BMC Research Notes

RESEARCH NOTE

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Comparative analysis of qPCR and metagenomics for detecting antimicrobial resistance in wastewater: a case study

William Taylor[2], Kristin Bohm[1], Kristin Dyet[1], Louise Weaver[2] and Isabelle Pattis[2*]

Abstract

Objective The World Health Organization (WHO) has declared antimicrobial resistance (AMR) as one of the top threats to global public health. While AMR surveillance of human clinical isolates is well-established in many countries, the increasing threat of AMR has intensified efforts to detect antibiotic resistance genes (ARGs) accurately and sensitively in environmental samples, wastewater, animals, and food. Using five ARGs and the 16S rRNA gene, we compared quantitative PCR (qPCR) and metagenomic sequencing (MGS), two commonly used methods to uncover the wastewater resistome. We compared both methods by evaluating ARG detection through a municipal wastewater treatment chain.

Results Our results demonstrate that qPCR was more sensitive than MGS, particularly in diluted samples with low ARG concentrations such as oxidation pond water. However, MGS was potentially more specific and has less risk of off-target binding in concentrated samples such as raw sewage. MGS analysis revealed multiple subtypes of each gene which could not be distinguished by qPCR; these subtypes varied across different sample types. Our findings affect the conclusions that can be drawn when comparing different sample types, particularly in terms of inferring removal rates or origins of genes. We conclude that both methods appear suitable to profile the resistome of wastewater and other environmental samples, depending on the research question and type of sample.

Keywords AMR, Antimicrobial resistance, Metagenomics, QPCR, Wastewater, Wastewater surveillance, Wastewater-based epidemiology.

Introduction

The World Health Organization (WHO) has declared antimicrobial resistance (AMR) as one of the top ten threats to global public health [1] and the Global Antimicrobial Resistance Surveillance System (GLASS) has highlighted the necessity for comprehensive global AMR surveillance and research [2]. Municipal wastewater plays a central role in the fight against AMR, offering insights into community-wide health questions. Wastewater analysis is key in monitoring drug use, detecting SARS-CoV2 variants and other pathogens, as well as in understanding population AMR profiles (resistome) [3, 4]. The discharge of treated wastewater into the environment is one of the main pathways for anthropogenic AMR dissemination into the environment, posing a potential risk of transmission to humans and animals [5].

Metagenomic sequencing (MGS) and quantitative PCR (qPCR) are two culture-independent methods that have been widely used to characterize and distinguish the resistome of environmental and wastewater samples

*Correspondence:
Isabelle Pattis
Isabelle.Pattis@esr.cri.nz
[1] Institute of Environmental Science and Research Ltd, Kenepuru, Wellington, New Zealand
[2] Institute of Environmental Science and Research Ltd, Christchurch, New Zealand

[6–9]. While qPCR offers higher sensitivity for detecting antibiotic resistance genes (ARGs), MGS provides a broader resistome profile [7].

Here, we applied both MGS and qPCR to analyse 33 samples collected over three consecutive days from a wastewater treatment plant (WWTP) [10], including raw sewage (influent), secondary treated sewage (effluent), oxidation (tertiary maturation) pond samples, and oxidation pond sediments. The genes chosen for this analysis were the 16S rRNA gene, and the *ermB*, *sul1*, *tetA*, *tetQ*, and *tetW* genes due to their value as common indicators for use in AMR surveillance and wide host ranges [11, 12]. This research note presents a comparative analysis of ARG prevalence throughout the wastewater treatment process using MGS and qPCR. This study is part of a larger research project investigating the presence and fate of resistant bacteria and ARGs through a municipal wastewater treatment plant in Aotearoa New Zealand [13].

Methods

Sample collection

As described previously [10], a total of 33 samples (including replicates) used in the current study were taken from different points in the wastewater treatment chain: post screen influent (INF), treated effluent, taken in the drop chamber after final clarifiers (EFF), tertiary maturation pond effluent, taken from the final pond prior to discharge into the ocean (POND), and base sediment from ponds (SED). Composite samples of INF, EFF, and POND (three replicates) were taken over a 24-hour period on three consecutive days in the spring/early summer of 2019 using an automatic water sampler (ISCO, Teledyne Technologies Inc., USA). For SED samples, three separate 10 g grab samples were taken on Days 2 and 3 only. All samples were immediately stored on ice and processed within 4 h.

DNA extraction and metagenomic sequencing

DNA extraction of wastewater samples was performed as described previously [10]. Briefly, samples were filtered through 0.22 µm polycarbonate filters (Merck, USA). The filters were immersed in LifeGuard™ solution (Qiagen, Germany) and stored at −80 °C. DNA extractions and blanks were performed using the PowerSoilPro DNA extraction kit (Qiagen), according to the manufacturer's protocol with some modifications. Lifeguard™ was removed from the stored filter, and each filter was subjected to bead beating for 3 min at 3,000 oscillations per minute using the kit's beads and lysis buffer (Mini-Beadbeater-24, Biospec, USA). After centrifugation at 3500 g for 5 min, the supernatant was used for DNA extraction. DNA concentrations were evaluated using a Nanodrop (Thermo Fisher Scientific, USA). The same extraction was used for MSG and qPCR. Sampling details can be found in Supplementary Table S4.

Sequencing and bioinformatic analysis

DNA was shipped frozen (10 µL per sample) to Macrogen Oceania (South Korea) for sequencing. Library preparation for MGS was performed using the TruSeq Nano DNA Library Prep kit (Illumina) and the prepared libraries were multiplexed and sequenced on the NovaSeq6000 platform (Illumina), using 2 × 150 bp paired end sequencing. Bioinformatic methods are detailed in the Supplementary Materials. Briefly, reads were trimmed and filtered using bbduk (v38.90) to remove adapters, reads containing ambiguous bases (N), and reads with a quality score less than Q20 [14]. Reads were aligned to the ResFinder (v2.1.1) [15] and SILVA 138.1 [16] databases with KMA (v1.4.9), as in Munk et al. [17, 18]. Visualisations and calculations were performed using R (v4.3.0 [19].

Quantitative PCRs

Five ARGs *ermB*, *sul1*, *tetA*, *tetQ*, *and tetW* and the 16S rRNA gene underwent qPCR with designed primers and probes (Supplementary Table S1–S3; [11]) using 5 ng input DNA. The 16S rRNA gene was quantified to normalize qPCR results of the ARGs [20]. All qPCRs were performed with a C1000 Touch CFX96 Real-time System (BioRad, NZ) as follows: 10-minute denaturation at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C with signal detected for 5 s at the end of each cycle in four technical replicates. The reaction efficiency ranged from 85 to 100%, with an R^2 value greater than 0.98. The detection limit threshold was set at $\sim 5 \times 10^1$ copies/µl for the analysis.

Alignment, qPCR comparisons and multiple sequence alignment

For MGS, of the ~1.2 billion input reads, ~5.8 million reads were aligned to ARGs, with a minimum identity of 80.65% and a minimum coverage of 98.98%. For presence-/absence comparisons, MGS gene subtype counts were combined for each gene, and subtype counts below 50 were omitted for both qPCR and MGS data. ARG per 16S rRNA values were generated for both qPCR and MGS gene counts [7].

Results

ARG detection rates with MGS and qPCR

All five ARGs were detected in all INF and EFF samples by both qPCR and MGS (Table 1 and Supplementary Table S5). *sul1* was the only ARG detected in all samples (four sample types and all replicates) using MGS, while both *sul1* and *tetA* were detected in all samples using

Table 1 ARG detection rates across sample types (% of samples where ARG was detected)

Sample type		*ermB*	*sul1*	*tetA*	*tetQ*	*tetW*
INF ($n=9$)	qPCR	100%	100%	100%	100%	100%
	MGS	100%	100%	100%	100%	100%
EFF ($n=9$)	qPCR	100%	100%	100%	100%	100%
	MGS	100%	100%	100%	100%	100%
POND ($n=9$)	qPCR	22.22%	100%	100%	22.22%	11.11%
	MGS	0%	100%	77.77%	0%	0%
SED ($n=6$)	qPCR	0%	100%	100%	0%	16.6%
	MGS	0%	100%	66.6%	0%	16.6%

qPCR. In POND samples, qPCR detected ermB, *tetA*, *tetQ*, and *tetW* in more samples than MGS. In SED samples, qPCR detected *tetA* in more samples than MGS, and both methods detected *tetW* at the same rate, albeit in different SED samples.

By qPCR, INF samples had the highest per 16S rRNA levels of ARGs (Fig. 1a), with *ermB* and *tetQ* contributing the highest proportions (Fig. 1b). However, in EFF samples, *sul1* and *tetW* were the most proportionally abundant. The relative abundance of *sul1* was highest in POND and SED samples, while the other genes were rarely detected.

MGS results showed that intra-day detections (replicates within one day) and detections by sample types (Fig. 1c) as well as proportional abundance (Fig. 1d) were relatively consistent. However, in contrast to qPCR results, ARGs per 16S rRNA were not diminished in EFF samples compared to INF samples, and the proportional abundance remained consistent (Fig. 1d). In POND and SED samples, *sul1* was dominant, with higher levels of *tetA* and *tetW* compared to those found by qPCR. POND and SED samples showed greater variability in proportional abundance compared with INF and EFF.

Metagenomic sequencing of ARGs

Further analysis of ARGs with MGS showed multiple subtypes for each gene detected by qPCR. The presence and proportions of specific gene subtypes varied between sample types. We identified six distinct *ermB* subtypes,

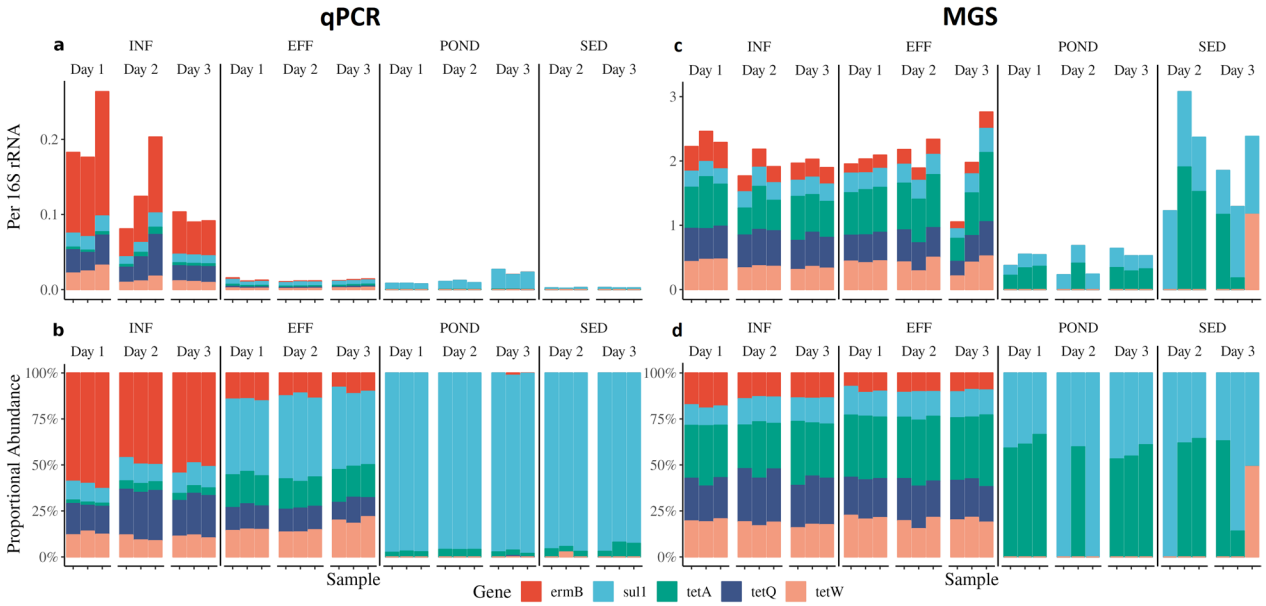

Fig. 1 ARG quantification relative to 16S rRNA gene copies as detected. Left qPCR: **a** Total counts of ARGs normalized to 16S rRNA gene copies; **b** Proportional abundance of each ARG relative to 16S rRNA gene copies. Right MGS: **c** Total counts of ARGs normalized to 16S rRNA gene copies; **d** Proportional abundance of each ARG relative to 16S rRNA gene copies

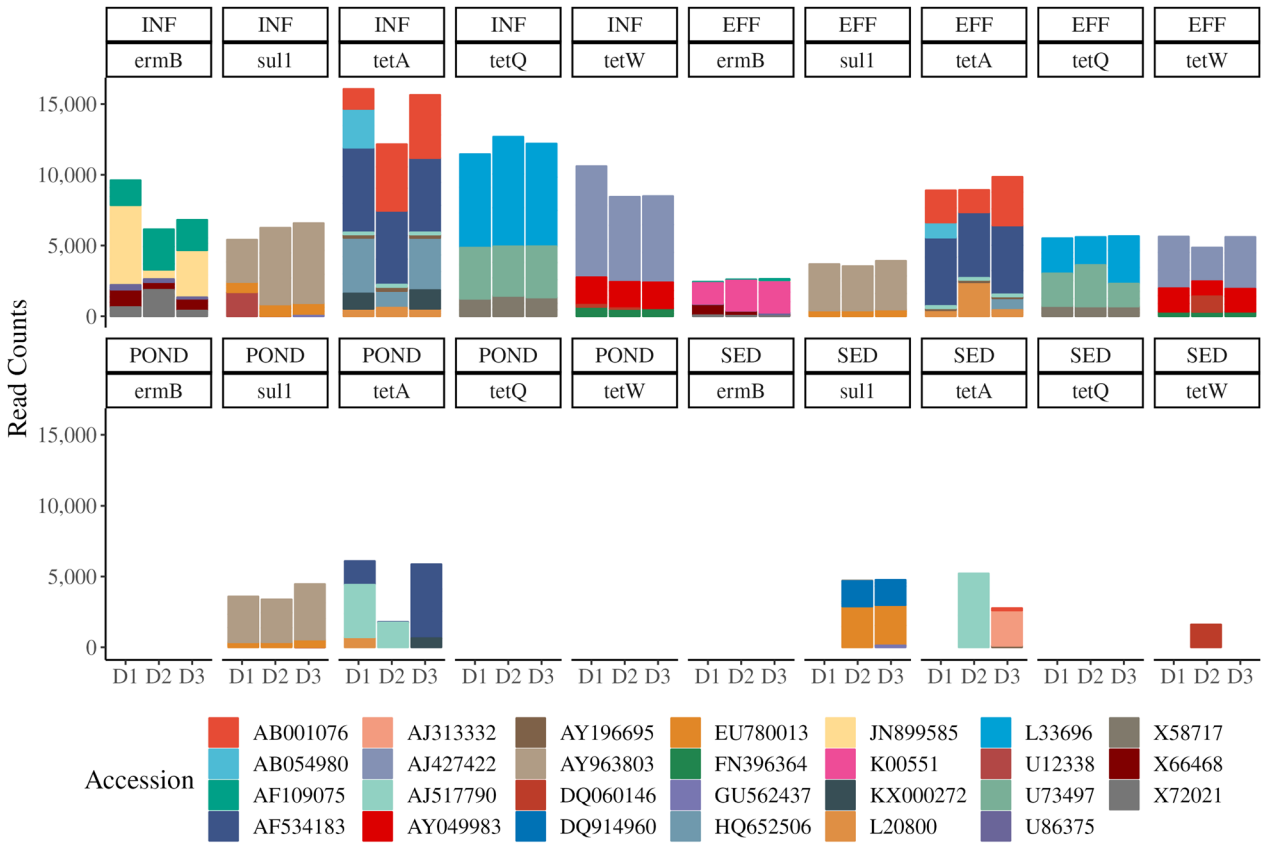

Fig. 2 Antimicrobial resistance gene read counts separated by subtype accession number. Shown are the combined counts for three replicates on each day (D1: Day 1; D2: Day 2; D3: Day3) for all sampling locations (Influent: INF; Effluent: EFF; oxidation pond water: POND; oxidation pond sediment: SED)

five *sul1* subtypes, nine *tetA* subtypes, four *tetQ* subtypes and four *tetW* subtypes (Fig. 2).

ermB was not detected in POND or SED by either MGS or qPCR. However, six *ermB* subtypes were detected, with two being specific to INF, one to EFF, and three present in both sample types (Fig. 2). In INF, the most abundant *ermB* subtype differed by sampling day, with JN899585 being most abundant on Days 1 and 3 and AF109075 on Day 2 (Supplementary Table S6).

Five *sul1* subtypes were detected, with one subtype found in all samples. Two subtypes were specific to INF and SED, while another subtype was present in all sample types except SED. One *sul1* subtype (EU780013) was detected in all sample types, although it was most abundant in SED, and another (AY963803) was prevalent in all other sample types. Additionally, two *sul1* subtypes (U12338 and GU560437) were only identified in single INF and SED samples, respectively.

Tetracycline resistance genes were detected across all sample types (Fig. 1). Nine *tetA* subtypes were detected: Three were present in both INF and EFF, two in INF, EFF, and POND, one in both INF and POND, one in both POND and SED, and two exclusively in SED. Interestingly, one subtype (L20800) was identified in greater abundance in EFF than INF, while two others (HQ652506 and KX000272) were found primarily in INF.

tetQ was identified only in INF and EFF, with three subtypes present in both sample types, and an additional one exclusively in INF. All subtypes were roughly half as abundant in EFF samples as in INF. Four *tetW* subtypes were detected: Three in both INF and EFF samples, and another in INF, EFF, and one SED sample. The most prevalent *tetW* subtype (AJ427422) was found only in INF and EFF.

Discussion

In tracking five ARGs through wastewater treatment, our results show that qPCR was more sensitive detecting ARG presence, particularly in diluted samples with low ARG concentrations such as oxidation pond water. This was expected as the higher number of qPCR cycles increases the number of copies of a specific target, while scarce ARGs in MGS data are not specifically amplified using the library preparation used here. Only a fraction

(~0.24%) of the total MGS reads aligned to ARGs. While qPCR could track ARGs through the system, MGS analysis revealed multiple ARG subtypes across sample types which were not distinguished by qPCR. While qPCR showed the relative and absolute prevalence of some genes decreased through treatment, they may not actually be the 'same' gene, such as *sul1* detected by qPCR was comprised of four distinct *sul1* subtypes that varied across sample types. This highlights that care should be taken when comparing different sample types based on qPCR analysis and inferring origins or removal rates of ARGs through wastewater treatment.

ARG levels per 16S rRNA gene were lower in EFF than INF samples using qPCR, while MGS found comparable levels between the two sample types. This disparity is likely due to MGS requiring a reference match (with sufficient coverage) leading to a lower proportional difference between 16S rRNA genes and ARGs between INF and EFF. However, in terms of read count, MGS detected higher ARG counts in INF than EFF samples, indicating that depending on the sample type, ARG per 16S rRNA ratios may be less reliable when using non-quantitative MGS. In addition, there is potential for qPCR to be affected by a greater diversity of DNA in INF for off-target primer and probe binding, and differences between subtypes may lead to the under estimation of some genes with both methods.

qPCR is fast and quantitative; however, a-priori knowledge is required, and it assumes that primers are binding to their intended target. Subtypes of each ARG were detected in different sample types with MGS, showing that while one ARG appears to be detected in different sample types or sample sites, one cannot infer that it is indeed the same gene subtype. Without specific qPCR primers or sequencing of the PCR product, qPCR, while more sensitive (in diluted samples), may lead to the intuitive assumption that a gene quantified in different samples, is the same ARG subtype, while in fact, it may have a different origin.

The results indicate that the chosen approach for ARG detection could be impacted by sample type more than previously considered. Gene subtype differences between samples, even in a continuous flow environment such as a WWTP, indicate that although qPCR can detect different ARGs with high sensitivity, the results of such an analysis may lead to erroneous conclusions about the continuity of their presence, reduction, or increase. Sequencing qPCR products could help alleviate misinterpretation of qPCR results. However, it is not practical to routinely sequence all qPCR products, highlighting one of the advantages of MGS. As our results show, the ARG subtype at the start of a treatment system, may not necessarily be that which is detected at the end. Thus, appropriate wording is important when communicating research outcomes of studies investigating ARGs in environmental samples. Additionally, the comparison between qPCR and MGS highlights the difficulties and parameters for direct comparisons between the methodologies.

A combination of qPCR and MGS could reduce the selection bias introduced by qPCR, while more costly MGS could serve as a baseline to select genes of interest for qPCR analysis. Hybrid capture approaches using targeted enrichment may be the future method of choice but are currently cost prohibitive [21]. Therefore, samples subjected to MGS (with or without hybrid-capture) could be used to achieve a higher resolution picture, in conjunction with more frequent targeted qPCR for real-time quantitative monitoring of detected genes of concern.

Limitations

Limitations of the study include qPCR being largely unaffected by gene length as only a specified region within a gene is amplified, while MGS are non-specifically generated, and a threshold of whole gene coverage is required. Therefore, MGS may be biased against longer genes such as 16S rRNA (~1500 bp), *tetQ* and *tetW* (~1900 bp), relative to qPCR, affecting ARG per 16S rRNA values. Additionally, if the point of differentiation between two subtypes has insufficient coverage, only one would be identified and affect subtype proportions.

Abbreviations

AMR	antimicrobial resistance
ARGs	antibiotic resistance genes
GLASS	Global Antimicrobial Resistance Surveillance System
MGS	metagenomic sequencing
INF	post screen influent
EFF	secondary treated effluent
POND	tertiary maturation pond effluent
SED	base sediment tertiary maturation pond
qPCR	quantitative PCR
WHO	World Health Organization
WWTP	wastewater treatment plant

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13104-024-07027-9.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Acknowledgements

The authors wish to thank the staff at the Christchurch City Council WWTP for their assistance in sampling and ongoing support. Many thanks also to Erin McGill for sample processing and DNA extraction and to Megan Devane and Zoe Yang for providing valuable comments on the manuscript.

Author contributions
W.T. methodology, analysis, data curation, writing-original draft, writing-review; K.B. methodology, analysis, writing-review & editing; K.D. writing-review & editing; L.W. writing-review & editing, funding acquisition; I.P. conceptualisation, writing-original draft, writing-review & editing, funding acquisition.

Funding
This research was funded by the Institute of Environmental Science & Research Ltd. as part of the Strategic Science Investment Funding from the New Zealand Ministry of Business, Innovation and Employment (MBIE).

Data availability
Raw sequencing reads have been uploaded to the NCBI sequence read archive bioproject: PRJNA904380.

Declarations

Ethics approval and consent to participate
An application to the NZ Health and Disability Ethics Committee (HDEC) indicated that the study research plan using human sewerage was out of the scope of HDEC review. We obtained permission from Christchurch City Council to access the wastewater treatment plant for sampling and to publish the findings in the current manuscript.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 30 January 2024 Accepted: 3 December 2024
Published online: 07 January 2025

References

1. WHO. Global action plan on Antimicrobial Resistance. Geneva, Switzerland: World Health Organization; 2015.
2. WHO. Global antimicrobial resistance and use surveillance system (GLASS) report 2022. Geneva: World Health Organization;; 2022.
3. Harrington A, Vo V, Papp K, Tillett RL, Chang C-L, Baker H, Shen S, Amei A, Lockett C, Gerrity D, Oh EC. Urban monitoring of antimicrobial resistance during a COVID-19 surge through wastewater surveillance. Sci Total Environ. 2022;853:158577.
4. Centazzo N, Frederick B-M, Jacox A, Cheng S-Y, Concheiro-Guisan M. Wastewater Analysis for Nicotine, Cocaine, Amphetamines, Opioids and Cannabis in New York City. Forensic Sci Res. 2019;4:152–67.
5. Londong J, Barth M, Söbke H. 2023. Reducing antimicrobial resistances by source separation of domestic wastewater. Front Environ Health 2:1151898
6. Keenum I, Liguori K, Calarco J, Davis BC, Milligan E, Harwood VJ, Pruden A. A framework for standardized qPCR-targets and protocols for quantifying antibiotic resistance in surface water, recycled water and wastewater. Crit Rev Environ Sci Technol. 2022;52:4395–419.
7. Ferreira C, Otani S, Aarestrup FM, Manaia CM. Quantitative PCR versus metagenomics for monitoring antibiotic resistance genes: balancing high sensitivity and broad coverage. FEMS Microbes. 2023;4:008.
8. Abramova A, Berendonk TU, Bengtsson-Palme J. A global baseline for qPCR-determined antimicrobial resistance gene prevalence across environments. Environ Int. 2023;178:108084.
9. Hendriksen RS, Munk P, Njage P, van Bunnik B, McNally L, Lukjancenko O, Röder T, Nieuwenhuijse D, Pedersen SK, Kjeldgaard J, Kaas RS, Clausen PTLC, Vogt JK, Leekitcharoenphon P, van de Schans MGM, Zuidema T, de Roda Husman AM, Rasmussen S, Petersen B, Bego A, Rees C, Cassar S, Coventry K, Collignon P, Allerberger F, Rahube TO, Oliveira G, Ivanov I, Vuthy Y, Sopheak T, Yost CK, Ke C, Zheng H, Baisheng L, Jiao X, Donado-Godoy P, Coulibaly KJ, Jergović M, Hrenovic J, Karpíšková R, Villacis JE, Legesse M, Eguale T, Heikinheimo A, Malania L, Nitsche A, Brinkmann A, Saba CKS, Kocsis B, Solymosi N, et al. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. Nat Commun. 2019;10:1124.
10. Ariyadasa S, Taylor W, Weaver L, McGill E, Billington C, Pattis I. Nonbacterial Microflora in Wastewater treatment plants: an underappreciated potential source of pathogens. Microbiol Spectr. 2023. https://doi.org/10.1128/spectrum.00481-23.
11. Bohm K, Taylor W, Gyawali P, Pattis I, Gutierrez-Gines MJ. Black soldier fly-based bioconversion of biosolids: microbial community dynamics and fate of antibiotic resistance genes. Sci Total Environ. 2024. https://doi.org/10.1016/j.scitotenv.2024.172823:172823.
12. Berglund B. Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics. Infect Ecol Epidemiol. 2015;5:28564.
13. Pattis I, Taylor W, Dyet K, McGill E, Dupont P-Y, Cornelius A, Straub C, Humphries B, Liaw L, Weaver L. 2022. Antimicrobial resistance in a New Zealand wastewater treatment plant: Metagenomics analysis and isolation of ESBL- and carbapenemase-producing Gram-negative bacteria, abstr Environmenal Dimension of Antibiotic Resistance EDAR 6, Gothenburg, Sweden, 22–27 September 2022.
14. Bushnell B. 2014. BBMap: A Fast, Accurate, Splice-Aware Aligner, 2014. https://www.osti.gov/servlets/purl/1241166.
15. Florensa AF, Kaas RS, Clausen P, Aytan-Aktug D, Aarestrup FM. ResFinder - an open online resource for identification of antimicrobial resistance genes in next-generation sequencing data and prediction of phenotypes from genotypes. Microb Genom. 2022;8:748.
16. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41:D590–6.
17. Clausen PTLC, Aarestrup FM, Lund O. Rapid and precise alignment of raw reads against redundant databases with KMA. BMC Bioinformatics. 2018;19:307.
18. Munk P, Brinch C, Møller FD, Petersen TN, Hendriksen RS, Seyfarth AM, Kjeldgaard JS, Svendsen CA, van Bunnik B, Berglund F, Larsson DGJ, Koopmans M, Woolhouse M, Aarestrup FM. Genomic analysis of sewage from 101 countries reveals global landscape of antimicrobial resistance. Nat Commun. 2022;13:7251.
19. Team RC. A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing; 2021.
20. Liu X, Xiao P, Guo Y, Liu L, Yang J. The impacts of different high-throughput profiling approaches on the understanding of bacterial antibiotic resistance genes in a freshwater reservoir. Sci Total Environ. 2019;693:133585.
21. Baba H, Kuroda M, Sekizuka T, Kanamori H. Highly sensitive detection of antimicrobial resistance genes in hospital wastewater using the multiplex hybrid capture target enrichment. mSphere. 2023;8:e00100–23.

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