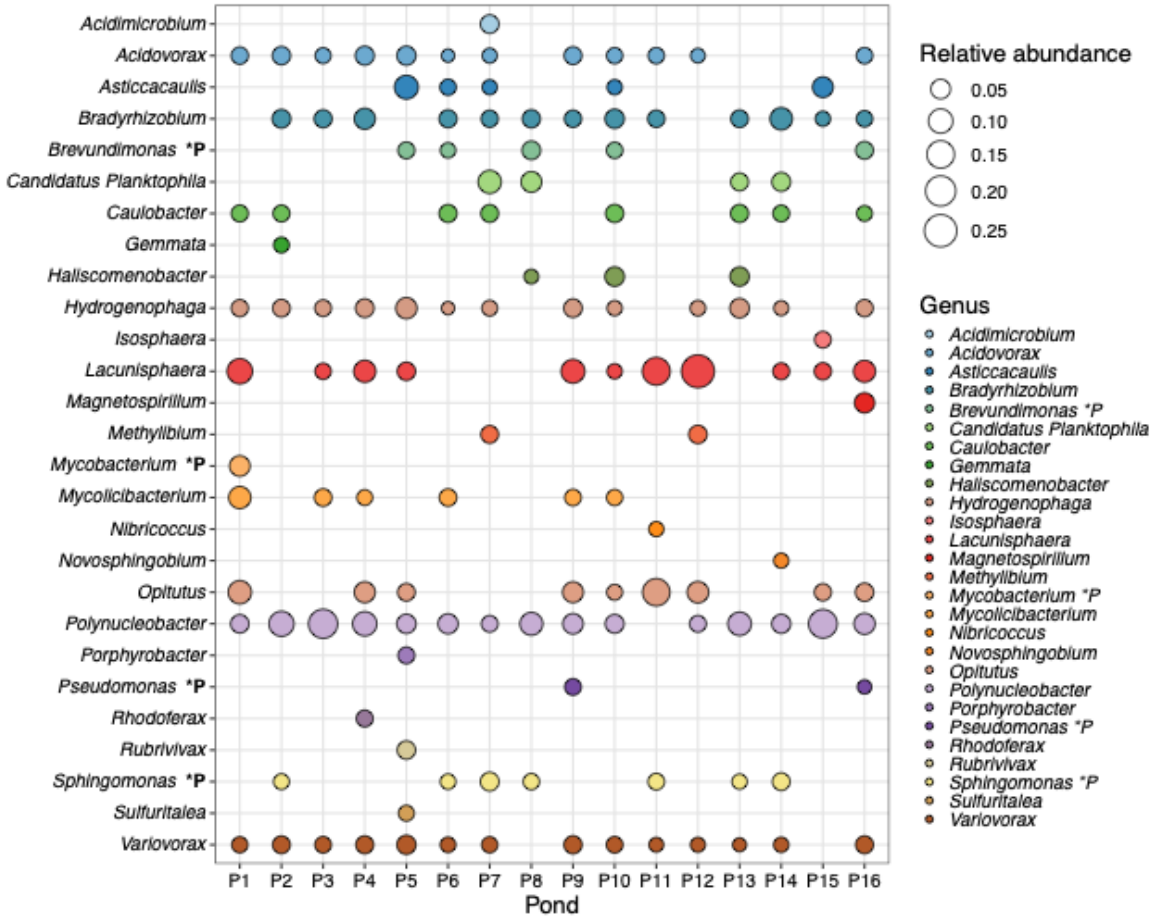


Figure 2 – Genus-level relative abundance of the read-based bacterial community across the 16 water harvesting ponds. Bubble size represents mean relative abundance of genera exceeding 2.5% relative abundance in at least one pond; colour denotes genus. Genera containing recognised opportunistic human pathogens, as defined by Bartlett et al. (2022) [47] are marked with *P.

Observed richness differed significantly among ponds (one-way ANOVA, $F_{(15,32)} = 2.93$, p = 0.005), with Pond 3 significantly higher than Ponds 4,5 and 7 (**Figure S1**). All other ponds were not significantly distinguishable from either extreme. Shannon diversity also differed significantly among ponds (Kruskal-Wallis, $\chi 2 = 37.42$, df =15, $p = 0.001$), with Pond 12 significantly higher than Pond 6 (Dunn's test, p = 0.023). No other pairwise comparison reached significance. Richness and Shannon diversity identifies largely non-overlapping ponds as extremes, indicating that differences among ponds reflect both taxon richness and evenness rather than either alone.

Community composition differed markedly among ponds (PERMANOVA, $R^2 = 0.852$, $F_{(15,32)} = 12.29$, p = 0.001), with pond identity explaining the majority of variance in the read-based community (**Figure S2A**). This separation was not attributable to differences in within-group dispersion, which did not differ significantly among ponds (betadisper, $F_{(15,32)} = 0.43$, p = 0.96), indicating that the PERMANOVA result reflects genuine compositional divergence between ponds rather than heterogeneity in variance (**Figure S2B**).

Overall bacterial community composition (genus level) and resistome composition (ARG subtype level) were significantly correlated at both the sample level and pond level (PROTEST r = 0.616, $p = 0.038$, Mantel $\rho = 0.478$, $p = 0.004$; $n = 48$) and pond level (PROTEST r = 0.618, $p = 0.003$, Mantel $\rho = 0.490$, $p < 0.001$; $n = 16$; **Figure S3**). At the coarser ARG type level, Mantel tests remained significant at both the sample level (Mantel $\rho = 0.335$, $p = 0.013$) and pond level (Mantel $\rho = 0.331$, $p = 0.009$). Procrustes correlations did not reach significance

(sample level: r = 0.445, p = 0.432; pond level: r = 0.463, p = 0.064), indicating that correspondence between community and resistome structure was more clearly detectable at the finer ARG-subtype resolution. Despite this overall structural correspondence, no individual genus-ARG subtype pair remained significant after correction for multiple testing among 2,370 pond-level comparisons, indicating this correspondence reflects broad, distributed covariation across many taxa and genes, rather than concentrated carriage by a small number of identifiable taxa.

Reads-based analysis revealed a total of 582 ARG subtypes, which belong to 27 ARG types, were detected in the water harvesting ponds (**Figure S4**). The most diverse ARG type was beta-lactam resistance genes, which contained 228 subtypes, followed by multidrug-resistance (88 subtypes; conferring resistance to multiple structurally unrelated antibiotic classes, rather than to a single drug class), aminoglycoside (54 subtypes), tetracycline (46 subtypes), and macrolide-lincosamide-streptogramin (MLS) (40 subtypes). Five abundant ARG types accounted for 90.6% of the total ARGs (**Figure 3**): bacitracin (47%, 7.99 × 10^{-2} copies/cell), multidrug (25.2%, 4.29 × 10^{-2} copies/cell), polymyxin (9.7%, 1.65 × 10^{-2} copies/cell), beta-lactam (4.4%, 7.43 × 10^{-2} copies/cell) and rifamycin (4.3%, 7.28 × 10^{-2} copies/cell). *bacA* (bacitracin; 7.91 × 10^{-2} copies/cell), *mexF* (multidrug; 7.57 × 10^{-3} copies/cell), *ugd* (polymyxin; 6.85 × 10^{-3} copies/cell), *rosA* (polymyxin; 5.60 × 10^{-3} copies/cell) and *rbpA* (rifamycin; 5.60 × 10^{-3} copies/cell) were identified as the most dominant ARG subtypes in the water harvesting ponds (**Figure S5**).

We further analysed the occurrence of last-resort ARGs (LARGs) conferring resistance to last-resort antibiotics (carbapenems, polymyxins, vancomycin and tigecycline) in the water harvesting ponds. There were 35 variants of *OXA*, 17 variants of *van*, 1 variant of *KPC*, 13 variants of *mcr* and 39 vairants of *IMP* (**Table S1**). No variants of *tet(X)* were detected in our dataset. Overall, the LARG group with the highest abundance across the ponds was *IMP* with an average of 7.61 copies/cell × 10^{-5} copies/cell, followed by *OXA* (4.03 × 10^{-5} copies/cell) and *mcr* (9.84 × 10^{-6} copies/cell) (**Figure S6**).

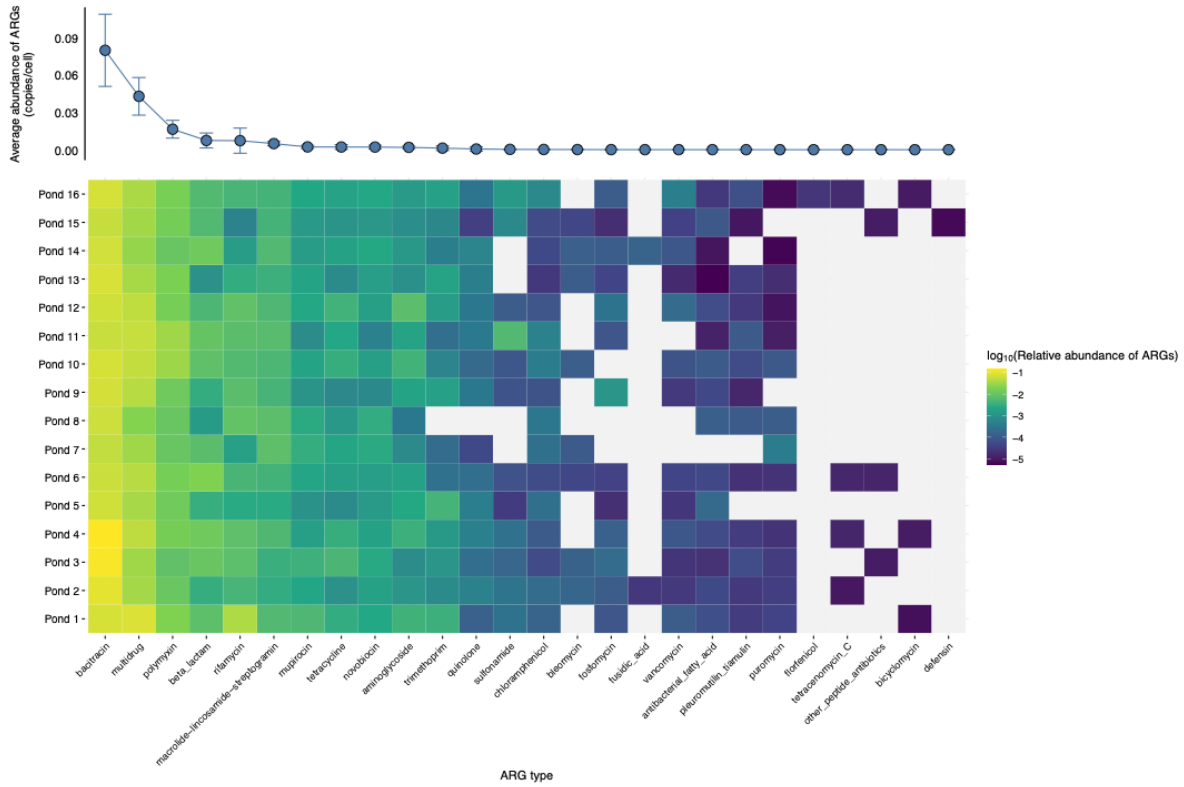


Figure 3 – Occurrence and abundance of antibiotic resistance gene (ARG) types detected in surface water samples from 16 water harvesting ponds. Top panel: average abundance (copies per cell) of each ARG type across all samples (±SD). Bottom panel: Heatmap of the log_{10}-transformed relative abundance of ARG types across ponds, coloured using a continuous viridus scale from low (dark purple) to high (yellow) relative abundance. Grey cells indicate ARG types that were not detected. ARG types are ordered by their average abundance across all samples (*n*=48). 'Multidrug' denotes genes conferring resistance to multiple, structurally unrelated antibiotics classes, rather than resistance to a single drug class.

None of the four nutrient ions correlated significantly with total ARG abundance across the 16 ponds (Spearman's ρ = -0.15 to -0.33, all *padj* > 0.5; **Table S2**). Exploratory comparisons against the five dominant ARG types identified a moderate negative association between sulphate and polymyxin resistance gene abundance (ρ = -0.53; *p* = 0.036) that did not survive correction for multiple testing (*padj* = 0.50). Resistome composition was similarly unrelated to the nutrient concentration. Neither the omnibus (R^2 = 0.30, *p* = 0.31) or the marginal PERMANOVA model identified a significant term (all p > 0.29; **Table S2**).

Taxonomic classification of metagenome-assembled genomes (MAGs)

Following co-assembly and binning, a total of 1,542 metagenome-assembled genomes (MAGs) were recovered from the water harvesting ponds, with genome sizes ranging from 0.54 Mb to 13.13 Mb, and GC% contents varying between 26.1% and 74.9%. MAGs had an average completeness of 86.03% (± 8.54%) and contamination of 3.50% (± 2.34%) (**Table S3**). These MAGs were assigned to 32 phyla, 75 classes, 164 orders, 287 families, 586 genera and 82 species (**Figure S7; Table S3**). The majority of MAGs assigned at the phylum level were affiliated with Pseudomonadota (59.98%), followed by Bacteroidota (14.23%), Actinomycetota (12.09%), Planctomycetota (9.81%) and Verrucomicrobiota (9.16%). The five genera identified with the highest number of classified MAGs were *Asticcacaulis* (2.08%), *Aurantibacillus* (1.17%), *JAFLN01* (1.17%), *ELB16-189* (1.17%) and *M3007* (1.10%). There was a total of 10 classes, 22 orders, 54 families, 108 genera and 331 species that did not have representatives in the GTDB (<95% average nucleotide identity), with the majority of MAGs (1,460) assigned to potentially novel species (**Table S3**).

Antibiotic-resistant bacteria (ARB) and potential pathogens in the water harvesting ponds

Of the 1,542 MAGs, 87 were identified as potentially antibiotic-resistant bacteria (ARB), as they possessed at least one ARG (**Table S4**). These ARGs encoded resistance to 15 classes of antibiotics, with bacitracin resistance being the most abundant (32 MAGs) (**Figure 4A**). The dominant resistance mechanism utilised by these ARB were antibiotic target alteration (28 MAGs), antibiotic efflux (10 MAGs) and antibiotic target protection (8 MAGs) (**Figure 4B**). The majority of ARBs were taxonomically assigned to Pseudomonadota (57.47%), followed by Actinomycetota (40.23%). Only a single MAG was taxonomically assigned to Verrucomicrobiota and Bdellovibrionota. Two MAGs (bin.749 and bin.1325) were predicted to encode the LARG subtype *IMP* and were both taxonomically assigned to the genus *Cellvibrio*.

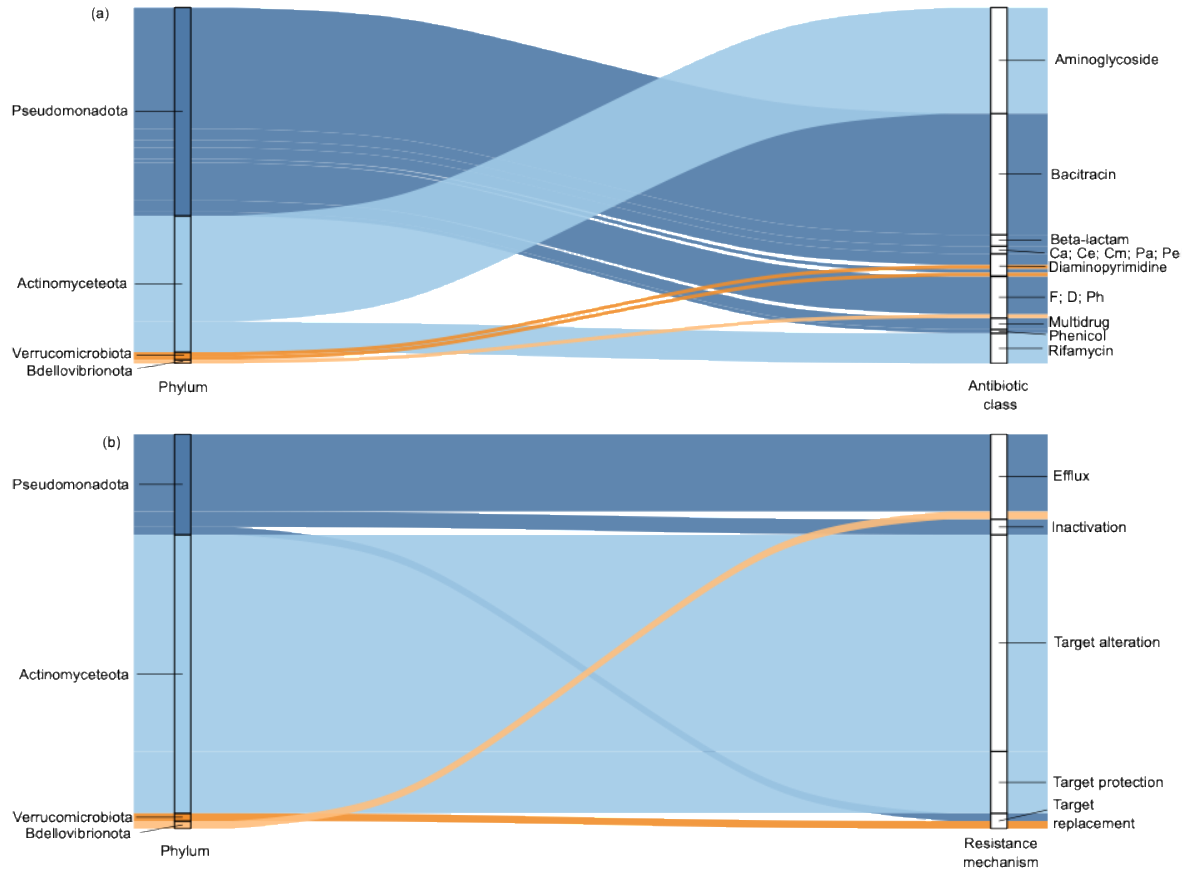


Figure 4 – Sankey diagrams showing the distribution of antibiotic resistance gene (ARGs) across bacterial phyla in metagenome-assembled genomes (MAGs) from water harvesting ponds. (a) Flow of ARGs from bacterial phyla to the corresponding antibiotic drug classes. Ca; Ce; Cm; Pa; Pe denotes an ARG that confers resistance to carbapenems, cephalosporins, cephamycins, penams and penems. F; D; Ph denotes an ARG that confers resistance to fluoroquinolones, diaminopyrimidines and phenicols. (b) Flow of ARGs from bacterial phyla to the resistance mechanisms they confer. The width of each flow is proportional the number of ARGs associated with each connection.

To assess the extent to which the read-based resistome was represented within the MAG-based analysis, we compared the set of ARG subtypes detected across the 87 antibiotic-resistant MAGs (**Table S4**) against the full read-based subtype abundance profile. Subtypes matching those detected in at least one MAG (*bacA*, *mexF*, *rbpA, dfrB2, dfrB3, dfrB9, IMP-11, IMP-14, ceoB, catB10 and bpeF*) together accounted for 56% of total read-based ARG abundance (0.095 of 0.170 mean copies/cell across all 582 subtypes), with bacA alone contributing 46.5%. Two further gene symbols detected in MAGs (*rsmA* and an unnumbered *IMP* hit) had no corresponding entry in the SARG database used for read-based quantification, likely reflecting differences in gene nomenclature between the CARD/RGI and SARG reference databases.

A total of 84 MAGs contained both ARGs and virulence factors (VFs) (**Figure 5; Table S5, S6**). The most abundant MAGs, based on cumulative genome copies per million reads (CPM) across all 16 ponds, were affiliated with the genera *Cellvibrio*, *Mycobacterium, UBA2093*, *Hylemonella* and *Rhodoferax*. *Cellvibrio* (Gammaproteobacteria), a genus of aerobic cellulolytic bacteria commonly found in soil and freshwater, was especially dominant contributing a total of 254.6 CPM across its MAGs. bin.1492 (*Cellvibrio*) was the single most abundant MAG (110.8 CPM) and encoded the highest number of predicted VFs (51). Other *Cellvibrio* MAGs, such as bin.1325 (68.9 CPM) and bin.749 (10.2 CPM), also carried high

numbers of VFs (24 and 29 VFs, respectively) and two ARGs each. *Mycobacterium* (Actinobacteria) was the second most abundant genus (124.9 CPM), with bin.1318 (34.1 CPM, 23 VFs) and bin.201 (22.6 CPM, 23 VFs) were among the most abundant representatives.

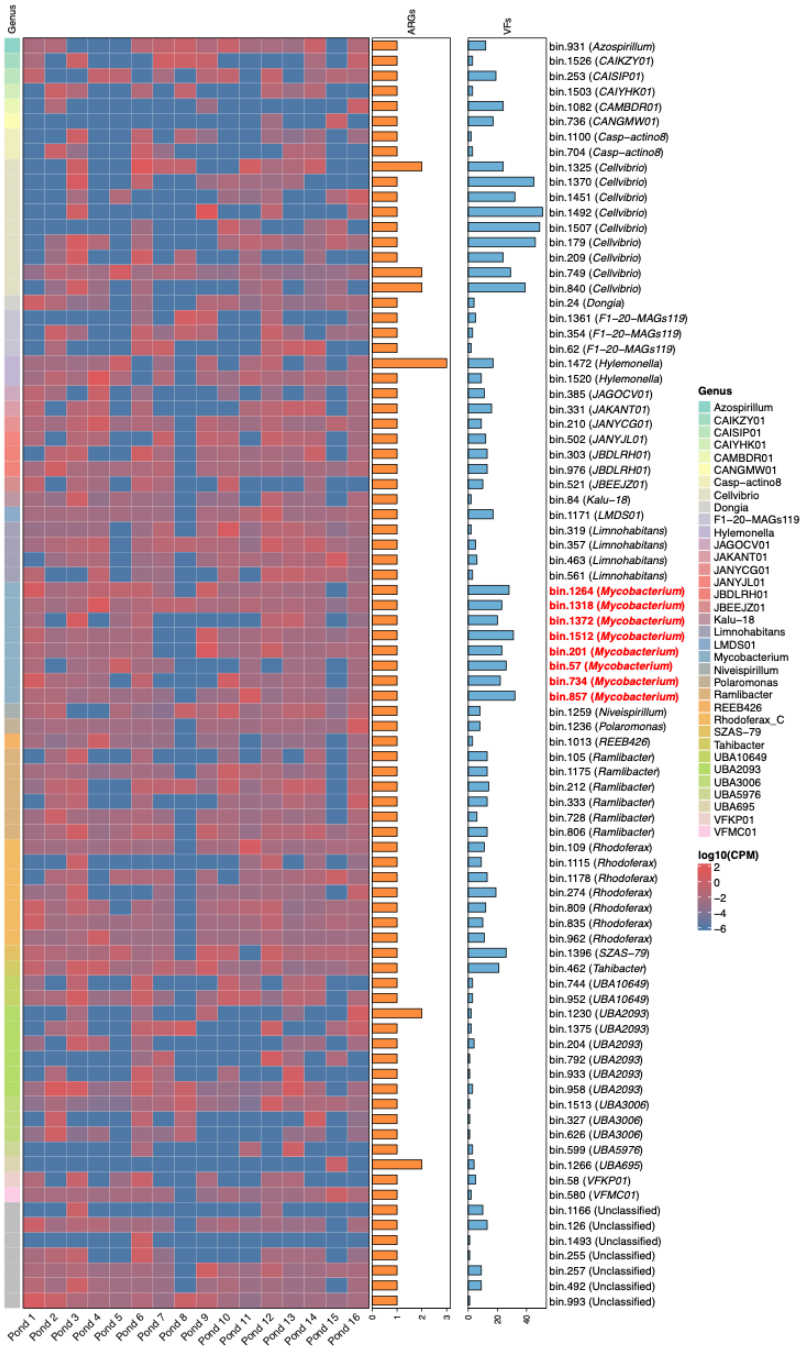


Figure 5 – Abundance of metagenome-assembled genomes (MAGs) that contain both virulence factors (VFs) and antibiotic resistance genes (ARGs). MAG abundance is log transformed genome copies per million reads (CPM). Right-side bar plots represent the total number of predicted VFs (orange bars) and ARGs (blue bars) identified in each MAG. Known human pathogenic genera, as defined by Bartlett et al. (2022) [47], are highlighted in red.

Other highly represented genera included *UBA2093* (98.7 CPM), a poorly characterised candidate lineage likely representing free-living aquatic heterotrophs; *Hylemonella*

(Betaproteobacteria; 60.5 CPM), a genus of motile freshwater bacteria with one known species, *Hylemonella gracilis*; and *Rhodoferax* (Betaproteobacteria; 40.7 CPM), facultative anaerobes within the purple nonsulfur bacteria which are well known for their versatile metabolism including their ability to degrade aromatic compounds. The majority of MAGs (93%) encoded only a single ARG. The highest ARG count was observed in bin.1472 (*Hylemonella*), which carried three ARGs. Virulence factor counts were more variable, ranging from 1 to 51 per MAG and were particularly high in some of the most abundant genera.

Eight MAGs that were originally taxonomically assigned to the genus *Mycobacterium* using GTDB-Tk, based on genome-wide phylogenetic placement. To refine these assignments, 5S and 16S rRNA sequences were extracted using Barnnap, allowing species-level comparisons with reference genomes. All eight MAGs were classified as nontuberculous mycobacteria (NTM) (**Figure 5**). One MAG (bin.57) lacked a detectable marker gene and could not be resolved beyond the genus level. Five MAGs were most closely related to environmental *Mycolicibacterium* species. bin.1318 and bin.1512 shared 98.7% identity with *Mycolicibacterium anyangense* (AP022620.1); a species originally isolated from cattle; bin.1372 had 100% identity to *Mycolicibacterium helvum* (APO22596.1), a species recovered from pitcher plants; and bin.857 showed 98.7% identity to *Mycolicibacterium diernhoferi* (CP080332.1), a soil bacterium. The remaining three MAGs correspond to NTM with known or suspected pathogenicity. bin.201 and bin 1264 both shared 100% sequence identity to *Mycolicibacterium litorale* (CP019882.10), a recently described species implicated in opportunistic human infections [48,49]. Similarly, bin.734 showed 100% identity to *Mycobacterium* sp EPa45 (CP011773.1), a strain whose genome encodes enzymes closely related to those found in pathogenic *Mycobacterium* species [50,51].

All eight MAGs encoded the antibiotic resistance gene *rbpA* (ARO:3000245), which codes for an RNA-polymerase conferring resistance to rifamycin (**Table S4**). In addition, each MAG contained 20 to 32 putative virulence factor genes (**Table S5**) spanning diverse functional categories including adherence, immune modulation, stress survival, regulation, effector delivery systems and nutritional/metabolic processes. With the exception of bin.1318, all MAGs carried genes encoding the antigen 85 complex (*fbpA, fbpB, fbpC*), a conserved set of mycobacterial adhesins. Multiple regulatory systems known to control mycobacterial pathogenicity were also present. These included two-component systems (*phoPR* in six MAGs; *mprAB* in seven MAGs), the iron uptake regulator *ideR* (six MAGs), and the dormancy survival regulator *dosR* (seven MAGs). Genes involved in host immune evasion were common and included components of the arabinogalactan/lipid biosynthesis pathway, such as *embC* (found in six MAGs). Notably, multiple MAGs carried components of Type VII secretion systems. For example, bin.1512 encoded ESX-1 secretion ATPases (*eccA1*, *eccCa1*/*eccCb1*), bin.857 contained an ESX-3 locus (*eccC3*, *esxG*/*esxH*), and two MAGs (bin.201 and bin.1264) harboured ESX-5 associated genes (*eccb5*/*eccC5* and PE/PPE-family effectors). These NTM genomes harbour a diverse array of virulence factors often orthologous to *M. tuberculosis* virulence determinants, indicating potential pathogenicity within the group.

Mobilome analysis and co-localisation of ARGS and mobile genetic elements (MGEs)

A total of 102 MGEs were identified in MAGs recovered from the water harvesting ponds, including plasmids (*n*=97) and insertion sequences (*n*=5) (**Table S7**). No integrons, phages or transposons were detected. MGEs were detected in 20 MAGs, of which two, bin.857 (*Mycobacterium*) and bin.931 (*Azospirillum*), also harboured ARGs (**Table S4**). bin.857 was the only MAG to contain multiple types of MGE (plasmid and insertion sequences). To assess horizontal gene transfer (HGT) potential, co-localisation analysis of ARGs and MGEs in these two MAGs found no ARGs located within 5kb of MGEs suggesting a low likelihood of ARG mobilisation within these genomes.

MetaCompare assesses resistome risk at the level of individual assembled contigs, scoring each contig for co-occurrence of ARG, MGE and pathogen taxonomy marker within a defined proximity window, before aggregating per-contig scores into a single composite risk score per

sample. As this operates directly on the full contig set generated by assembly, rather than genome bins, it inherently captures ARGs and MGEs on contigs that were not subsequently recovered as MAGs, in addition to those that were. All pond samples had MetaCompare resistome scores <25 (mean±SE: 22.42±0.14) placing them in the 'moderate-risk' category for aquatic environments as defined by Yang et al. (2025) [52] (**Table S8**). No significant differences were observed between most ponds, with the exception of Pond 9 (21.35±0.15), which had a significantly lower score compared to Pond 7 (23.61±0.68) and Pond 8 (23.59±0.22) (ANOVA, $F(15,32) = 3.561$, $P = 0.00125$) (**Figure S8; Table S8**). This elevated risk in Ponds 7 and 8 was associated with a 1.58-fold higher proportion of contigs containing ARGs relative to Pond 9 (**Figure S9; Table S8**).

To contextualise these results, we compared resistome scores in our samples MetaCompare scores reported by Yang et al. (2025) [52] (**Figure 6**). The ponds had an average resistome risk score of 22.4±0.14 which was significantly lower than those of slaughterhouse wastewater (59.6) and hospital wastewater (32.5) (Tukey's HSD, $P < 0.05$), but did not differ significantly from those of municipal wastewater (30.6), swine wastewater (26.9), groundwater (25.7), drinking water (23.2) or marine water (20.3). Pond scores therefore fell within the range reported for natural waters (20.3-25.7). Together with the absence of ARG-MGE co-localisation within MAGs and the limited detection of MGEs, this suggests a low potential for HGT in the water harvesting ponds sampled.

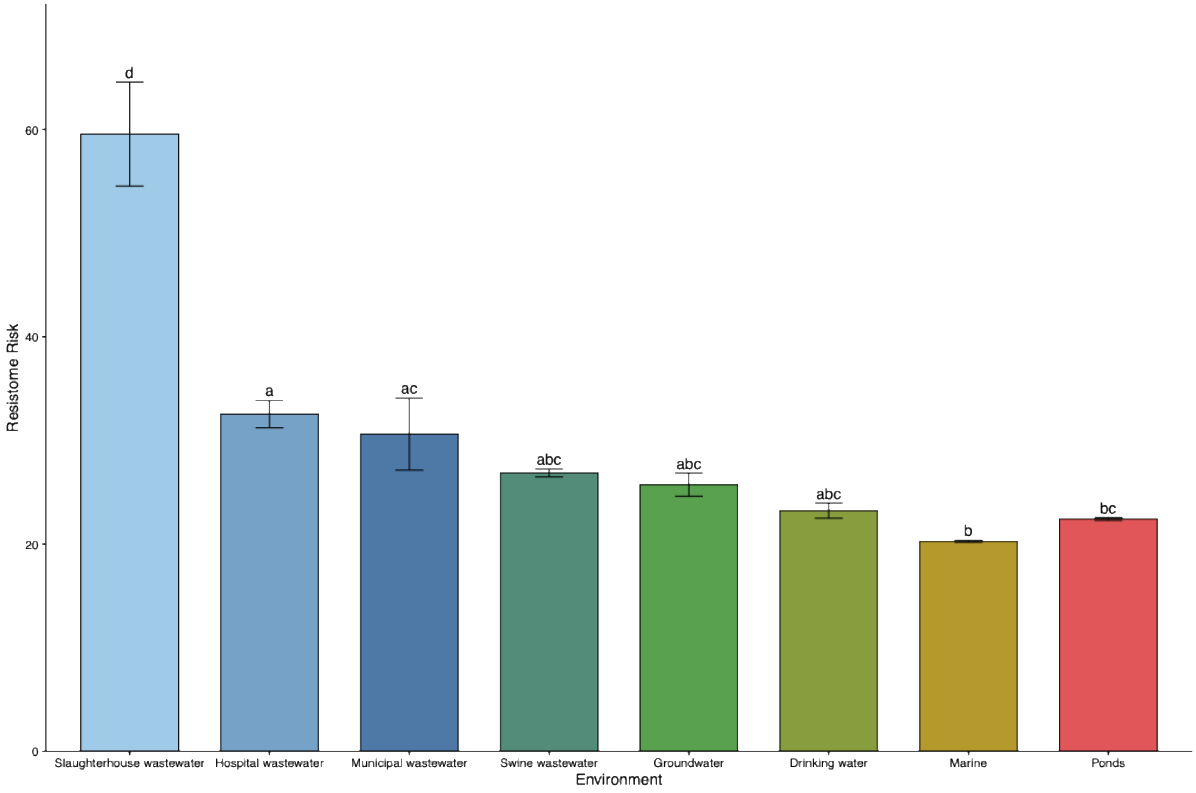


Figure 6 – Comparison of resistome risk scores (±SE) in different aquatic environments with established resistome risk rankings calculated using MetaCompare. The ponds samples in this study are shown in red; reference environments, each shown in a distinct colour, are from Yang et al. (2025) [52]. Bars sharing the same letter are not significantly different based on Tukey's HSD test ($P < 0.05$).

Discussion

Antimicrobial resistance (AMR) in water harvesting ponds reflects local agricultural use

Our analysis reveals that antimicrobial resistance patterns in smallholder managed water harvesting ponds reflects the antibiotics commonly used in local livestock farming. Among the 582 ARG subtypes identified, bacitracin resistance genes, particularly *bacA*, dominated the resistome, accounting for nearly half of the total ARG reads across all sampled ponds. This finding aligns with field studies indicating widespread and often unregulated use of bacitracin in Kenyan poultry farming, where it is commonly used as a prophylactic feed additive and growth promoter. Recent nationwide and county surveys found ~80% of farming households used antimicrobials, with most administering them without veterinary prescription [53,54]. In many rural areas, bacitracin (in the form of zinc bacitracin) is inexpensive, easily accessible through agrovets without a prescription, and considered a routine supplement for broilers and layers. *bacA* is not typically carried on plasmids and is usually a chromosomal, intrinsic gene [55]. Therefore, the detection of *bacA* likely reflects intrinsic bacitracin resistance of the bacterial community, rather than recent acquisition via mobile elements.

Consistent with *bacA* being an intrinsic, chromosomally encoded gene rather than one associated with recent horizontal acquisition, resistome composition broadly tracked overall bacterial community composition rather than being concentrated within a small number of taxa. Bray-Curtis dissimilarities in genus-level community composition and resistome composition were significantly correlated at both the sample level and pond level, with the clearest correspondence at ARG-subtype resolution. However, no individual genus was significantly associated with any specific ARG subtype after correction for multiple testing among 2,370 pond level comparisons. This indicates that the correspondence reflects broad, distributed covariation across many taxa and genes, rather than concentrated carriage by a small number of identifiable taxa. This pattern is consistent with the resistome being shaped by a taxonomically widespread, intrinsic resistance profile within the bacterial community, rather than acquisition or carriage within one or a few dominant lineages.

Consistent with this, neither total ARG abundance or resistome composition was significantly associated with pond nutrient chemistry (ammonium, nitrate, phosphate, sulphate). This absence of a nutrient signal is more consistent with the resistome being shaped by antimicrobial usage practice and the intrinsic resistance profile of the resident bacterial community, than by variation in ambient nutrient status.

Interestingly, resistance to β-lactams, one of the most widely used antibiotic classes in both human and veterinary medicine, was rare in the ponds, comprising <4% of total ARG reads. This is consistent with previous studies that show resistance genes encoding extended-spectrum β-lactamases (ESBLs) are more common in urban areas, but occur at lower levels in rural, low density agricultural environments with reduced pharmaceutical usage [56,57]. For example, in a Kenyan village, only ~10% of environmental *E. coli* isolates from soil or water samples carried any *bla_CTX-M* variants [58]. The low detection in these rural ponds likely reflects both reduced β-lactam usage in smallholder settings and potential environmental degradation of these compounds before they reach the ponds. Similarly, no tetracycline destructase genes (e.g. *tet(X)*) were detected. In contrast, 12 variants of the mobile colistin resistance gene *mcr* were detected in the read-based resistome, albeit at very low abundance (9.84×10^{-6} copies/cell; <0.01% of total ARG abundance), and none were recovered in MAGs. As short-read annotation cannot resolve full-length genes or their genomic context, these hits may include chromosomal *mcr*-like phosphoethanolamine transferases of environmental bacteria rather than plasmid-borne *mcr*, and their hosts and mobility could not be determined.

Across all reconstructed MAGs, only two showed any co-localisation of ARGs with mobile genetic elements, and none showed ARGS integrated within 5 kb of integrases, plasmid markers, or phage sequences. This suggests horizontal gene transfer plays a minor role in these habitats. While this could imply a reduced risk of ARG exchange and spread, it also points to persistent environmental reservoirs that could mobilise under future selection pressures, particularly if antimicrobial use or agricultural runoff increases.

This is consistent with the ARG content of individual MAGs, which was low and uniform. 93% of MAGs encoded only a single ARG, with a maximum of three in bin.1472 (*Hylemonella*), a pattern typical of intrinsic, chromosomally encoded resistance rather than genes recently acquired via horizontal transfer. The dominant ARG in this dataset, *bacA*, encodes undecaprenyl pyrophosphate phosphatase (UppP), an enzyme required for regenerating the lipid carrier used in peptidoglycan and cell-wall polymer synthesis [59]. Its role in bacitracin resistance is a coincidental consequence of this essential housekeeping function, which explains its broad conservation as a single-copy chromosomal gene rather than an amplified or mobile determinant. This is supported by previous studies which show resistomes in freshwater environments are strongly influenced by the composition of indigenous bacterial populations and local environmental conditions, with a comparatively small proportion of ARGs associated with plasmids or other indicators of recent horizontal transfer [60,61].

In contrast, virulence factor (VF) counts varied widely among MAGs (1-51 VFs), including within the most abundant genera (e.g. bin.1492, Cellvibrio, 51 VFs). Virulence factors are disproportionately encoded in the bacterial accessory genome and are frequently acquired via pathogenicity islands and other mobile elements, which can introduce substantial differences in virulence gene content even between closely related lineages [62]. Many of the genes classified as virulence factors in environmental, non-pathogenic taxa are dual function traits, including motility, secretion systems and adherence factors, that are more plausibly under selection for competition or resistance to protozoan predation than for pathogenesis [63,64]. The high VF count in *Cellvibrio,* is consistent with this genus's extensive carbohydrate-active enzyme and secretion system used for polysaccharide degradation in the environment, rather than an indication of pathogenic potential. This asymmetry between low, uniform ARG counts and highly variable VF counts across MAGs has also been reported in other environmental surveys, including a study of MAGs recovered from an agricultural pond, in which virulence factors comprised largely flagellar secretion-systems and adherence genes, rather than genes associated with pathogenicity [65].

Environmental mycobacteria pose One Health risks

Eight of the MAGs recovered from the water harvesting ponds were assigned to the non-tuberculosis mycobacterial (NTM) genus *Mycolicibacterium*. Like other NTM bacteria, *Mycolicibacterium* spp. are environmentally robust and common across a wide range of environments, for example animal impacted freshwaters [66] and have also previously been detected in drinking water systems [67–69]. *Mycolicibacterium* spp. are frequently isolated from clinical samples of humans and animals [70–74], highlighting their significance as an opportunistic pathogen that bridges across all One-Health contexts. *Mycolicibacterium* spp. are commonly resistant to a range of antibiotics, in particular macrolides, beta-lactams, aminoglycosides, and rifampin [75,76]. They have also been shown to have resilience in chlorinated drinking water [68], making their detection in water harvesting ponds here (that are used for crop watering as well as animal and potential human drinking water) a concern, as removal might be challenging. All the *Mycolicibacterium* MAGs recovered here contained a range of virulence genes (**Table S5**), including Type VII secretion systems (ESX-1, ESX-3, ESX-5) which are implicated in the virulence of *Mycolicibacterium* spp. and previously identified in genomic analysis of *Mycolicibacterium fortuitum* [75]. All MAGs also contained the *rbpA* gene, conferring resistance to rifampin. This gene was also found to be ubiquitous in *Mycolicibacterium fortuitum* genomes investigated from a range of environments (human, soil, fish, companion animal) with samples covering every continent except Europe [75]. Therefore, our study further strengthens evidence that *Mycolicibacterium* spp. have high rates of (or even innate) resistance to rifampin. Across this study MAGs assigned to *Mycolicibacterium* spp. were detected in every pond, with all MAGs containing a range of resistance and virulence genes. Given their ubiquity here, and as outlined previously, their wide-ranging prevalence in the environment (including in drinking water), potential resistance to chlorine treatment, resistance to antibiotics, and records of infections in humans and animals there is a clear risk to health across the One-Health continuum, with water harvesting ponds acting as a conduit

for transmission and infection. The increase resistance to rifamycin is also particularly alarming in the context of the usage of these antibiotics to treat severe cases of carbapenem-resistant bacterial infections [77]. In the last few years, Kenyan hospitals have seen an increase of carbapenem- resistant infection between 7-17% with a third of the cases classified as difficult to treat [78]. It should be noted that a further challenge to mitigating this risk is that *Mycolicibacterium* were recently reclassified from the *Mycobacterium* genus (e.g. *Mycobacterium tuberculosis*) [79]. The reclassification of *Mycobacterium* into five new genera may be justified based on genomic analysis but has the potential confusion [80] in clinical and regulatory contexts.

Policy and on-farm mitigation strategies

Given that bacitracin resistance (*bacA*) was the dominant ARG signal in this study, and reflects widespread, unregulated use of bacitracin as an unprescribed poultry feed additive, the most directly relevant policy lever is stronger enforcement of Kenya's existing National Action Plan for AMR [81]. This plan already prohibits antibiotic use for growth promotion and mandates prescription-only veterinary access. Enhanced licensing and inspection of agrovet outlets, combined with expansion of One Health AMR surveillance to include environmental compartments, such as water harvesting ponds, would directly target the informal antibiotic distribution pathways implicated by our findings [53][82,83].

At the farm level, our field survey found that only one of the samples sites had any barrier preventing livestock access, despite this being a recommended safety measure in pond construction guidance. Given the limited evidence for horizontal gene transfer or mobile ARG carriage in this study, restricting direct livestock contact, through fencing and alternative watering points, both shown to reduce faecal and bacterial contamination of surface water [84,85], represents the most directly evidenced intervention for these ponds. Composting of livestock manure [86,87] and vegetated buffer strips [88,89] are both established interventions for reducing ARG loading in agricultural runoff and could plausibly be adapted to smallholder settings in Kenya. As this study did not directly assess manure management or runoff pathways at these sites, we present these as general, literature-supported recommendations, rather than measures directly evidenced by our data.

Together these measures, targeting the specific point source (unregulated bacitracin use) and transmission pathway (livestock access) identified in this study, alongside broader, literature supported farm level practices, offer a strategy for reducing AMR dissemination from smallholder water harvesting ponds in rural Kenya.

Limitations

This study has several limitations. Firstly, the narrow range of filtered volumes (6.54-15.3 ml) most likely reflects rapid clogging of the 0.2 µm filters by suspended solids or organic matter. Turbidity and total suspended solids were not measured for these ponds, so this could not be formally tested. ARG abundances were normalised to copies per cell, which controls for variation in DNA input regardless of its cause, so this is not expected to bias the resistome comparisons reported here. Secondly, no negative or positive extraction/sequencing controls were processed alongside the samples. While sterile single-use filters and per-replicate filter changes limit cross-contamination, low biomass contamination cannot be fully excluded, and this should be considered when interpreting low-abundance ARG and LARG signals. ARG and MAG recovery relied on a co-assembly of all 48 samples rather than per-sample assemblies. This approach improves recovery of genomes shared across ponds but present at low individual coverage. However, it may favour recovery of taxa abundant across multiple ponds and could reduce sensitivity to strain-level variation within individual ponds.

Conclusions

In conclusion, this study provides one of the first high-resolution resistome profiles of smallholder managed water harvesting ponds in sub-Saharan Africa. We show that while overall resistome risk is moderate, these ponds consistently harbour ARGs linked to

agricultural use, particularly *bacA*, conferring resistance to bacitracin. The identification of multidrug-resistance and virulence-associated *Mycolicibacterium* spp. underscores the potential for these water systems to act as environmental reservoirs and transmission points for opportunistic pathogens. Although mobile genetic elements and B-lactamases were rare, the persistence of ARGs in core environmental taxa highlights the role of unmanaged agricultural runoff and livestock in shaping aquatic microbiomes.

As water harvesting ponds continue to expand across drought prone regions of Africa, minimising the presence of pathogens and AMR is crucial. Practical low-cost interventions, such as composting livestock waste, installing buffer vegetation and better management of livestock should reduce the presence of ARB. At the policy level, sustained investment in antimicrobial stewardship, environmental surveillance and farmer education will be essential to reduce AMR. Our findings demonstrate the value of integrating environmental metagenomics with One Health approaches to inform policy, improve rural water management and reduce AMR transmission risks in smallholder farming systems.

Ethics declarations

Ethics approval and consent to participate

This study involved environmental surface water sampling from private water harvesting ponds in Laikipia County, Kenya. The overarching project received institutional ethical approval from the University of Essex (approved 12/11/2015 and 17/02/17 for data collection spanning 2016–2019) and research permits from local agricultural ministry offices, authorized by the County Director of Agriculture. Audio-recorded verbal informed consent for site access and sampling was obtained from all landowners prior to collection, and all farm visits were conducted in the presence of local agricultural extension officers.

Consent for publication

All authors consent to the publication of this article

Competing interests

The authors declare they have no competing interest

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Other information

Clinical trial number: not applicable

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Data availability

Shotgun metagenomic data can be accessed in the NCBI Sequence Read Archive under the following link: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1371399/

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