FEMS Microbiology Ecology, 2024, **100**, fiae155
DOI: 10.1093/femsec/fiae155
Advance access publication date: 9 November 2024
Research Article

MetaCompare 2.0: differential ranking of ecological and human health resistome risks

Monjura Afrin Rumi[1], Min Oh[1,2], Benjamin C. Davis[3,4], Connor L. Brown[4], Adheesh Juvekar[1], Peter J. Vikesland[4], Amy Pruden[4], Liqing Zhang[1,*]

[1]Department of Computer Science, Virginia Tech, Blacksburg, VA 24060, USA
[2]Microsoft Research, Redmond, 98052 WA, USA
[3]Office of Research and Development, US Environmental Protection Agency, Cincinnati, OH 45268, USA
[4]Department of Civil & Environmental Engineering, Virginia Tech, Blacksburg, VA 24060, USA
*Corresponding author. Department of Computer Science, Virginia Tech, Blacksburg, VA 24060, USA. E-mail: lqzhang@vt.edu
Editor: [Max Haggblom]

Abstract

While numerous environmental factors contribute to the spread of antibiotic resistance genes (ARGs), quantifying their relative contributions remains a fundamental challenge. Similarly, it is important to differentiate acute human health risks from environmental exposure, versus broader ecological risk of ARG evolution and spread across microbial taxa. Recent studies have proposed various methods for achieving such aims. Here, we introduce MetaCompare 2.0, which improves upon original MetaCompare pipeline by differentiating indicators of human health resistome risk (potential for human pathogens of acute resistance concern to acquire ARGs) from ecological resistome risk (overall mobility of ARGs and potential for pathogen acquisition). The updated pipeline's sensitivity was demonstrated by analyzing diverse publicly-available metagenomes from wastewater, surface water, soil, sediment, human gut, and synthetic microbial communities. MetaCompare 2.0 provided distinct rankings of the metagenomes according to both human health resistome risk and ecological resistome risk, with both scores trending higher when influenced by anthropogenic impact or other stress. We evaluated the robustness of the pipeline to sequence assembly methods, sequencing depth, contig count, and metagenomic library coverage bias. The risk scores were remarkably consistent despite variations in these technological aspects. We packaged the improved pipeline into a publicly-available web service (http://metacompare.cs.vt.edu/) that provides an easy-to-use interface for computing resistome risk scores and visualizing results.

Keywords: antibiotic resistance gene; assembly method; ecological resistome risk; human health resistome risk; resistome risk; sequencing depth

Introduction

Antibiotic resistance is a global public health threat, resulting in an increasing rate of human morbidity and mortality worldwide (O'Neill 2016). There are numerous sources, pathways, and factors that contribute to the evolution and spread of antibiotic resistance, which makes it difficult to pinpoint precise interventions that can help attenuate the carriage of antibiotic resistance genes (ARGs) by human pathogens. It is increasingly being recognized that mitigation efforts must move beyond a myopic focus on clinical settings and must address environmental sources and ecological processes that contribute to the spread of resistance (Berendonk et al. 2015; UNEP 2023). Environmental sources of concern include untreated sewage, wastewater treatment plant (WWTP) effluent, livestock waste, surface water runoff, landfill leachate, and pharmaceutical manufacturing waste (Bengtsson-Palme et al. 2018).

To effectively inform strategies to mitigate the spread of antibiotic resistance, a systematic and quantitative means of comparing putative sources of ARGs and their potential to be acquired by pathogens is required (Martínez et al. 2015, Hernando-Amado et al. 2019). For this purpose, MetaCompare (Oh et al. 2018), herein referred to as MetaCompare 1.0, was introduced as the first computational pipeline to quantify and rank the "resistome risk" of various environments. "Resistome risk" refers to the conceptual framework introduced by Martínez et al. (2015), in which it is assumed that ARGs that (i) confer resistance to antibiotics currently used for therapeutic purposes, (ii) are associated with mobile genetic elements (MGEs), and (iii) are carried by human pathogens represent the greatest public health risk. MetaCompare 1.0 was developed as a pipeline to put this concept into practice and introduced a reified resistome risk metric (Oh et al. 2018). Comparing the resistome risk metric across environments can serve as a means to identify potential "hot spots" for mobilization of antibiotic resistance to pathogens, which can then be prioritized for targeted mitigation. A proof-of-concept experiment using publicly available metagenomic datasets demonstrated that MetaCompare 1.0 provided a ranking of resistome risk consistent with expectations. Specifically, the pipeline ranked resistomes in order of hospital sewage as having the highest risk scores, dairy lagoons as having moderate risk scores, and WWTP effluent as having the lowest risk scores (Oh et al. 2018). MetaCompare 1.0 has now been widely applied, providing insight into potential critical control points for ARG transmission to pathogens across a wide variety of agricultural, wastewater, and other environmental systems, e.g. (Karkman et al. 2019, Rice et al. 2020, Keenum et al. 2021, Majeed et al. 2021, Wind et al. 2021a, Wu et al. 2022, Zhang et al. 2022a).

Received 14 March 2024; **revised** 27 June 2024; **accepted** 8 November 2024

Other approaches have been proposed to assess and rank the risks of individual ARGs in various environments. For example, Slizovskiy et al. (2020) proposed a metric called the "mobility index," which considers colocalization of ARGs with MGE-markers on the same contig in a sample, but does not take into account the presence of pathogens. Zhang et al. (2021) developed a method to rank ARGs in terms of their anthropogenic enrichment, association with MGEs (mobility), and human pathogens (host pathogenicity) into four categories, with Rank I being the highest risk category and Rank IV being the lowest. Subsequently, Zhang et al. (2022c) defined a "risk index" to categorize ARGs prevalent only in human-associated environments considering the clinical availability of antibiotics, mobility, host pathogenicity, and potential of transmission of ARGs from environment to humans. A key distinction relative to other such ARG risk ranking systems is that MetaCompare resistome risk scores are defined for the entire collection of ARGs detected in a sample, whereas the latter provides a ranking system only for individual ARGs. In other words, MetaCompare incorporates multiple lines of evidence into a single metric reflecting the overall resistome risk of a particular environment.

While it is widely recognized that a risk assessment framework is needed to address environmental dimensions of antibiotic resistance (UNEP 2023), a challenge is that it does not fit the mold of conventional microbial risk assessment (Ashbolt et al. 2013). Specifically, there are multiple bacterial pathogens of concern and thousands of ARGs. Considering exposures to individual resistant pathogens can inform quantitative microbial risk assessment (Garner et al. 2021, Schoen et al. 2021), but evolution and horizontal gene transfer of ARGs moving across microbial communities, including both pathogens and non-pathogens, is arguably of equal concern if the aim is to mitigate the acquisition of ARGs by pathogens in the first place. Here we use the term "risk" broadly as a general relative comparison, as frameworks remain to be adapted to move towards estimating probabilities of resistant infections from dose-response of various resistome exposures. From a human health risk standpoint, the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), have been recognized as World Health Organization priority pathogens that tend to be highly virulent and antibiotic resistant (WHO 2017). ESKAPE pathogens have also been shown to be enriched with a specific subset of acquired (i.e., not belonging to their core genome), mobile ARGs in anthropogenically impacted environments (Zhang et al. 2021). From an ecological standpoint, a major limitation has been a lack of a suitable database for accurate annotation of MGEs. Inclusion of accessory or cargo sequences, including ARGs, in public MGE databases, such as A CLAssification of Mobile genetic Elements (ACLAME) (Leplae et al. 2010), has undoubtedly led to false positives (e.g., an ARG being annotated as an MGE) (Slizovskiy et al. 2020). Finally, it is not clear how differing sequencing and analysis approaches affect the risk scores. In the case of MetaCompare 1.0, *de novo* assembly of contigs is required, the representativeness of which is directly affected by sequencing depth, chosen assembler, and microbial diversity associated with the sample complexity.

Here we introduce MetaCompare 2.0, which incorporates several improvements to address the above-noted limitations of MetaCompare 1.0 and other ARG risk ranking approaches. Specifically, we introduce two distinct resistome risk scores, one corresponding to the ecological resistome risk (ERR) and the other to the human health resistome risk (HHRR). The ERR score factors in a wide-ranging array of pathogens and ARGs in order to broadly represent the potential for ARGs to mobilize in a given environment. The HHRR, on the other hand, focuses more specifically on pathogens that are of most acute concern with regard to antibiotic-resistant infections in humans (i.e,. ESKAPEE pathogens) and Rank I ARGs (Zhang et al. 2021). For both indices, the annotation methodology for the taxonomic assignment of potential pathogens was improved by using "many-against-many" sequence searching (MMseqs2), a tool designed specifically for taxonomy assignment (Steinegger and Söding 2017). Inaccuracies in MGE annotation were addressed by incorporating an updated MGE database (mobileOG-DB) (Brown et al. 2022). To provide a more intuitive output for comparison, the range of possible values for risk scores was set using a 0–100 scale. In the case of the ERR specifically, DeepARG database (DeepARG-DB) (Arango-Argoty et al. 2018), a database built specifically to capture environmental ARGs, was applied to broadly consider the potential for ARGs to evolve and spread. To assess an expanded range of feasible output resistome risk values, the updated pipeline was applied to publicly-available metagenomes representing a wide range of environments, including wastewater, surface water, soil, sediment, and gut microbiomes. MetaCompare 2.0 was further validated by applying it to samples representing a range of sequencing depths, assembler methods, and assembly sizes. Lastly, the pipeline was made more accessible through a web service that allows users to compute the risk scores of their metagenomic data and visualize annotations of assembled sequences in various dimensions.

Methods

Overview of pipeline

MetaCompare 2.0 employs two computational branches that can be selected by the user (Fig. 1). One branch assesses the ERR and the other assesses the HHRR. The ERR evaluates a broad array of both known and putative ARGs, their co-occurrence with MGEs, and a full range of human bacterial pathogens annotated in the metagenome to capture their probable contribution to the proliferation of antibiotic resistance in corresponding environments. The HHRR focuses on a narrower set of ARGs, defined by Zhang et al. (2021) as Rank I ARGs, that are (i) demonstrated to be enriched in "human-associated" environments, (ii) mobile (carried by MGE), and (iii) can be carried by ESKAPE pathogens. The range of pathogens used in HHRR was confined to the ESKAPE pathogens as well as any contig annotated as Enterobacteriaceae. This was done to include *Escherichia coli* (as in the "ESKAPEE") (Yu et al. 2020, Ruekit et al. 2022) as well as to ensure that contigs without complete taxonomic annotations, but which are likely derived from the ESKAPE organisms, would be captured. This is common for contigs originating from ESKAPE-associated plasmids and MGEs (Brown et al. 2023, Ji et al. 2023). This approach also covers genetically related non-pathogenic strains that have the greatest potential to participate in gene exchange with pathogenic ESKAPEE strains.

MetaCompare 2.0 follows the computational approach adopted in MetaCompare 1.0, with some modifications. MetaCompare 1.0 takes assembled contigs as input and annotates ARGs, MGEs, and putative association with known human pathogens. Subsequently, these contigs are classified into three categories: (i) those containing one or more ARGs, (ii) those containing one or more ARGs and one or more MGEs, or (iii) those containing one or more ARGs, MGEs, and alignment to known human pathogens. The numbers of contigs belonging to these three categories are subsequently normalized by the total number of contigs using Equations 1–3. The normalization is unweighted in terms of the

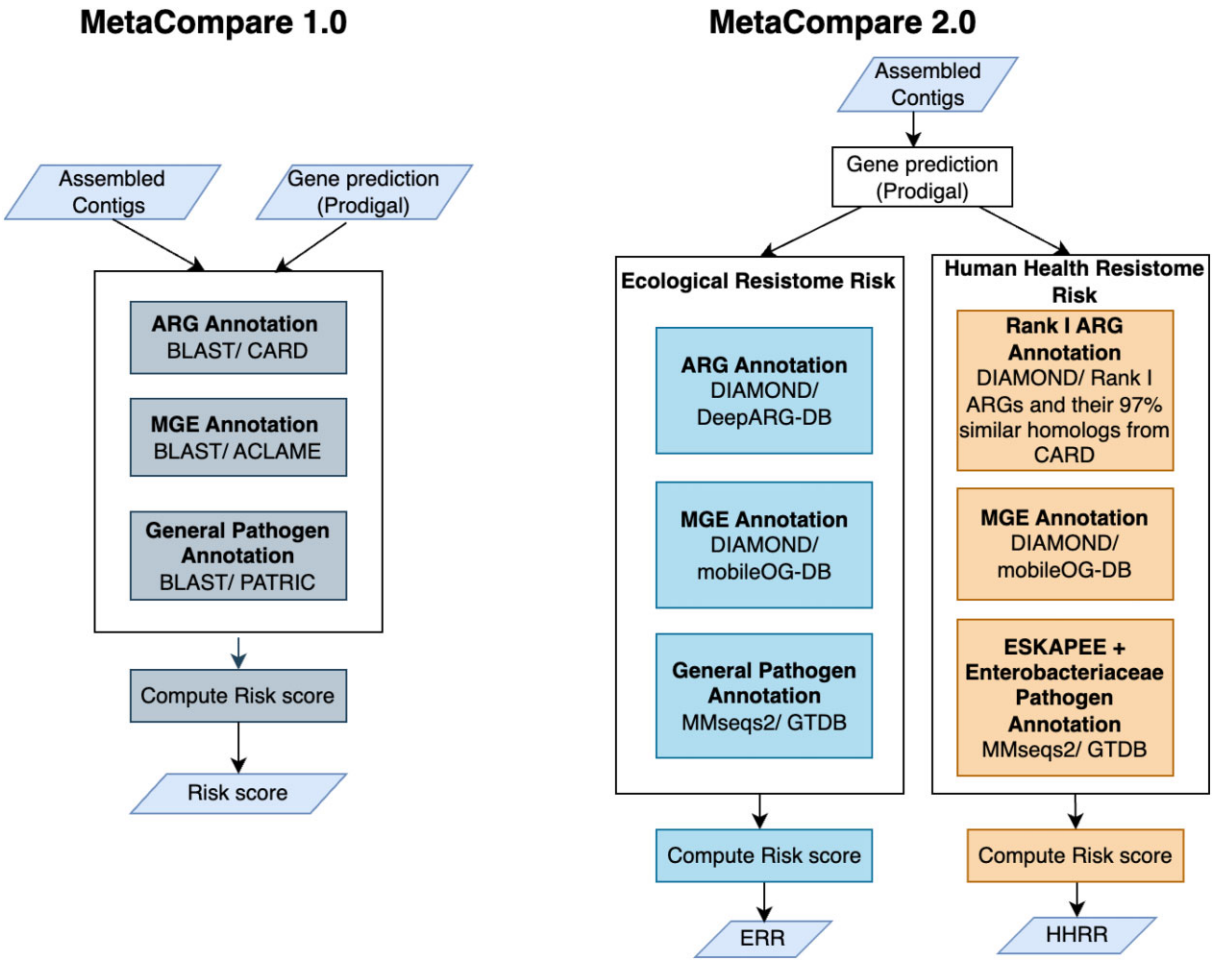

Figure 1. Modifications of the MetaCompare 2.0 pipeline relative to the MetaCompare 1.0 pipeline.

number of ARGs or MGEs co-occurring on a contig. Since a pathogen with an ARG is still a human health concern, even if it cannot be demonstrated mobile, a new category has been added in MetaCompare 2.0 for contigs containing one or more ARGs and alignment to known human pathogens (Equation 4).

$$Q_{ARG} = N_{ARG}/N_{Contigs}, \quad (1)$$

$$Q_{ARG,\,MGE} = N_{ARG,\,MGE}/N_{Contigs}, \quad (2)$$

$$Q_{ARG,\,MGE,\,PAT} = N_{ARG,\,MGE,\,PAT}/N_{Contigs}, \textit{ and} \quad (3)$$

$$Q_{ARG,\,PAT} = N_{ARG,\,PAT}/N_{Contigs}. \quad (4)$$

Here, $N_{Contigs}$ is the total number of contigs in the sample and N_{ARG}, $N_{ARG,\,MGE}$, $N_{ARG,\,MGE,\,PAT}$, and $N_{ARG,\,PAT}$ are the numbers of contigs that contain regions annotated as ARGs only, contain annotated regions indicating that ARGs are proximal to MGEs, contain annotated regions indicating that MGE-associated ARGs are carried within a pathogen, and contain annotated regions indicating that an ARG is carried by a pathogen, respectively.

Additional modifications were introduced to make the risk score output more intuitive. MetaCompare 1.0 calculates the risk score by projecting the samples in a 3-dimensional (3D) space termed as "hazard space," each dimension corresponding to the proportions of contigs annotated as carrying ARGs, carrying ARGs and MGEs, and carrying ARGs, MGEs, and having alignment to known human pathogens, respectively. An empirical theoretical maximum point, indicating the highest value any Q in Equations 1–3 can reach, was set to (0.01, 0.01, and 0.01) based on the result of a simulation utilizing the prevalence data in the Comprehensive Antibiotic Resistance Database (CARD) (Jia et al. 2017). However, subsequent studies obtained values exceeding this maximum threshold (Qin et al. 2020). Therefore, we reset the maximum point to (1.0, 1.0, and 1.0) to calculate d_s, the Euclidean distance of the sample to the maximal point in the 3D hazard space with dimensions Q_{ARG}, $Q_{ARG,\,MGE}$, $Q_{ARG,\,MGE,\,and\,PAT}$. We also calculated d_w, the Euclidean distance of a sample containing no ARGs (i.e. Q_{ARG}, $Q_{ARG,\,MGE}$, $Q_{ARG,\,MGE,\,and\,PAT}$ are all 0), and used d_w to normalize d_s. The basic framework of the 3D hazard space was maintained in the calculation of ERR in MetaCompare 2.0. However, a 4th dimension, QARG, PAT, was added for HHRR calculation. The risk score is simplified as follows:

$$\text{Risk Score} = (1 - d_s/d_w) \times 10^4. \quad (5)$$

By removing the distance inversion and logarithmic scaling used in MetaCompare 1.0, we have alleviated the problem of producing a theoretically infinite score. Instead, we have set the minimum score to 0, where it was originally 17.57. The maximum score is now set at 100, by incorporating a multiplication factor of 10^4 (Equation 5).

Table 1. Summary of updates in MetaCompare 2.0 relative to MetaCompare 1.0.

		MetaCompare 2.0	
Databases	**MetaCompare 1.0**	**Ecological resistome risk**	**Human health resistome risk**
ARG annotation	CARD (Jia et al. 2017)	DeepARG-DB (Arango-Argoty et al. 2018)	799 genes from CARD (Rank 1 ARGs and their 97% similar homologs)
MGE annotation	ACLAME (Leplae et al. 2010)	MobileOG-DB (Brown et al. 2022) (All reference proteins)	
Pathogen annotation	24 human bacterial pathogens in PATRIC (Wattam et al. 2017)	GTDB (Parks et al. 2022) to 538 human bacterial pathogens (Li et al. 2015a, Woolhouse et al. 2012)	ESKAPEE + Enterobacteriaceae pathogens
Alignment algorithm	BLAST (Boratyn et al. 2013)	DIAMOND (Buchfink et al. 2015) + MMseqs2 (Steinegger and Söding 2017)	

Updates in tools and databases

Updates incorporated into MetaCompare 2.0 are summarized in Table 1. While MetaCompare 1.0 used the standard Basic Local Alignment Search Tool (BLAST) (Boratyn et al. 2013), MetaCompare 2.0 incorporates DIAMOND BLASTx (Buchfink et al. 2015) for ARG and MGE annotation. DIAMOND dramatically improves processing time compared to BLAST through its employment of double indexing for search and alignment, which is more efficient for large environmental microbiome datasets. Prior studies have demonstrated consistent and highly comparable results of DIAMOND and BLAST alignment (Buchfink et al. 2015, Hernández-Salmerón and Moreno-Hagelsieb 2020). For pathogen annotation, MMseqs2 (Steinegger and Söding 2017) replaces BLAST in MetaCompare 2.0. MMseqs2 uses k-mer searches based on similarity instead of exact matches which enables it to use long k-mers without losing sensitivity. Elimination of random memory access and parallelization on multiple levels makes the runtime of MMseq2 highly scalable and thus it has become a popular tool for species/taxonomy annotation (Steinegger and Söding 2017).

The sensitivity and relevance of annotations have also been improved by updating the associated databases queried by MetaCompare 2.0. DeepARG-DB was employed for ARG annotation because it was constructed using a deep learning algorithm to capture all known and putative ARGs in a metagenome, including ones that may not yet be reported in public databases (Buchfink et al. 2015). DeepARG-DB was built by integrating multiple databases [CARD, ARGs Database (Liu and Pop 2009), and Universal Protein Resource (Apweiler et al. 2004)] in a non-redundant fashion. Expanded ARG detection is especially important for the calculation of ERR, where the aim is to assess the potential of antibiotic resistance to evolve and mobilize in a given environment. Metacompare 2.0 focuses specifically on ARGs and does not consider biocide or metal resistance genes. For MGEs, the mobile orthologous groups database (mobileOG-DB) was incorporated. Extensive manual curation was employed in mobileOG-DB to comprehensively include multiple MGE types (plasmids, transposons, integrons, etc.) while excluding accessory and cargo genes to avoid false positive annotations (Brown et al. 2022). The Genome Taxonomy Database (GTDB) (Parks et al. 2022) was used to annotate contigs for pathogens since it is currently the largest curated collection of bacterial and archaeal genome diversity. Such an extensive database is valuable in ensuring detection of essentially all potential bacterial pathogens, and, also in confidently assigning taxonomic annotation. Also, including off-target references during taxonomic annotation is crucial, as failing to do so can lead to erroneous inferences (Gihawi et al. 2023). Pathogens were classified against GTDB and filtered from MMseqs2 output using a predetermined list of 538 known, emerging, and re-emerging bacterial pathogens (Woolhouse et al. 2012, Li et al. 2015a), to expand upon the previous list of 24 pathogens queried in MetCompare 1.0 in the Pathosystems Resource Integration Center (PATRIC) database (Wattam et al. 2017). The list of included pathogens is presented in Table S1. To expand the capabilities of MetaCompare 2.0, we downloaded the list of 122 Rank I ARG references from (Zhang et al. 2021) and aligned them to the protein homolog model database of CARD (v3.2.0) (Jia et al. 2017) using BLASTp (Buchfink et al. 2015). The alignments were then filtered at ≥97% identity to expand the list of Rank I ARGs to include the original set plus their closest homologs, resulting in 799 total ARGs. The expanded list of ARGs included in HHRS calculation is reported in Table S2.

Web service

MetaCompare 2.0 has been made publicly available as a web service to increase accessibility and ease of use (http://metacompare.cs.vt.edu/). Users can upload assembled metagenomic FASTA files associated with samples of interest and can process the pipeline directly from the web server using a user-friendly graphical interface. A back-end server performs all necessary computational analysis while the front-end service presents the results in tabular format, which can be downloaded as a CSV file. In the command-line interface of MetaCompare 1.0, users were required to provide two FASTA input files: one containing the assembled contigs and the other containing their predicted protein-coding regions. In the web platform, users only need to upload the assembled contigs, and the burden of computing gene prediction is taken care of in the back-end. Finally, to provide informative output and allow users to inspect further and investigate annotated contigs, a new visualization functionality has been incorporated. Specifically, the visual output allows users to inspect which specific ARGs, MGEs, and pathogens are annotated and their relative positions on the contigs. Researchers can zoom in/out and extract corresponding ARG/MGE/pathogen DNA sequences for further analysis (Fig. 2).

MetaCompare 2.0 validation

The performance of MetaCompare 2.0 was first evaluated using the same dataset applied for MetaCompare 1.0: hospital sewage, WWTP effluent, and agricultural lagoon water (Table 2). To compare the output difference between the two versions, we first applied the Shapiro-Wilk test to ensure that the scores are normally distributed and then calculated Pearson's correlation coefficient between risk scores from MetaCompare 1.0 and MetaCompare 2.0 for this dataset.

Several areas of uncertainty in model robustness were raised during the development of the MetaCompare 1.0 pipeline. Specifically, it was uncertain whether different sample types (i.e. sample

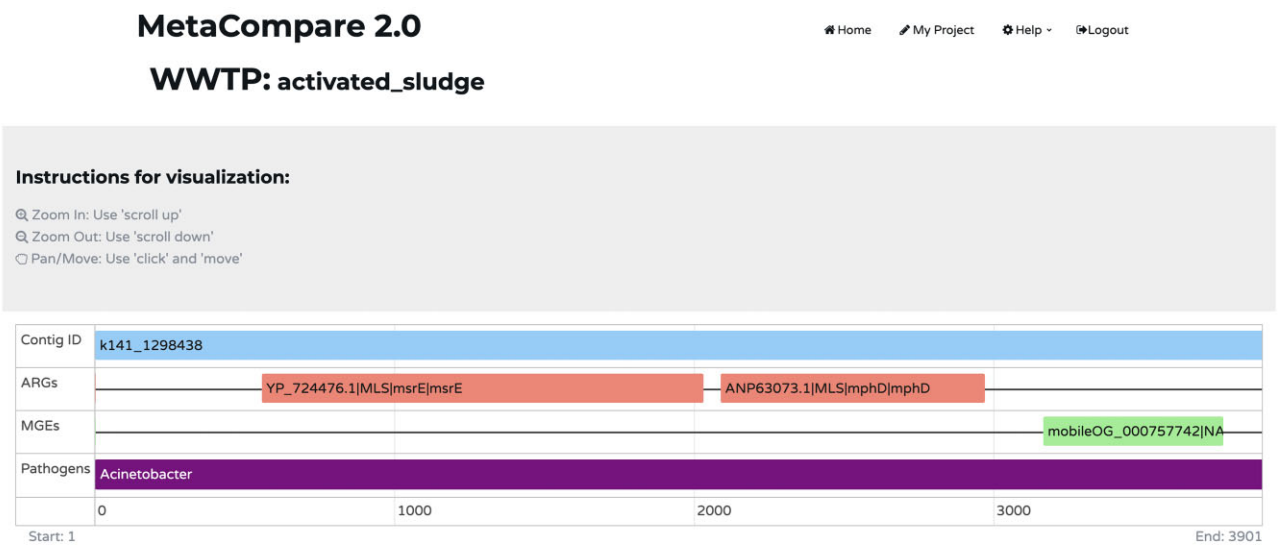

Figure 2. Visualization of ARG, MGE, and pathogen annotation of a representative contig via MetaCompare 2.0 web service.

Table 2. MetaCompare 2.0 risk scores were obtained from the original MetaCompare 1.0 validation study.

ENA accession	Sample type	MetaCompare 1.0	MetaCompare 2.0 (ERR)	MetaCompare 2.0 (HHRR)
ERS1019924	Hospital sewage	43.00	23.19	1.8
ERS1019927	Hospital sewage	39.47	18.58	1.22
ERS1019928	Hospital sewage	39.24	20.25	1.42
ERS1019923	Hospital sewage	36.23	19.24	1.52
ERS1019925	Hospital sewage	34.62	17.05	1.03
ERS1019926	Hospital sewage	34.47	16.88	1.34
ERS1019959	Dairy lagoon	29.02	10.27	1.01
ERS1019958	Dairy lagoon	26.84	11.15	0.5
ERS1019922	Dairy lagoon	24.82	6.79	0.74
ERS1019955	Dairy lagoon	24.20	6.6	0.44
ERS1019956	Dairy lagoon	22.71	7.59	0.36
ERS1019920	WWTP effluent	22.77	6.5	0.34
ERS1019947	WWTP effluent	21.59	3.96	0.17
ERS1019948	WWTP effluent	18.42	5.87	0.22

complexity), assemblers, library coverage, and the total number of contigs have an effect on the computed risk scores (Brown et al. 2021, Wind et al. 2021b). To challenge both ERR and HHRR models, we collected publicly available Illumina short-reads from NCBI, along with an internal archive of deeply sequenced wastewater metagenomes. The criteria for collection are explained in more detail in section 3.1. All samples were initially cleaned using fastp (Chen et al. 2018) with default parameters and assembled using MEGAHIT (Li et al. 2015b), unless otherwise specified. Prodigal (Hyatt et al. 2010), a prokaryotic gene recognition tool, was applied for predicting protein-coding genes in contigs.

Survey of diverse environments

We aimed to establish the range of scores encountered when MetaCompare 2.0 was applied to a wide range of environments: soil, sediment, surface water, gut microbiome, WWTP, as well as a mock microbial community and deionized lab water (categorized as "Lab generated" in latter sections) as control samples. For each environment, we identified contrasting sub-sets of samples based on available Sequence Read Archive (SRA) metadata and contextual evidence from their accompanying research articles. For each sub-set, we sought to identify 10 samples. We defined the sub-set coming from human-influenced environments as an "impacted/polluted" set and the sub-set coming from less human-influenced environments as "unimpacted/remediated." For example, we refer to samples collected from Arctic soil (Zhang et al. 2022b) as unimpacted and soil collected from dairy farms (PRJNA379303) as impacted. We contrasted sediment samples collected from the river bed of a mountain stream (Kneis et al. 2022) (unimpacted) with river sediments contaminated by pharmaceutical discharge (polluted) (PRJEB28019). For surface water, we compared freshwater samples (unimpacted) (PRJNA626373) with water from ditches (polluted) in densely populated regions (PRJEB13833). For gut microbiomes, we compared samples collected from healthy people (unimpacted) and samples from COVID-19 patients (impacted) (Zuo et al. 2020) since it was found that COVID-19 significantly alters gut microbiome composition (Yeoh et al. 2021). We also tested edge cases (samples with a high probability of generating extremely low/high-risk scores) using deionized water (i.e., negative controls) which have a near zero probability of ARG presence and Zymo Mock Microbial Community (catalog number D6300, zymoresearch.com)

which contain mixtures of 10 organisms, 7 of which are bacterial pathogens (*Listeria monocytogenes, Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli, Salmonella enterica, Enterococcus faecalis*, and *Staphylococcus aureus*). For WWTPs, we contrasted raw influent (PRJEB13831) (impacted) and treated effluent samples (remediated) (PRJNA438174, PRJNA490743, PRJNA904380, PRJNA505617, PRJEB14051, PRJNA532678, and PRJEB15519). All samples, their BioProjects, and SRA accessions used in model validation are provided in Table S3. MetaCompare 2.0 was applied to determine resistome risk scores of these samples. Wilcoxon rank-sum tests were performed to examine whether the differences between the ERR and HHRR scores of the paired categories were statistically significant. Additionally, Nonpareil3 (Rodriguez-R et al. 2018) was used to estimate library coverage for each sample using options––T kmer and recorded in Table S3.

Evaluation of the effect of assembly method on resulting risk scores

The original MetaCompare 1.0 pipeline used IDBA-UD (Peng et al. 2012) for short-read assembly. However, it has been demonstrated that assemblers can vary in their accuracy (Brown et al. 2021), which could potentially affect downstream risk scores. To examine the effect of assembler choices on the risk scores, we applied three commonly used assemblers: IDBA-UD (Peng et al. 2012), MEGAHIT (Li et al. 2015b), and metaSpades (Nurk et al. 2017) to five samples from five different environments. The detailed metadata for these samples are provided in Table S4. All samples were quality filtered using fastp (Chen et al. 2018) prior to assembly. For each sample, three sets of contigs were analyzed, one for each assembly approach, prior to MetaCompare 2.0 analysis. The assembly statistics for these samples were calculated using SeqKit (Shen et al. 2016) (Table S4).

Evaluation of the effect of sequencing depth and assembly size

To assess the effect of sequencing depth, coverage, and assembly size on resistome risk scores, we subsampled both short-reads and assembled contigs from deeply sequenced Illumina datasets for this purpose using Seqtk (Li 2024). Short-reads were subsampled from three deeply sequenced wastewater samples that we generated internally from influent, activated sludge, and secondary effluent samples each containing ∼4 billion reads (∼1 TB) sequenced on an Illumina NovaSeq6000. The samples were subsampled at discrete intervals of 1 M (million), 10 M, 50 M, 100 M, 125 M, and 250 M, then assembled with MEGAHIT (Li et al. 2015b) and scored. The subsampling was performed 20 times for smaller sized samples (1 M, 10 M, and 50 M) and 10 times for larger sized ones (100 M, 125 M, and 250 M). Nonpareil3 (Rodriguez-R et al. 2018) was used on the pre-assembled short-read data to estimate their library coverage. For contig simulation, we assembled the largest activated sludge sample (250 M reads resulting in 6 645 925 contigs) and subsampled sets of contigs containing 2.5k (1k = 1000), 5k, 10k, 50k, 100k, 500k, and 1 M contigs, which were then annotated and scored. For each subsampling, we generated 50 sets of contigs.

Results

Correlation between MetaCompare 2.0 and MetaCompare 1.0 scores

The performance of MetaCompare 2.0 was first evaluated using the same dataset applied for MetaCompare 1.0: hospital sewage, WWTP effluent, and agricultural lagoon water. To compare the output difference between the two versions, we first applied the Shapiro-Wilk test to ensure that the scores are normally distributed and then calculated Pearson's correlation coefficient between risk scores from MetaCompare 1.0 and MetaCompare 2.0 for this dataset (Table 2).

Range of risk scores encountered across diverse environments

Figure 3 illustrates the range of risk scores encountered across a broad range of environmental metagenomes. The highest resistome risk score obtained was 63.52, generated by the Zymo Mock Microbial Community. The lowest resistome risk score was 0, generated by a deionized water sample. For each environment, the average ERR score and HHRR scores were higher in the "impacted/polluted" datasets compared to the corresponding "unimpacted/remediated" datasets (Wilcoxon rank sum test; P-value $<$ .0003).

Effect of different assembly programs

The effect of assembly methodology on resistome risk score was tested by applying the MetaCompare 2.0 pipeline on contig sets obtained from the same samples using three different assemblers. This test was performed on influent wastewater, effluent wastewater, polluted soil, sediment, and surface water samples. Although the resistome risk scores differed for a given sample as a function of the assembler used to generate the contigs, the same general trend in the ranks of the resulting resistome risk scores was produced (Fig. 4). This analysis indicates that overall trends in resistome risk scores are likely to hold true, even if different assemblers are applied. However, because the scores themselves differ, we recommend that users make resistome risk score comparisons among samples computed from contigs generated by the same assemblers.

Effect of sequencing depth and coverage

Given the complexity of environmental metagenomes, we further assessed the effect of sequencing depth and coverage on resistome risk scores and estimated the "saturation point" for converging scores. Simulated metagenomes containing short-reads at different depths of 1 M, 10 M, 50 M, 100 M, 125 M, and 250 M reads were derived from sub-sampling Influent, Activated Sludge, and Effluent samples as representative aquatic environments of distinct microbial complexity. These sub-sampled metagenomes were assembled and then run through the pipeline. Figure 5A and B demonstrates that, although the ERR and HHRR scores vary with sequencing depth for the same environment, MetaCompare 2.0 was able to distinguish and produce the correct ranking of resistome risks among influent, activated sludge, and secondary effluent environments over the broad range of sequencing depths. As expected, risk scores tend to vary more with low sequencing depth, e.g. 1 M, 10 M with coverage ≥50%, but become stable at 50 M depth and ≤60% coverage. This suggests that samples of similar sequencing depths are still comparable via MetaCompare 2.0, even with relatively shallow sequencing of 10 M (Fig. 5C). However, to be able to compare metagenomes of different depths, they should ideally be sequenced at >60% coverage.

Noticing the high variability of scores generated using MetaCompare 1.0 among air samples reported in a recent study (Qin et al. 2020), we ran another experiment where multiple samples containing different numbers of contigs were generated from the largest activated sludge sample assembled in the previous

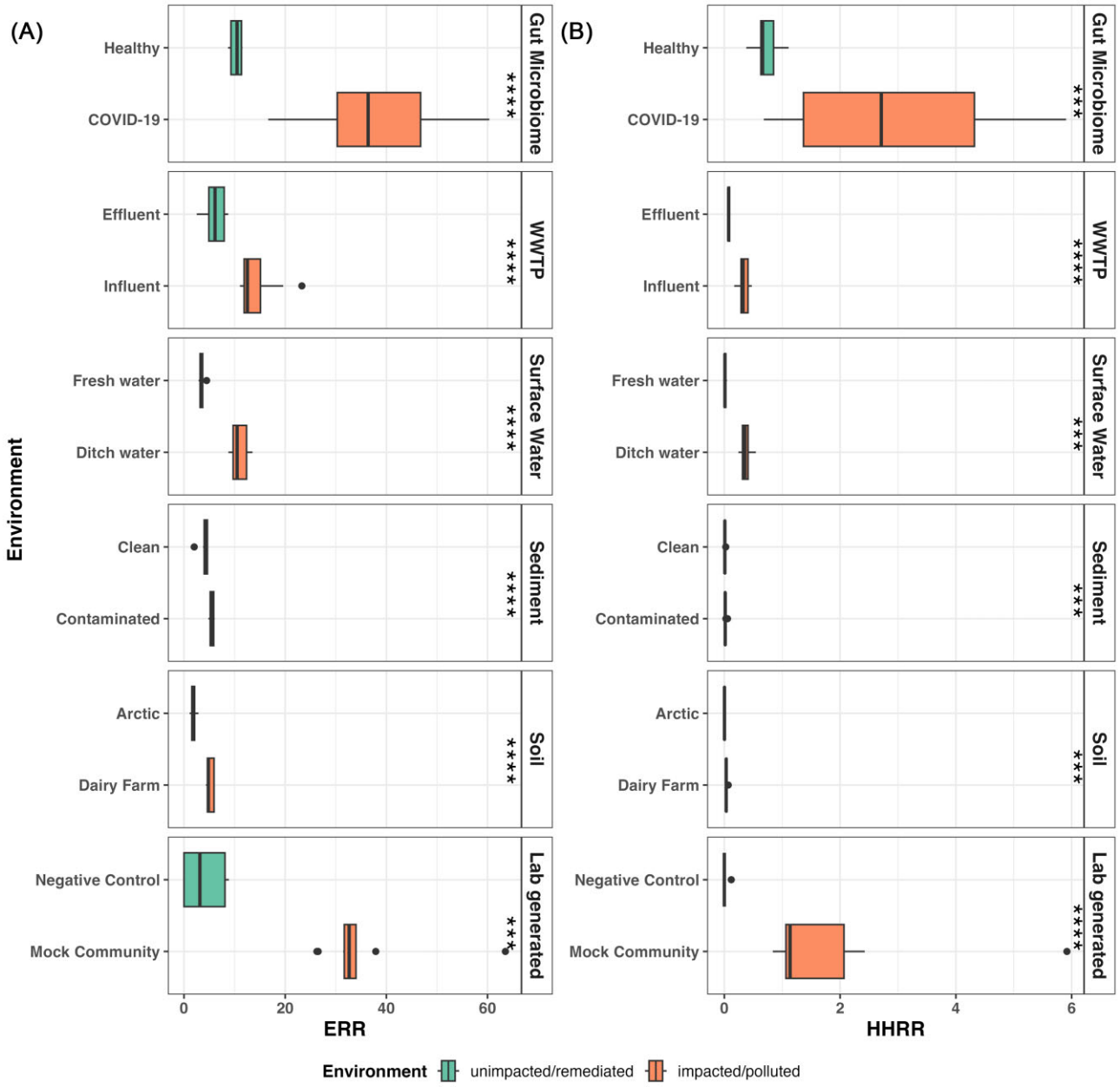

Figure 3. Range of (A) ERR and (B) HHRR scores obtained for six distinct environments and contrasting impact levels, with $n = 10$ samples selected from available public data as representative of each category. Here, "impacted/polluted" and "unimpacted/remediated" were broadly defined according to availability of metadata, corresponding to reported input of anthropogenic pollutants or infection with a pathogen versus a relatively pristine or remediated environment. We also included laboratory blanks and the Zymo Mock Community of 10 bacteria (7 of which are pathogens), as control samples. *$P < .05$; **$P < .01$, ***$P < .001$, and ****$P < .0001$ according to Wilcoxon rank-sum test comparing scores between the paired categories.

experiment. We generated 50 sub-samples for each n, where n is the number of contigs. Figure 6A and B shows that the range of scores can trend higher for samples generating fewer contigs (≤10k) and the scores converge for samples with a higher number of contigs (≥50k).

Discussion

Antibiotic resistance is a pandemic driven by microbial ecology and evolution. As metagenomic data grows in volume and scope, increasingly drawing the attention of research and regulatory institutions, there is an urgent need to translate complex metagenomic data into meaningful metrics for comparison. Here, we introduce an upgraded tool, MetaCompare 2.0, for describing the genetic context of ARGs in environmental systems and relaying the corresponding resistome risk. MetaCompare 2.0 provides several improvements over MetaCompare 1.0, including a revamped algorithm, faster annotation tools, updated databases, and a publicly-available and user-friendly interface. The determination of two distinct risk scores, ERR and HHRR, provides greater resolution in comparing the relative resistome risks across various environment of interest. The HHRR score focuses on specific antibiotic-resistant pathogens of concern (i.e. ESKAPEE pathogens) and the acute human health hazards that they and their genotypes represent. The ERR, on the other hand, can be applied in scenarios where the concern is much broader in terms of assessing the

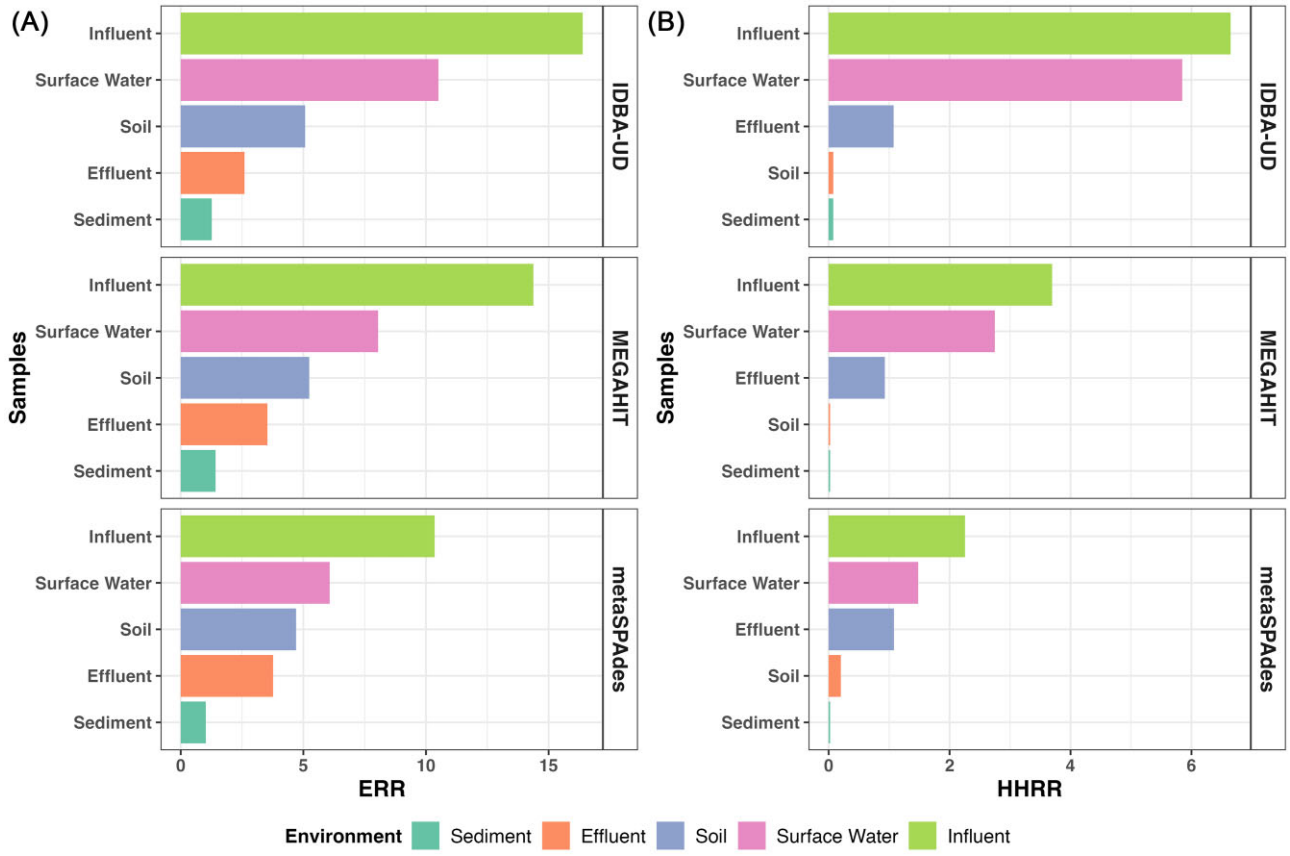

Figure 4. Effect of the assembler on the resistome risk score. Ranking of the resistome risk scores of the samples remains consistent irrespective of the assembly method used.

potential for antibiotic resistance to evolve and spread, e.g. during biological treatment of wastewater, surface water discharge pharmaceutical wastewaters, or application of biosolids to soil. In sum, both the ERR and HRR can provide a comparative metric across a system or environment of interest to identify potential "hot spots" worthy of additional attention from a mitigation standpoint. MetaCompare can also be used to assess trends with time, e.g. in response to a targeted intervention or the assessment of experimental data comparing time series effects of variables of interest.

We tested MetaCompare 2.0 over a wide range of sample types in order to characterize the encountered distribution of risk scores. Consistent with expectation, sample sets with greater anthropogenic "impact/pollution" yielded correspondingly higher resistome risk scores. Interestingly, the gut microbiome samples exhibited the highest resistome risk scores. Even the resistome risk scores for healthy humans produced higher scores than raw influent wastewater (i.e., sewage) samples. This is consistent with the well established understanding that human feces is enriched in pathogens (Zhang et al. 2022c), while these pathogen markers essentially become diluted with endogenous microbes in the sewer as well as dissipate due to dramatic turnovers in ambient physicochemical states through the conveyance network (Bengtsson-Palme et al. 2016). We also noticed higher risk scores in aquatic environments (e.g., wastewater, surface water) compared to terrestrial environments (e.g., soil, sediment). This suggests that diversity/complexity of the metagenome could generally act to lower the resistome risk scores. Recent work has shown that greater microbiome diversity in natural communities can act as a barrier to the invasion of antibiotic-resistant bacteria and subsequent accumulation and horizontal transfer of ARGs due to niche exclusivity, potentially corroborating this observation (Chen et al. 2019, Klümper et al. 2024). A cross-environmental study can provide more insights into the composition of ARGs, MGEs, pathogens, and their association with resistome risk scores in such environments.

While the theoretical range of both ERR and HHRR scores is 0–100, this study provides insight into the actual range of scores encountered across different environments and sample types. We found that the general range of ERR scores was observed to be <40 for "impacted/polluted" samples, and <10 for "unimpacted/remediated" samples. The corresponding range of HHRR scores were <10 and ≈ 0, respectively.

We further evaluated the effect of assemblers on the risk score calculation. For that purpose, we employed three assemblers, namely IDBA-UD (Peng et al. 2012), MEGAHIT (Li et al. 2015b), and MetaSPAdes (Nurk et al. 2017), all of which are widely used in publicly-available pipelines. We observed in our experiment that the final scores were different for the contig sets generated by different assembly algorithms, but the ranking of resistome risk scores of the samples remained the same (Fig. 4). Considering the required computing resources, speed, and the tendency to produce more accurate assemblies (Vollmers et al. 2017, Brown et al. 2021), we used MEGAHIT as the default assembler for all other experiments in this project. Though users are free to choose any assembly pipeline based on their requirements and preferences, we recommend the use of a single assembler when comparing and ranking risk scores of samples.

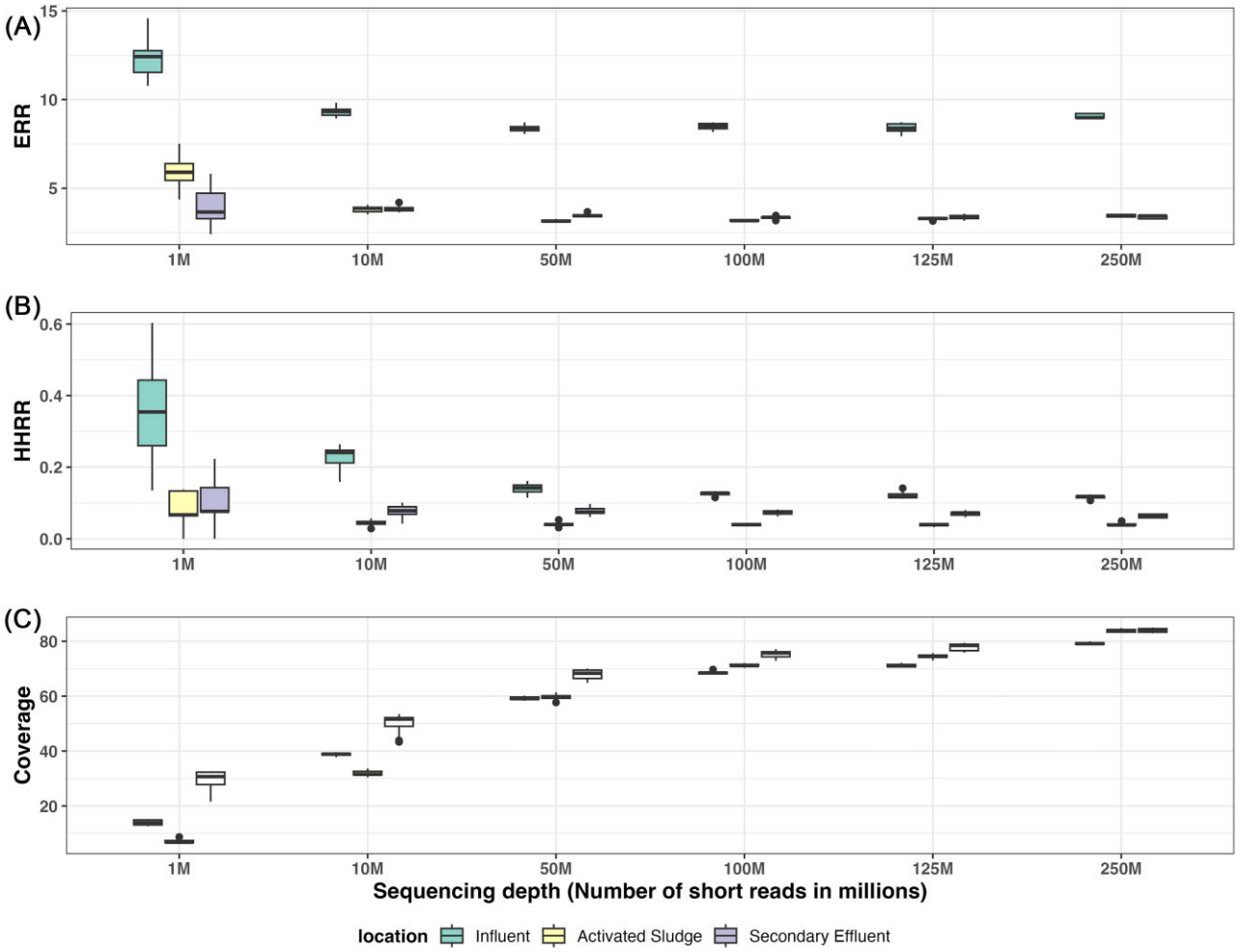

Figure 5. Effect of sequencing depth and library coverage on (A) ERR and (B) HHRR scores of a subset of influent, activated sludge, and secondary effluent sample metagenomes. The metagenomes were generated by subsampling 20 × (1 M, 10 M, and 50 M)/10 × (100 M, 125 M, and 250 M) from one large sample (~4 billion short-reads) of each category. (C) Coverage of all metagenomes analyzed.

We assessed how differences in sequencing depth might affect the scores, which can be particularly important when analyzing genetically complex samples. Theoretically, MetaCompare 2.0 can be applied to any sample irrespective of coverage and sequencing depth. However, it is noted that the risk scores for samples with either lower coverage or sequencing depth tended to have higher variance (Fig. 5). This result is not surprising due to the highly stochastic nature of the set of genes or sequences that happen to be generated in the sequencing experiment. In fact, low coverage or sequencing depth can adversely affect contig assembly and thus downstream analyses resulting from the assembly (Rizzetto et al. 2017). Based on our experiments, we suggest that applying our pipeline to samples with ≥60% coverage would be the best practice. This should also support the application of MetaCompare 2.0 to high-complexity samples, given that stable resistome risk scores can be achieved beyond 60% coverage.

In recent work, there have been multiple attempts in ranking risks associated with environmental aspects of antibiotic resistance. Even though similar aspects have been considered in corresponding risk score computations, such as the mobility and pathogenicity of ARGs, there remains an important distinction between the MetaCompare framework and that proposed by others. MetaCompare computes a "resistome risk" score for the full range of ARGs identified across a metagenome, whereas other approaches (Zhang et al. 2021, 2022c) assign a risk score or rank for individual ARGs. Both frameworks have pros and cons, depending on the aim of the application. If one wants to compare samples derived from different monitoring points in terms of aggregate risk of antibiotic resistance evolution and spread, having a single metric that captures the full range of ARGs is more relevant than focusing in on individual ARGs. However, if one is interested in zooming in on a specific subset of ARGs of concern, then ranking and scoring individual ARGs may be more useful.

There were a number of decision points in updating the algorithms and databases for MetaCompare 2.0. We selected Rank I ARGs for inclusion in the HHRR pipeline given that these are increasingly being adopted in the literature as encompassing ARGs of most immediate clinical concern in terms of their likelihood to cause complications with antibiotic treatment (Gwenzi et al. 2022, Tarek and Garner 2023, Kerkvliet et al. 2024). At the same time, this list is subject to debate and evolving as the antibiotic resistance itself continues to evolve. In the future, this database can be readily updated, highlighting the advantage of modular risk assessment pipelines. We likewise chose to focus on the ESKAPEE pathogens in the HHRR pipeline, given that they are widely recognized to cause infections that are complicated by antibiotic resistance (De Oliveira et al. 2020, He et al. 2023). We clarify, however, that strain-level, whole-genome resolution is typically required to

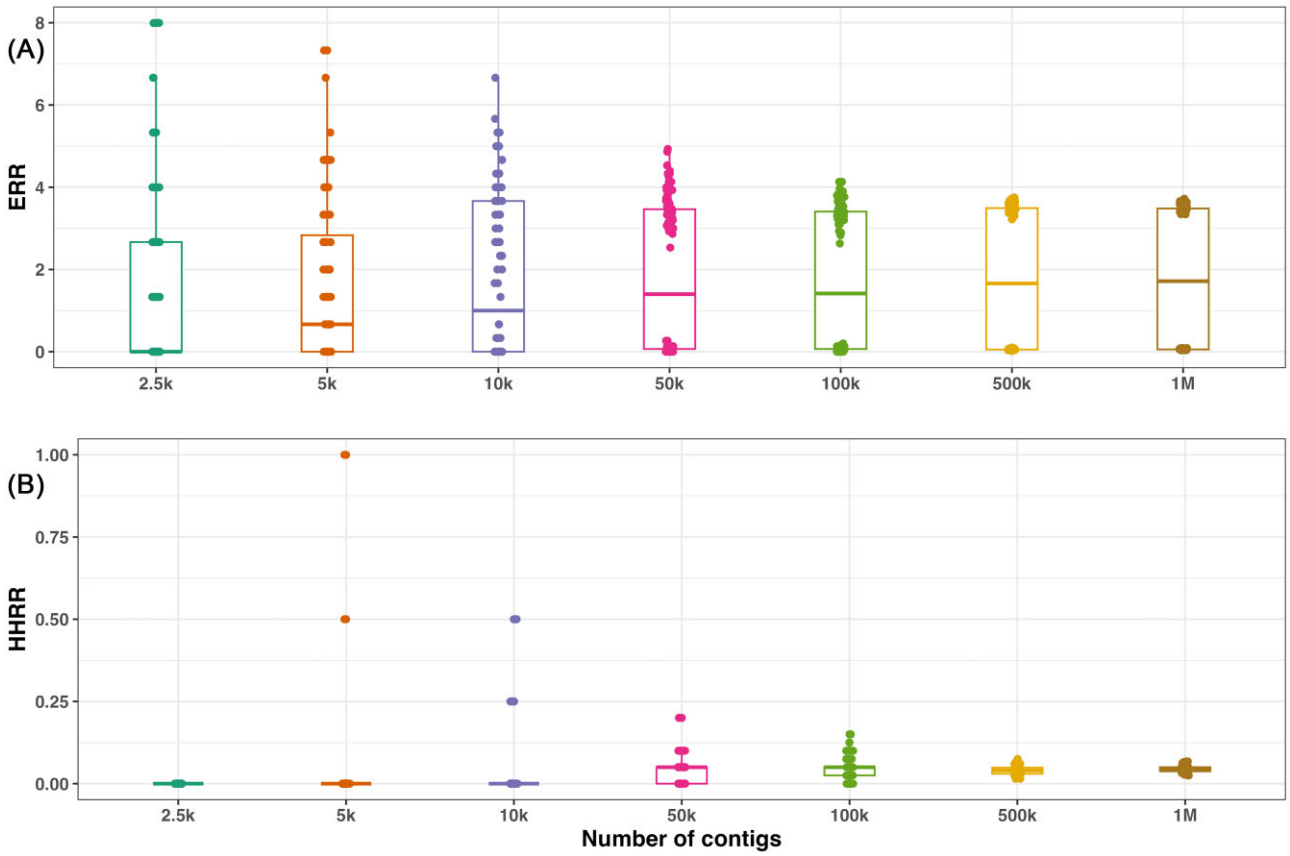

Figure 6. (A) ERR and (B) HHRR scores were obtained over a range of metagenomic assembly sizes, as indicated by the number of assembled contigs yielded. The samples were generated from an activated sludge sample by subsampling it 50 times for each corresponding assembly size.

differentiate pathogenic and non-pathogenic forms of ESKAPEE organisms. We also note that contigs containing only ARGs or only ARGs and an MGE are also factored into the risk scores, thus representing potential contributions of non-pathogens to the spread of antibiotic resistance.

Finally, we note that there remain some limitations in determining resistome risk. Specifically, two bottlenecks are noted for metagenomic characterization of antibiotic resistance and MetaCompare 2.0. First, many important ARGs are present in low concentrations in some environments, necessitating deep sequencing for recovery (Davis et al. 2023). This is further complicated in multi-environment studies, where different degrees of sequencing effort may be needed for comparability. Second, metagenomic assembly of co-occurring strains and genetic contexts remains a fundamental challenge (Miller and Arias 2024). Long-read sequencing can theoretically bypass the need for metagenomic assembly. However, we did not consider long-read sequencing data in MetaCompare 2.0 as it has yet to supplant short-read sequencing. It should be noted that the increased incidence of frameshift errors in intermediate fidelity long reads can disrupt open reading frame prediction, thus leading to an underestimation of ARG and MGE annotation. In MetaCompare's current form, the resulting risk scores obtained from long-read data might not be directly translatable given that reads will likely be much longer than assembled contigs and thus may capture multiple ARGs and multiple MGEs within the same genome. The lower coverage that is typical when long-read sequencing is applied for shotgun metagenomics could also be an issue, as it would not likely be feasible to achieve ≥60% coverage recommended here. Thus, at this time, it is not recommended to use long reads for MetaCompare2. Further validation of MetaCompare 2.0 for long-read metagenomics and benchmarking to Illumina sequencing would be recommended. Future adaptations to MetaCompare 2.0 for long-read data might be necessary as long-read bioinformatic software continues to be developed and we envision subsequent iterations for long-read data including plasmid identification/typing and a biologically conscious scoring system across MGE categories.

Acknowledgments

We thank James Stoll for helping with the webserver setup. The research presented was not performed or funded by EPA and was not subject to EPA's quality system requirements. The views expressed in this article are those of the author(s) and do not necessarily represent the views or the policies of the US Environmental Protection Agency.

Author contributions

Monjura Afrin Rumi (Data curation, Formal analysis, Software, Validation, Visualization, Writing – original draft, Writing – review & editing), Min Oh (Methodology, Software), Benjamin C. Davis (Conceptualization, Validation, Writing – original draft), Connor L. Brown (Conceptualization, Validation), Adheesh Juvekar (Software, Visualization), Peter J. Vikesland (Supervision, Writing – review & editing), Amy Pruden (Supervision, Writing – review & editing), and Liqing Zhang (Supervision, Writing – review & editing)

Supplementary data

Supplementary data is available at FEMSEC *Journal* online.

Conflict of interest: The authors declare that no conflict of interest exists.

Funding

This work was supported by the US National Science Foundation (NSF) AWARDS #2004751, #2125798, and #2319522 and Water Research Foundation Project 4813.

Data availability

The web service for MetaCompare 2.0 is available at http://metacompare.cs.vt.edu/. The source code for the entire pipeline can be found at https://github.com/mrumi/MetaCompare2.0. Users are required to create an account before uploading sequences to the platform.

References

Apweiler R, Bairoch A, Wu CH *et al.* UniProt: the universal protein knowledgebase. *Nucleic Acids Res* 2004;**32**:D115–9. https://doi.org/10.1093/nar/gkh131.

Arango-Argoty G, Garner E, Pruden A *et al.* DeepARG: a deep learning approach for predicting antibiotic resistance genes from metagenomic data. *Microbiome* 2018;**6**:23. https://doi.org/10.1186/s40168-018-0401-z.

Ashbolt NJ, Amézquita A, Backhaus T *et al.* Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. *Environ Health Perspect* 2013;**121**:993–1001. https://doi.org/10.1289/ehp.1206316.

Bengtsson-Palme J, Hammarén R, Pal C *et al.* Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. *Sci Total Environ* 2016;**572**:697–712. https://doi.org/10.1016/j.scitotenv.2016.06.228.

Bengtsson-Palme J, Kristiansson E, Larsson DGJ. Environmental factors influencing the development and spread of antibiotic resistance. *FEMS Microbiol Rev* 2018;**42**:fux053. https://doi.org/10.1093/femsre/fux053.

Berendonk TU, Manaia CM, Merlin C *et al.* Tackling antibiotic resistance: the environmental framework. *Nat Rev Micro* 2015;**13**:310–7. https://doi.org/10.1038/nrmicro3439.

Boratyn GM, Camacho C, Cooper PS *et al.* BLAST: a more efficient report with usability improvements. *Nucleic Acids Res* 2013;**41**:W29–33. https://doi.org/10.1093/nar/gkt282.

Brown CL, Cheung YF, Song H *et al.* Kairos infers in situ horizontal gene transfer in longitudinally sampled microbiomes through microdiversity-aware sequence analysis. *bioRxiv* 2023. https://doi.org/10.1101/2023.10.24.563791

Brown CL, Keenum IM, Dai D *et al.* Critical evaluation of short, long, and hybrid assembly for contextual analysis of antibiotic resistance genes in complex environmental metagenomes. *Sci Rep* 2021;**11**:3753. https://doi.org/10.1038/s41598-021-83081-8.

Brown CL, Mullet J, Hindi F *et al.* mobileOG-DB: a manually curated database of protein families mediating the life cycle of bacterial mobile genetic elements. *Appl Environ Microbiol* 2022;**88**:e00991–22. https://doi.org/10.1128/aem.00991-22.

Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. *Nat Methods* 2015;**12**:59–60. https://doi.org/10.1038/nmeth.3176.

Chen Q-L, An X-L, Zheng B-X *et al.* Loss of soil microbial diversity exacerbates spread of antibiotic resistance. *Soil Ecol Lett* 2019;**1**:3–13. https://doi.org/10.1007/s42832-019-0011-0.

Chen S, Zhou Y, Chen Y *et al.* fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 2018;**34**:i884–90. https://doi.org/10.1093/bioinformatics/bty560.

Davis BC, Brown C, Gupta S *et al.* Recommendations for the use of metagenomics for routine monitoring of antibiotic resistance in wastewater and impacted aquatic environments. *Crit Rev Environ Sci Technol* 2023;**53**:1731–56. https://doi.org/10.1080/10643389.2023.2181620.

De Oliveira DMP, Forde BM, Kidd TJ *et al.* Antimicrobial resistance in ESKAPE pathogens. *Clin Microbiol Rev* 2020;**33**:e00181–19. https://doi.org/10.1128/cmr.00181-19.

Garner E, Organiscak M, Dieter L *et al.* Towards risk assessment for antibiotic resistant pathogens in recycled water: a systematic review and summary of research needs. *Environ Microbiol* 2021;**23**:7355–72. https://doi.org/10.1111/1462-2920.15804.

Gihawi A, Ge Y, Lu J *et al.* Major data analysis errors invalidate cancer microbiome findings. *mBio* 2023;**14**:e01607–23. https://doi.org/10.1128/mbio.01607-23.

Gwenzi W, Shamsizadeh Z, Gholipour S *et al.* The air-borne antibiotic resistome: occurrence, health risks, and future directions. *Sci Total Environ* 2022;**804**:150154. https://doi.org/10.1016/j.scitotenv.2021.150154.

He L-X, He L-Y, Gao F-Z *et al.* Mariculture affects antibiotic resistome and microbiome in the coastal environment. *J Hazard Mater* 2023;**452**:131208. https://doi.org/10.1016/j.jhazmat.2023.131208.

Hernández-Salmerón JE, Moreno-Hagelsieb G. Progress in quickly finding orthologs as reciprocal best hits: comparing blast, last, diamond and MMseqs2. *BMC Genomics [Electronic Resource]* 2020;**21**:741. https://doi.org/10.1186/s12864-020-07132-6.

Hernando-Amado S, Coque TM, Baquero F *et al.* Defining and combating antibiotic resistance from one health and global health perspectives. *Nat Microbiol* 2019;**4**:1432–42. https://doi.org/10.1038/s41564-019-0503-9.

Hyatt D, Chen G-L, LoCascio PF *et al.* Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinf* 2010;**11**:119. https://doi.org/10.1186/1471-2105-11-119.

Ji Y, Shang J, Tang X *et al.* HOTSPOT: hierarchical host prediction for assembled plasmid contigs with transformer. *Bioinformatics* 2023;**39**:btad283. https://doi.org/10.1093/bioinformatics/btad283.

Jia B, Raphenya AR, Alcock B *et al.* CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res* 2017;**45**:D566–73. https://doi.org/10.1093/nar/gkw1004.

Karkman A, Pärnänen K, Larsson DGJ. Fecal pollution can explain antibiotic resistance gene abundances in anthropogenically impacted environments. *Nat Commun* 2019;**10**:80. https://doi.org/10.1038/s41467-018-07992-3.

Keenum I, Williams RK, Ray P *et al.* Combined effects of composting and antibiotic administration on cattle manure–borne antibiotic resistance genes. *Microbiome* 2021;**9**:81. https://doi.org/10.1186/s40168-021-01006-z.

Kerkvliet JJ, Bossers A, Kers JG *et al.* Metagenomic assembly is the main bottleneck in the identification of mobile genetic elements. *PeerJ* 2024;**12**:e16695. https://doi.org/10.7717/peerj.16695.

Klümper U, Gionchetta G, Catão E *et al.* Environmental microbiome diversity and stability is a barrier to antimicrobial resistance gene accumulation. *Commun Biol* 2024;**7**:1–13. https://doi.org/10.1038/s42003-024-06338-8.

Kneis D, Berendonk TU, Forslund SK *et al.* Antibiotic resistance genes in river biofilms: a metagenomic approach toward the identification of sources and candidate hosts. *Environ Sci Technol* 2022;**56**:14913–22. https://doi.org/10.1021/acs.est.2c00370.

Leplae R, Lima-Mendez G, Toussaint A. ACLAME: a CLAssification of mobile genetic elements, update 2010. *Nucleic Acids Res* 2010;**38**:D57–61. https://doi.org/10.1093/nar/gkh084.

Li B, Ju F, Cai L *et al.* Profile and fate of bacterial pathogens in sewage treatment plants revealed by high-throughput metagenomic approach. *Environ Sci Technol* 2015a;**49**:10492–502. https://doi.org/10.1021/acs.est.5b02345.

Li D, Liu C-M, Luo R *et al.* MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* 2015b;**31**:1674–6. https://doi.org/10.1093/bioinformatics/btv033.

Li H. lh3/seqtk: toolkit for processing sequences in FASTA/Q formats. 2024.

Liu B, Pop M. ARDB—antibiotic resistance genes database. *Nucleic Acids Res* 2009;**37**:D443–7. https://doi.org/10.1093/nar/gkn656.

Majeed HJ, Riquelme MV, Davis BC *et al.* Evaluation of metagenomic-enabled antibiotic resistance surveillance at a conventional wastewater treatment plant. *Front Microbiol* 2021;**12**. https://doi.org/10.3389/fmicb.2021.657954.

Martínez JL, Coque TM, Baquero F. What is a resistance gene? Ranking risk in resistomes. *Nat Rev Micro* 2015;**13**:116–23. https://doi.org/10.1038/nrmicro3399.

Miller WR, Arias CA. ESKAPE pathogens: antimicrobial resistance, epidemiology, clinical impact and therapeutics. *Nat Rev Micro* 2024;**22**:598–616. https://doi.org/10.1038/s41579-024-01054-w.

Nurk S, Meleshko D, Korobeynikov A *et al.* metaSPAdes: a new versatile metagenomic assembler. *Genome Res* 2017;**27**:824–34. https://doi.org/10.1101/gr.213959.116.

O'Neill J. Tackling Drug-Resistant Infections Globally: Final report and recommendations. Government of the United Kingdom, 2016.

Oh M, Pruden A, Chen C *et al.* MetaCompare: a computational pipeline for prioritizing environmental resistome risk. *FEMS Microbiol Ecol* 2018;**94**:fiy079. https://doi.org/10.1093/femsec/fiy079.

Parks DH, Chuvochina M, Rinke C *et al.* GTDB: an ongoing census of bacterial and archaeal diversity through a phylogenetically consistent, rank normalized and complete genome-based taxonomy. *Nucleic Acids Res* 2022;**50**:D785–94. https://doi.org/10.1093/nar/gkab776.

Peng Y, Leung HCM, Yiu SM *et al.* IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics* 2012;**28**:1420–8. https://doi.org/10.1093/bioinformatics/bts174.

Qin N, Liang P, Wu C *et al.* Longitudinal survey of microbiome associated with particulate matter in a megacity. *Genome Biol* 2020;**21**:55. https://doi.org/10.1186/s13059-020-01964-x.

Rice EW, Wang P, Smith AL *et al.* Determining hosts of antibiotic resistance genes: a review of methodological advances. *Environ Sci Technol Lett* 2020;**7**:282–91. https://doi.org/10.1021/acs.estlett.0c00202.

Rizzetto S, Eltahla AA, Lin P *et al.* Impact of sequencing depth and read length on single cell RNA sequencing data of T cells. *Sci Rep* 2017;**7**:12781. https://doi.org/10.1038/s41598-017-12989-x.

Rodriguez-R LM, Gunturu S, Tiedje JM *et al.* Nonpareil 3: fast estimation of metagenomic coverage and sequence diversity. *mSystems* 2018;**3**. https://doi.org/10.1128/msystems.00039-18.

Ruekit S, Srijan A, Serichantalergs O *et al.* Molecular characterization of multidrug-resistant ESKAPEE pathogens from clinical samples in Chonburi, Thailand (2017–2018). *BMC Infect Dis* 2022;**22**:695. https://doi.org/10.1186/s12879-022-07678-8.

Schoen ME, Jahne MA, Garland J *et al.* Quantitative microbial risk assessment of antimicrobial resistant and susceptible Staphylococcus aureus in reclaimed wastewaters. *Environ Sci Technol* 2021;**55**:15246–55. https://doi.org/10.1021/acs.est.1c04038.

Shen W, Le S, Li Y *et al.* SeqKit: a cross-platform and ultrafast toolkit for FASTA/Q file manipulation. *PLoS One* 2016;**11**:e0163962. https://doi.org/10.1371/journal.pone.0163962.

Slizovskiy IB, Mukherjee K, Dean CJ *et al.* Mobilization of antibiotic resistance: are current approaches for colocalizing resistomes and mobilomes useful? *Front Microbiol* 2020;**11**. https://doi.org/10.3389/fmicb.2020.01376. https://doi.org/10.3389/fmicb.2020.01376

Steinegger M, Söding J. MMseqs2 enables sensitive protein sequence searching for the analysis of massive data sets. *Nat Biotechnol* 2017;**35**:1026–8. https://doi.org/10.1038/nbt.3988.

Tarek MH, Garner E. A proposed framework for the identification of indicator genes for monitoring antibiotic resistance in wastewater: insights from metagenomic sequencing. *Sci Total Environ* 2023;**854**:158698. https://doi.org/10.1016/j.scitotenv.2022.158698.

UNEP. Bracing for superbugs: strengthening environmental action in the one health response to antimicrobial resistance. Online report. UNEP—UN Environment Programme, 2023.

Vollmers J, Wiegand S, Kaster A-K. Comparing and evaluating metagenome assembly tools from a microbiologist's perspective—not only size matters! *PLoS One* 2017;**12**:e0169662. https://doi.org/https://doi.org/10.1371/journal.pone.0169662.

Wattam AR, Davis JJ, Assaf R *et al.* Improvements to PATRIC, the all-bacterial Bioinformatics Database and Analysis Resource Center. *Nucleic Acids Res* 2017;**45**:D535–42. https://doi.org/10.1093/nar/gkw1017.

WHO. WHO publishes list of bacteria for which new antibiotics are urgently needed. Online report. World Health Organization, 2017.

Wind L, Keenum I, Gupta S *et al.* Integrated metagenomic assessment of multiple pre-harvest control points on lettuce resistomes at field-scale. *Front Microbiol* 2021a;**12**. https://doi.org/10.3389/fmicb.2021.683410.

Wind L, Krometis L-A, Hession WC *et al.* Cross-comparison of methods for quantifying antibiotic resistance in agricultural soils amended with dairy manure and compost. *Sci Total Environ* 2021b;**766**:144321. https://doi.org/10.1016/j.scitotenv.2020.144321.

Woolhouse M, Gowtage-Sequeria S, Evans B. T16: quantitative analysis of the characteristics of emerging and re-emerging human pathogens. In: *Infectious Diseases: Preparing for the Future*. Office of Science and Innovation, 2012.

Wu D, Jin L, Xie J *et al.* Inhalable antibiotic resistomes emitted from hospitals: metagenomic insights into bacterial hosts, clinical relevance, and environmental risks. *Microbiome* 2022;**10**:19. https://doi.org/10.1186/s40168-021-01197-5.

Yeoh YK, Zuo T, Lui GC-Y *et al.* Gut microbiota composition reflects disease severity and dysfunctional immune responses in patients with COVID-19. *Gut* 2021;**70**:698–706. https://doi.org/10.1136/gutjnl-2020-323020.

Yu Z, Tang J, Khare T *et al.* The alarming antimicrobial resistance in ESKAPEE pathogens: can essential oils come to the rescue? *Fitoterapia* 2020;**140**:104433. https://doi.org/10.1016/j.fitote.2019.104433.

Zhang A-N, Gaston JM, Dai CL *et al.* An omics-based framework for assessing the health risk of antimicrobial resistance genes. *Nat Commun* 2021;**12**:4765. https://doi.org/10.1038/s41467-021-25096-3.

Zhang L, Ji L, Liu X *et al.* Linkage and driving mechanisms of antibiotic resistome in surface and ground water: their responses to land use and seasonal variation. *Water Res* 2022a;**215**:118279. https://doi.org/10.1016/j.watres.2022.118279.

Zhang T, Li J, Wang N *et al.* Metagenomic analysis reveals microbiome and resistome in the seawater and sediments of Kongsfjorden (Svalbard, High Arctic). *Sci Total Environ* 2022b;**809**:151937. https://doi.org/10.1016/j.scitotenv.2021.151937.

Zhang Z, Zhang Q, Wang T *et al.* Assessment of global health risk of antibiotic resistance genes. *Nat Commun* 2022c;**13**:1553. https://doi.org/10.1038/s41467-022-29283-8.

Zuo T, Zhang F, Lui GCY *et al.* Alterations in gut microbiota of patients with COVID-19 during time of hospitalization. *Gastroenterology* 2020;**159**:944–55. https://doi.org/10.1053/j.gastro.2020.05.048.

Received 14 March 2024; **revised** 27 June 2024; **accepted** 8 November 2024