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Candidate biomarkers of antibiotic resistance for the monitoring of wastewater and the downstream environment

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ABSTRACT

Urban wastewater treatment plants (UWTPs) are essential for reducing the pollutants load and protecting water bodies. However, wastewater catchment areas and UWTPs emit continuously antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs), with recognized impacts on the downstream environments. Recently, the European Commission recommended to monitor antibiotic resistance in UWTPs serving more than 100 000 population equivalents. Antibiotic resistance monitoring in environmental samples can be challenging. The expected complexity of these systems can jeopardize the interpretation capacity regarding, for instance, wastewater treatment efficiency, impacts of environmental contamination, or risks due to human exposure. Simplified monitoring frameworks will be essential for the successful implementation of analytical procedures, data analysis, and data sharing. This study aimed to test a set of biomarkers representative of ARG contamination, selected based on their frequent human association and, simultaneously, rare presence in pristine environments. In addition to the 16S rRNA gene, ten potential biomarkers (*intI1, sul1, ermB, ermF, aph(3'')-Ib, qacEΔ1, uidA, mefC, tetX*, and crAssphage) were monitored in DNA extracts ($n = 116$) from raw wastewater, activated sludge, treated wastewater, and surface water (upstream and downstream of UWTPs) samples collected in the Czech Republic, Denmark, Israel, the Netherlands, and Portugal. Each biomarker was sensitive enough to measure decreases (on average by up to 2.5 log-units gene copy/mL) from raw wastewater to surface water, with variations in the same order of magnitude as for the 16S rRNA gene. The use of the 10 biomarkers allowed the typing of water samples whose origin or quality could be predicted in a blind test. The results show that, based on appropriate biomarkers, qPCR can be used for a cost-effective and technically accessible approach to monitoring wastewater and the downstream environment.

1. Introduction

Human sewage is a major source of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Munk et al., 2022). In most world regions, human sewage is collected along with domestic liquid wastes. These raw wastewaters should be treated before returning to the environment. According to the European Commission, in Europe, over 90% of urban wastewater is dealt accomplishing the EU standards. Still, 10 million Europeans and half of the world population do not have access to adequate sanitation systems (European Commission, 2023;

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UN-Water, 2020). Even in regions where urban wastewater treatment plants (UWTPs) are implemented and operating properly, it is demonstrated that antibiotic resistance emissions may have noticeable impacts on the receiving environment (Mukherjee et al., 2021; Manaia 2023). However, the scientific community and regulators recognize that it is still difficult to assess the risks associated with exposure. Simplified and low-cost monitoring methods might contribute to mapping the distribution of ARGs, measuring their removal during wastewater treatment, and assessing potential impacts on the receiving environment. Over the last years, metagenomic-based studies have unveiled the impressive diversity of ARGs that can be found in wastewaters (Numberger et al., 2019; Hendriksen et al., 2019; Munk et al., 2022). However, it has been argued that not all genes are equally important or relevant in terms of human health risks (Manaia 2017). Recognizing that some prioritization is needed, Zhang et al. (2021) proposed a list of 37 ARGs to be considered regarding potential human-health risk. This list was inspired by the World Health Organization pipeline for Antibacterial Agents in Clinical Development (WHO, 2019). Genetic assays used for tracking antibiotic resistance across One-Health compartments, assessing environmental impacts or measuring wastewater treatment efficiency, may be also useful for supporting the development of quality criteria and policy for controlling the spreading of antibiotic resistance in water systems (Manaia 2023; Miłobedzka et al., 2022). UWTPs may have an essential role on the prevention of ARGs dissemination (Manaia 2023). However, while one primary role of sewage collection and treatment is to limit the spreading of pathogens, UWTPs have not been primarily designed to remove ARB or ARGs. The recent proposal of the European Parliament and Council to revise the 30-years-old EU Directive 91/271/EEC concerning urban wastewater treatment (European Commission, 2022) addresses the need to monitor antibiotic resistance emissions by UWTPs larger than 100 000 population equivalent (p.e.). Such an effort should contribute to reduce the impacts of antibiotic resistance release from UWTPs in downstream environments. Environmental impacts of antibiotic resistance are well documented in recreational waters, wild-life, or agriculture soils, enhancing the risks of dissemination and human exposure (Han et al., 2022; Laborda et al., 2022; Leonard et al., 2018; Christou et al., 2017; Vredenburg et al., 2014). Selective culturing, qPCR assays, and shotgun metagenomics have been frequently used for monitoring antibiotic resistance in the environment (Franklin et al., 2021). However, these procedures need to be customized and harmonized for routine monitoring, as it must be feasible and cost-effective, enabling wide implementation, objective interpretation, and consistent data sharing. The most successful example of microbiological routine monitoring is based on the detection and enumeration of *Escherichia coli*, used as an indicator of faecal contamination (e.g., ISO 9308–1:2014) (Harwood et al., 2017). Nevertheless, this indicator is of little value for antibiotic resistance monitoring, as a myriad of culture and non-culturable bacteria belonging to a wide range of taxonomic groups may harbour ARGs in interest. For this reason, the detection and quantification of ARGs based on culture-independent methods has been considered the best option to investigate or monitor antibiotic resistance in the environment (Grenni, 2022; Manaia et al., 2018). Quantitative PCR (qPCR) has been suggested as an interesting option for the customized monitoring of ARGs in wastewater environments (Keenum et al., 2022; Ferreira et al., 2023; Manaia, 2023). As a targeted method, qPCR-based monitoring relies on the selection of a set of genes representative of the contamination of interest. In the case of ARGs this is challenging, since more than 2500 genes are reported and frequently updated in public databases like CARD (Comprehensive Antibiotic Resistance Database) (Yao and Yiu, 2019). As recently proposed, the selection of adequate biomarkers that represent the diversity of wastewater ARGs should follow some criteria: (*i*) be present in every sample suspected to contain clinically relevant ARGs; (*ii*) be frequently associated with mobile genetic elements; (*iii*) be sufficiently abundant to permit a reliable quantification even after wastewater treatment or dilution; and (*iv*) be stable in the environmental resistome, i.e., not emerging or in process of extinction (Manaia, 2023).

The present study aimed to establish a set of genes that could be used as representative biomarkers of antibiotic resistance contamination for monitoring wastewater and downstream aquatic environments. The selection of potential biomarkers relied on a risk prioritization list of ARGs (Zhang et al., 2021), trimmed for genes with frequent occurrence in human-impacted areas and simultaneous absence in pristine water sources. This rationale allowed the shortlisting of ten candidate genetic biomarkers, which were tested on wastewater and surface water samples collected in five countries, namely the Czech Republic, Denmark, Israel, the Netherlands, and Portugal. The study focused on evaluating the variation in biomarker abundance in different types of wastewater, and also aimed at antibiotic resistance typing of raw sewage, treated sewage, and surface water samples.

2. Material and methods

2.1. Biomarkers selection

The selection process (Fig. 1) started with a group of 60 antibiotic resistance and housekeeping genes and mobile genetic elements (MGEs) that have been frequently associated with anthropogenic impact, have been classified as being clinically relevant, and/or observed to occur in wastewater environments (Manaia 2023; Karkman et al., 2019; Pärnänen et al., 2019; Zhang et al., 2021). To be considered as a potential biomarker, each of these genes was classified according to their frequent occurrence in humans or in environments with human contamination (e.g. human gut, wastewater, sewage), and that were not reported in clean environments, i.e. not expected to be under strong human impact (such as clean freshwater, marine water, or soil). The occurrence of each gene in these different environments was assessed based on metagenomes querying using the public database MGnify (Richardson et al., 2023) available in https://www.ebi.ac.uk/metagenomics/ (hosted by EMBL-EBI, accessed between September 21 and October 28, 2021 - supplementary Table S1_annex). The 33 827 metagenomic analyses present in the database were about 54 % from human's; 19 % environmental - 14 % aquatic environments, specifically 1 % of freshwater, and 4 % of engineered habitats, specifically 2 % of wastewater. To perform the *blastP* query, for each genetic determinant, the complete deduced amino acid sequence was collected from CARD, NCBI (National center for Biotechnology Information) database, or the respective publication. According to the MGnify BLAST requirements, fragments of less than 600 amino acids were used for search and when the gene allelic variant was not indicated, the most frequent variant was used as reference. For aminoacid sequences longer than 600 amino acids (e.g., *tetQ* and *tetM*) was used a segment corresponding to the amplicon frequently used for qPCR measurements (Pärnänen et al., 2019).The search criteria required a query coverage of >75 % and an *e*-value <1.0 e^{-25}. The 15 best hits (lowest *e*-values and highest scores found) were polled according with the metagenome source (Fig. 1, Table S1) and each query gene was labelled as "Human", if mainly reported in the human gut, wastewater and sludge, or as "Human + Environment" when reported in both human associated metagenomes and in environmental metagenomes not expected to have anthropogenic contamination (e.g. glaciers, soil, deep ocean or marine environments). In no situation, did the first 15 entries list only, or mainly, pristine sites. Genes labelled as "Human" ($n = 21$) constituted the preliminary list of potential biomarkers and were screened for a second query round, based on the search of similar sequences in freshwater metagenomes, leading to a short list of five genes (*ermB; tetX; mefC; aph(3'')-Ib;* crAssphage) associated with humans and not detected in freshwater. The crAssphage gene was already previously describe as a suitable indicator of humn faecal contamination (Park et al., 2020). Aiming to benchmark these with other potential indicators, other genes were included. The genes *intI1* (class 1 integron integrase), *sul1* (sulfonamide resistance), and *qacEΔ1* (quaternary ammonium compound resistance) that may occur

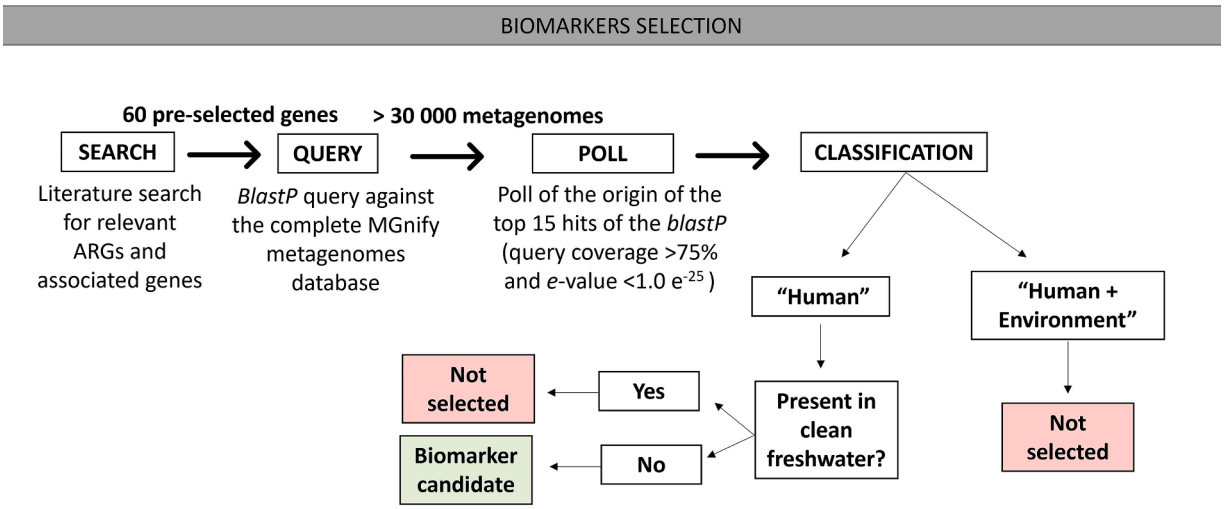

Fig. 1. Pipeline for the biomarkers selection. Step 1: search in the literature for relevant genes associated with anthropogenic pollution, that resulted in the pre-selection of 60 genes. Step 2: *BlastP* query of the 60 genes against > 30 000 metagenomes in the MGnify public database. Step 3: Poll of the results obtained. And Step 4: classification of the gene occurrence according to type of samples where the gene was detected.

Table 1
Characterization of the water samples collected in different countries and analysed in this study.

WATER TYPE	SAMPLE	DESCRIPTION/TREATMENT	SAMPLING DATE	POPULATION EQUIVALENT (INHABITANTS – P.E.)
Influent (I)	PT_RWW_AIRPT	airport UWTP influent	March 29, 2017	N.A.
(no. of DNA	PT_RWW_WWTP	municipal UWTP influent	July 26, 2017	80 000
extracts = 41)	PT_HE	hospital effluent	March 11, 2019	N.A.
	PT_RWW_UWTP	municipal UWTP influent	March 12, 2019	170 513
	NL_RWW1/2/3	municipal UWTP influent	February 24, 26, 28, 2020	308 333
	CZ_RWW1/2/3	municipal UWTP influent	February 1, 2, 3, 2022	800 000
	DK_RWW1/2/3	municipal UWTP influent	February 8, 9, 10, 2022	265 000
	IL_SWG_1/2/3	raw sewage	February 7, 8, 9, 2022	N.A.
Sludge (S)	PT_S	activated sludge - based secondary treatment	July 26, 2017	80 000
(no. of DNA	NL_S	activated sludge - (municipal) – conventional UWTP	November 23, 2021	814 800
extracts = 4)	CZ_S	activated sludge - sludge recirculation	February 25, 2020	800 000
Effluent (E)	PT_TWW_AIRPT	secondary airport effluent - preliminary treatment, primary treatment, and activated sludge-based secondary treatment	March 29, 2017	N.A.
(no. of DNA extracts = 47)	PT_TWW_WWTP	secondary municipal effluent - preliminary treatment, primary treatment, and activated sludge-based secondary treatment	July 26, 2017	80 000
	PT_TWW_UWTP	secondary municipal effluent - preliminary treatment, primary treatment, and activated sludge-based secondary treatment	March 14, 2019	170 513
	NL_TWW1/2/3	pre-treatment, Nereda technology - aerated granular sludge plant, sand filtration	February 24, 26, 28, 2020	308 333
	CZ_TWW1/2/3	secondary clarifier effluent - activation tanks (fine-bubble aeration), secondary clarification, sludge recirculation	February 1, 2, 3, 2022	800 000
	DK_TWW1/2/3	secondary municipal effluent	February 8, 9, 10, 2022	265 000
	IL_MABR_1/2/3	membrane aerated bioreactor (MABR)	February 7, 8, 9, 2022	N.A.
	IL_RES_1/2/3	4500 L polypropylene reservoir	February 7, 8, 9, 2022	N.A.
Surface water (SW)	PT_RA	river upstream UWTP (450 m)	March 14, 2019	170 513
(no. of DNA	PT_RB	river downstream UWTP (850 m)	March 14, 2019	170 513
extracts = 24)	CZ_RA1/2/3	river upstream UWTP (200 m)	February 7, 8, 9, 2022	800 000
	CZ_RB1/2/3	river downstream UWTP (800 m)	February 7, 8, 9, 2022	800 000

N.A. – not available.
1/2/3: sampling day one, two and three.
Countries: PT – Portugal; NL - The Netherlands; CZ - Czech Republic; DK – Denmark; and IL - Israel.
Note: Heavy crosswind (50–70 km/h) on Feb 1–2, possible admixing of WWTP effluent into sample #3 stream water, in Czech Republic (CZ_RA2).

independently (Table S1), although are part of the conserved regions of class 1 integrons, a recognized proxy for anthropogenic pollution (Gillings 2014). In addition, the gene encoding the beta-glucuronidase (*uidA*), a molecular tag for *E. coli* (Chern et al., 2009), and the gene *ermF* (MLSB: macrolide, lincosamide, streptogramin B), sporadically found in clean freshwater (2 metagenomes) were included.

Based on this approach six ARGs (*ermB; ermF; tetX; mefC; aph(3″)-Ib; sul1*), one gene associated with genetic recombination (*intI*1), an efflux pump gene (*qacEΔ1*) and two indicators of faecal contamination (*uidA, E. coli*; and crAssphage, *Bacteroides intestinalis*) were selected to be tested as potential biomarkers (Table S1 in SI).

2.2. Samples' collection and processing

A total of 41 water samples from 5 different countries (the Czech Republic, Denmark, Israel, the Netherlands, and Portugal) were available for the study. These included influent (I; airport, hospital, municipal UWTPs); effluent (E; secondary effluents of UWTPs as well as a tertiary effluent (tE) from a 4500 L polypropylene reservoir receiving secondary effluent (IL_RES samples)); sludge (S; UWTPs activated sludge); and surface water (SW; river water, upstream and downstream of UWTPs) (Table 1). A total of 116 DNA extracts obtained from duplicate or triplicate sample replicates was used to measure the abundance of each of the selected potential biomarkers and the bacterial 16S rRNA gene by qPCR. The collection and processing of samples were performed at the origin country, according to a standard protocol (Table S2 in SI). The DNA extracts, whenever needed, were shipped to the laboratory where qPCR assays were performed, in Portugal. All DNA extracts were preserved at −20 °C until being analysed.

2.3. Genes quantification using quantitative polymerase chain reaction (qPCR)

The concentration of all DNA extracts was determined using a Qubit fluorometer (Thermo Fisher Scientific, USA) in the laboratory conducting the qPCR analyses. The qPCR primers and conditions to test the biomarkers candidates (*ermB; ermF; tetX; mefC; aph(3″)-Ib; qacEΔ1; intI1; uidA; sul1* and crAssphage) and the 16S rRNA gene are indicated in Table S3 in SI. Each qPCR reaction was run in duplicate and performed in StepOne™ Real-Time PCR System (Life Technologies, Carlsbad, CA, USA), using reaction mixtures of 20 μL. The gene copy numbers were interpolated from the Ct values in the respective standard curve built with known concentrations of the target gene. The standard curve method was implemented as described in Brankatschk et al. (2012), using gBlocks Gene Fragments (Integrated DNA Technologies, Inc), except the standard curve for the 16S rRNA gene that used genomic DNA from *E. coli* ATCC 25922. The four quality criteria for acceptance of qPCR measurements included: (*i*) standard curve efficiency between 90 and 110 % (as an exception, we accepted 88 % of efficiency for two samples analysed for *ermB* and two original river samples with low copy number for *tetX*); (*ii*) Ct values in the technical replicates could be interpolated in the standard curve and differed by less than <0.5 cycles; (*iii*) each amplification provided a single and expected melting temperature; and (*iv*) absence of shoulders (increased signal in the baseline), e.g., due to primer dimers.

2.4. Blind test samples

A blind test was performed with 15 samples whose source was unknown to the operator (including biological triplicates of raw wastewater, secondary treated wastewater, wastewater after UV disinfection, and surface water) and three negative control food samples (sausage, ham, and grilled chicken). The qPCR operator received the DNA extracts and processed and analysed them as previously described for the other samples.

2.5. Statistical analysis

The qPCR results were expressed as the logarithm of gene copy number per volume of sample (log (gene copy/mL)) or per 16S rRNA gene copy number (log (gene copy/16S rRNA gene copy)). The one-way analysis of variance (ANOVA) and Tukey's and Bonferroni post hoc tests were used to infer statistically significant differences ($p < 0.05$) in the abundance of total bacteria and genes by using the SPSS Statistics for Windows v.28.0 (IBM Corp., Armonk, NY, USA). A dendrogram and a Pearson's correlation were created, also with SPSS resources, based on biomarkers abundance hierarchical cluster method (between-groups linkage) with interval squared Euclidean distance and bivariate analysis, respectively. A Principal Component Analysis (PCA) was performed using the *prcomp* and *biplot* commands and a heatmap was performed with *heatmap ()* function of the RStudio statistical software for Windows v.2022.12.0 Build 353 (Posit Software, PBC).

3. Results

3.1. Abundance and prevalence of biomarkers

Quantitative PCR analyses were expressed as abundance (log-units gene copy / mL) and prevalence (log-units gene copy / gene copy of 16 rRNA) (Fig. 2). The total bacterial abundance assessed using the 16S rRNA gene ranged between 6.3 and 8.7 log-units gene copy/mL in influent DNA extracts ($n = 41$) and 6.9–8.0 log-units gene copy/mL in sludge DNA extracts ($n = 4$), and it was significantly lower ($p<0.05$) in effluent (5.8–7.8 log-units gene copy/mL; $n = 47$) and surface water (4.9–7.6 log-units gene copy/mL; $n = 24$) DNA extracts.

The abundance of the ten biomarker candidates (*intI1, sul1, ermB, ermF, aph(3″)-Ib, uidA, qacEΔ1, tetX, mefC*, and crAssphage) ranged between 1.0 and 7.9 log-units gene copy/mL; *intI1* and *uidA* were the most and least abundant genes, respectively (Fig. 2a and Figure S1 in SI). When expressed as a ratio to the 16S rRNA gene, the prevalence of the 10 genes varied (in average values) between −4.7 ± 0.5 and −0.9 ± 0.5 log-units gene copy number/16S rRNA gene copy number; as expected, *intI1* and *uidA* were the most and least prevalent genes, respectively (Fig. 2b and S2).

In influent samples, the gene abundance values ranged from 4.3 ± 0.5 to 6.5 ± 0.4 log-units gene copy number/mL; in decreasing abundance, the genes organized as *intI1* > *ermB* > *ermF* > *qacEΔ1* > *mefC* > *aph(3″)-Ib* > *sul1* > *tetX* > crAssphage > *uidA*. The abundance values for activated sludge samples ranged from 2.8 ± 0.6 to 6.6 ± 0.7 log-units gene copy number/mL sample, with *intI1* > *sul1* > *qacEΔ1* > *aph(3″)-Ib* > *ermF* > *tetX* > crAssphage > *ermB* > *mefC* > *uidA* (Fig. 2a). In effluent (E and tE) samples, values varied between 2.2 ± 0.8 and 4.9 ± 0.8 log-units gene copy number/mL sample, and the genes abundance ranking was similar to although slightly different from the activated sludge: *intI1* > *sul1* > *qacEΔ1* > *aph(3″)-Ib* > *ermF* > *mefC* > *ermB* > *tetX* > crAssphage > *uidA*. In surface water, the biomarker candidates were ranked as *intI1* > *mefC* > *sul1* > *ermB* > *ermF* > *qacEΔ1* > *aph(3″)-Ib* > crAssphage > *tetX* > *uidA*, with lower average values of abundance (1.7 ± 1.0 to 4.1 ± 0.7 log-units gene copy number/mL sample) than in effluent samples (Fig. 2a). No statistically significant differences on biomarkers' abundance were observed in surface water samples, upstream and downstream the UWTPs discharge point (Figure S3 in SI).

Collectively, these results show that the abundance of all the selected biomarker candidates significantly decreased from raw wastewater to surface water, although not all of them showed the same pattern of variation (Fig. 2 and Figures S1-S3 in SI). The Pearson's correlation coefficient between the abundance (per volume of sample) of each of the ten putative biomarkers in the different types of samples and the respective 16S rRNA gene abundance values ranged from 0.78 (*tetX*) to 0.88 (*ermB* and *ermF*), and the determination coefficient (R^2) values ranged from 0.61 to 0.77 (Fig. S4 in SI). These results suggest that the fate of bacteria harbouring the biomarker candidates follows the same

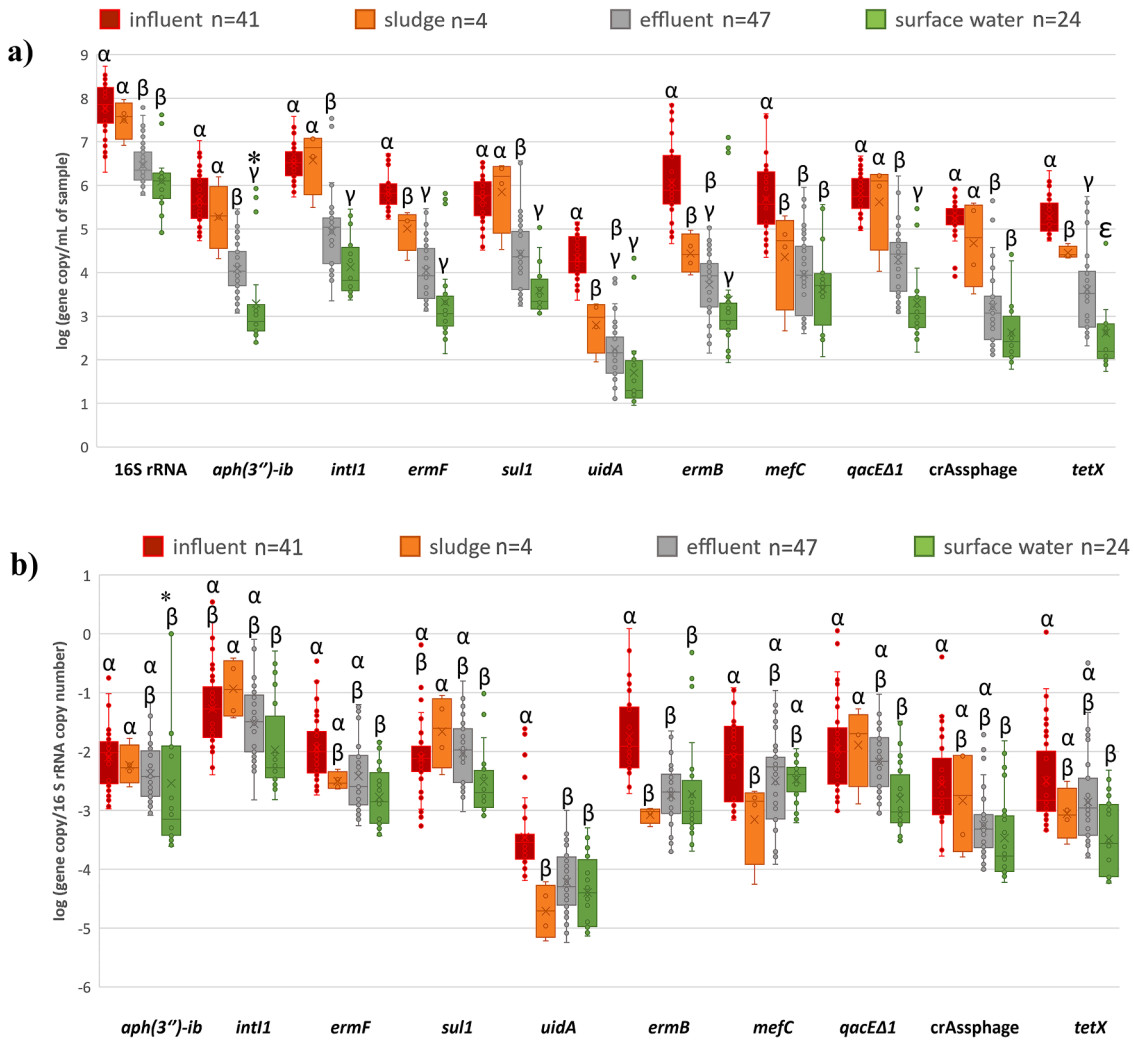

Fig. 2. Abundance and prevalence of biomarkers across samples. a) Abundance (log (gene copy/mL of sample)) and b) prevalence (log (gene copy/16S rRNA gene copy number)) of the 16S rRNA gene and selected biomarkers (*intI1, sul1, ermB, ermF, aph(3′′)-Ib, uidA, qacEΔ1, tetX, mefC* and crAssphage) in different DNA extracts from different samples. Influent (raw wastewater), sludge, effluent (treated wastewater), and surface water (upstream and downstream of WWTPs) samples are indicated in red, orange, grey and green, respectively. The asterisk (*) indicates the presence of unspecific amplification for PT_RA sample, in surface water sample group (green). α, β, γ and ε indicate significantly ($p < 0.05$) different Tukeyś groups comparing the genotype of samples: influent, sludge, effluent, and surface water samples.

trend as total bacteria. The strongest Pearson's correlation values (>0.95) were observed between the gene *uidA* and the genes *ermF* and *ermB*. The weakest values (<0.75) were observed between the gene *mefC* and the genes *intI1, sul1, qacEΔ1* and crAssphage, suggesting that *mefC* can be considered a good biomarker for clean waters samples (Table S5 in SI).

3.2. Biomarkers removal variation's

To evaluate the sensitivity of the biomarker candidates to assess treatment efficiency and downstream impacts, the biomarkers removal variations, in terms of reduction log values (per volume of sample), were determined between influent - effluent (I-E) and effluent – surface water samples (E-SW) (Table 2). These values ranged from 1.2 to 2.3 for wastewater treatment and 0.3 to 1.0 log-units gene copy/mL for impacts in the downstream environment. The biomarker candidates showing the highest I-E variation were *ermB, uidA* and crAssphage (2.3–2.0 log-units removal), while *sul1* showed the lowest variation (1.2 log-units gene copy/mL). Regarding the E-SW log reduction values, which would indicate mainly the dilution of the UWTP effluent in the receiving environment, the variations were in general lower than between influent (I) and effluent (E). The highest E-SW log reduction values were

Table 2
Removal of biomarkers within the wastewater treatment system and at the discharge interphase. Log-reduction (per volume of sample) differences values between influent (I) – effluent (E) and effluent (E) - surface water (SW) samples. Measurements in bold and in red indicate the highest and the lowest values, respectively.

	aph(3′′)-Ib	*intI1*	*ermF*	*sul1*	*uidA*	*ermB*	*mefC*	*qacEΔ1*	crAssphage	*tetX*	Average log-reduction of all biomarkers
I-E	1.6	1.6	1.8	1.2	**2.1**	**2.3**	1.7	1.5	**2.0**	1.7	1.8
E - SW	0.8	0.8	0.7	0.9	0.5	0.4	0.3	**1.0**	0.6	**1.0**	0.7
Average log- reduction across samples	1.2	1.2	1.3	1.1	1.3	**1.4**	1.0	1.3	1.3	**1.4**	

observed for *qacEΔ1* and *tetX* (1.0 log-units gene copy/mL) and the lowest for *mefC* and *ermB* (0.3–0.4 log-units gene copy/mL) (Table 2). The log-removal of the genes (log-units gene copy/mL) was also determined for each UWTP, between the influent - effluent (I-E) (Table S4 in SI). The average log-removal values observed for the different UWTPs ranged for all biomarkers (1.7–1.9 log-units gene copy/mL), except the Netherlands (1.0 log-units gene copy/mL). Despite UWTP-dependant variations, the biomarker candidate genes that presented log removal values above the average were the ARG *ermB* and the faecal indicator *uidA*.

3.3. Wastewater and water antibiotic resistance-based typing

The system-dependant variations (e.g., country, UWTP) were minor when the different types of sample (I, E, S, SW) were compared by Principal Component Analysis (PCA), in which the dimensions 1 and 2 explained ~ 93 % of the variance (Fig. 3). As expected, in general, the influent (I) samples were separated from effluent (E) and surface water (SW) samples along PC1 (~88 % variance), confirming major differences amongst sample types. Moreover, the biomarkers had a similar weight (vectors) amongst them and varied between 8.2 % (*mefC*) to 10.9 % (*ermF*). In particular, the vectors with the highest percentage values in this order: *ermF* > *uidA* > *qacEΔ1* > *tetX* > crAssphage, hence those with highest potential as biomarker candidates. In the PCA, effluent (E) and surface water (SW) were separated along axis 1 in most situations, suggesting the usefulness of the biomarkers also for typing these water samples and for indicating a high degree of ARG contamination of surface waters (displayed by samples SWup_CZ, in Fig. 3), while the tE profile was similar to SW samples. The potential of the tested biomarkers for typing water types was demonstrated in the heatmap (Fig. 4), where a major group represented influent (I) samples and another joined two sub-clusters of effluent (E) and surface water (SW) samples. The analysis was sensitive enough to identify effluents inadequately treated or contaminated surface waters, i.e. treated wastewater clustering with raw wastewater or surface water clustering with treated wastewater (e.g. PT_TWW_WWTP and CZ_RA2).

3.4. Blind test analysis

The usefulness of biomarkers for typing water quality was further tested through a blind test, in which the operator was challenged to correctly identify 15 blind test (BT) samples, based on the inspection of the 10 biomarkers. The presumable origin of the 12 of the BT water samples could be identified based on the clustering analysis (Fig. 4 and Fig. S5, in SI). Three of the BT samples (2, 6, 15) were of influent (I), three (1, 9 e 11) were of effluent (E), three (3, 7, 14) UV treated effluent

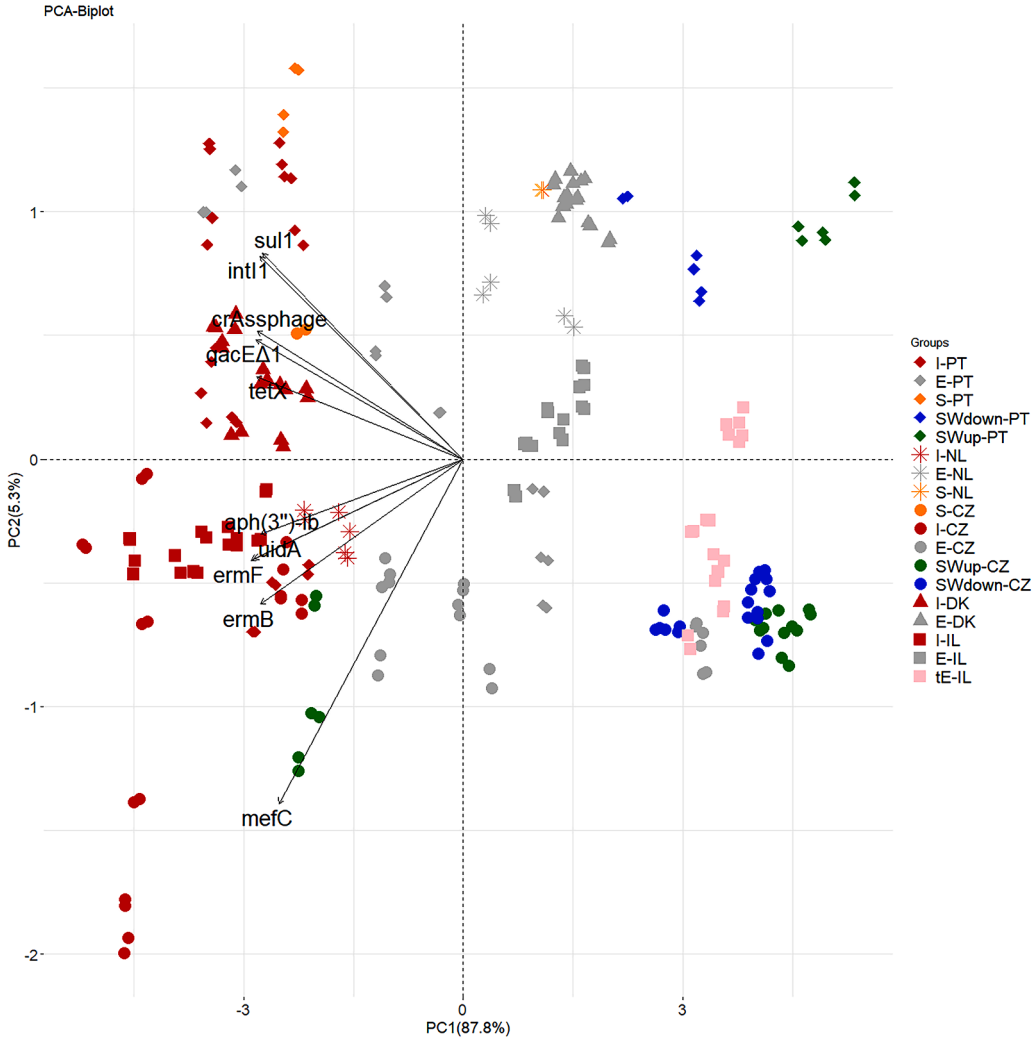

Fig. 3. Biomarkers Principal Component Analysis (PCA). Biplot showing the distribution of the different types of samples: influent (I), effluent (E), sludge (S) and surface water (SW) samples, based on the quantification by qPCR of the ten putative biomarkers (*intI1, sul1, ermB, ermF, aph(3')-Ib, uidA, qacEΔ1, tetX,* crAssphage and *mefC*), for the five countries in the study (NL = The Netherlands, CZ= Czech Republic, DK = Denmark, IL=Israel, and PT= Portugal).

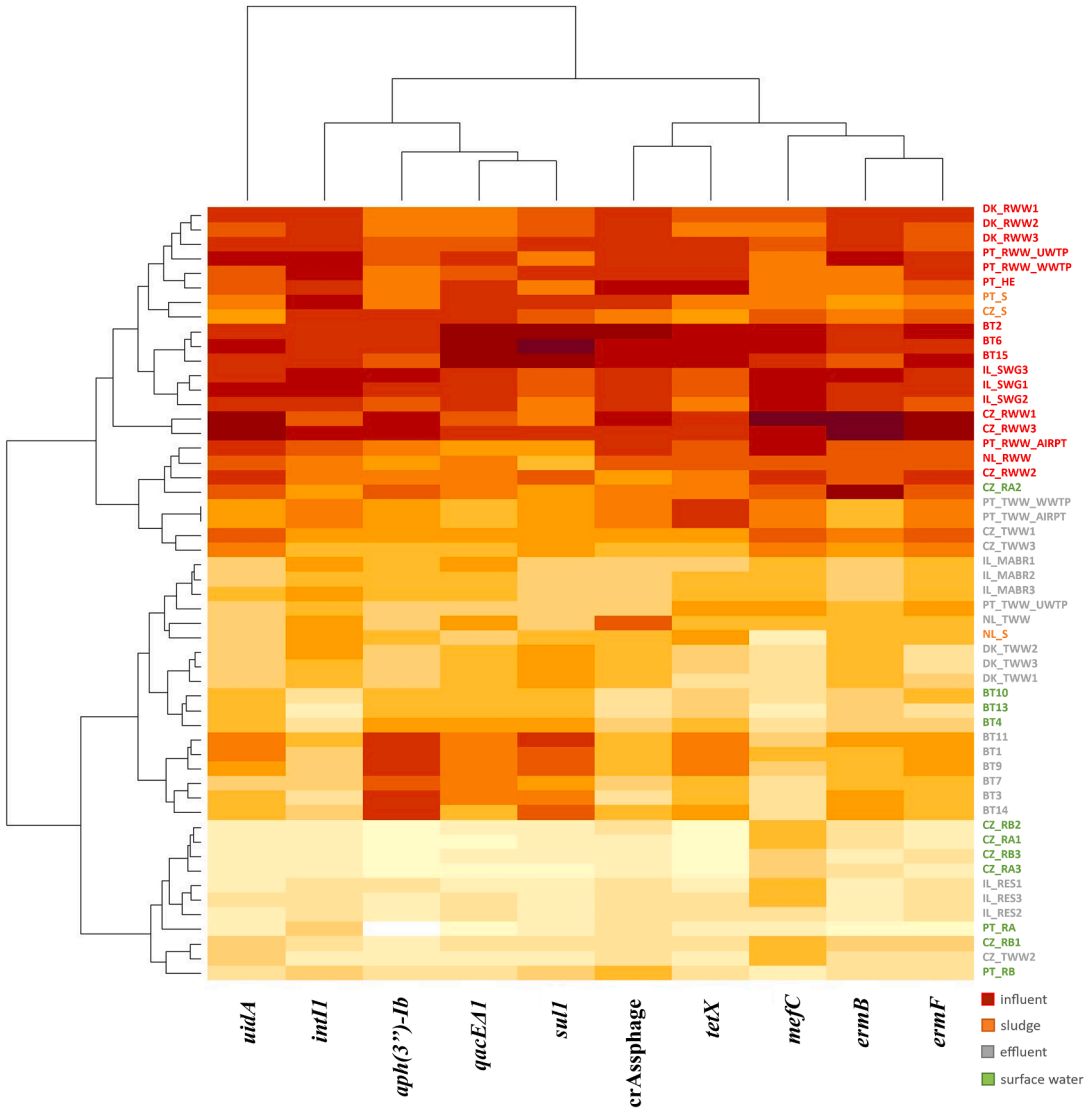

Fig. 4. Heatmap representation with corresponding dendrograms of concentration (log (gene copy/mL of sample)) of the ten putative biomarkers (*intI1, sul1, ermB, ermF, aph(3'')-Ib, uidA, qacEΔ1, tetX,* crAssphage and *mefC*), where colours represent the absolute abundance of each gene found in the different samples.

samples, and three (4, 10, 13) were surface water (SW) samples. Other three BT samples (5, 8, 12) represented negative controls selected from street food products, in which most of the analysed biomarkers were below the quantification or detection limits (data not shown). The BT analysis confirmed the reliability of biomarker candidates for typing water quality, and for hinting heavily contaminated surface waters, as was confirmed by the BT samples 4, 10, and 13. Conventional analysis showed that these samples tested positive for coliforms (data not shown).

4. Discussion

Wastewater-based epidemiology has gained a renewed interest with the recent COVID-19 epidemics (Fuschi et al., 2021; Monteiro et al., 2022; Riquelme et al., 2022). The concept has been proposed to survey antibiotic resistance (Aarestrup and Woolhouse, 2020) and the Global sewage project (www.globalsurveillance.eu) has been investigating in this area for some years. Recently, Munk et al. (2022) have profiled ARGs in more than 750 sewage samples from 101 countries. However, the monitoring of antibiotic resistance in wastewater has implications far beyond its epidemiological value. Indeed, it is essential to assess the effectiveness of UWTPs in removing human pathogens and the impact of wastewater treatment plant discharges on the receiving environment. Moreover regular and integrated monitoring processes have the potential to build a realiable body of information that maps antibiotic resistance in the environment and the possible relationships with geographic or socioeconomic contexts (Manaia, 2023). Nonetheless, although feasible, such monitoring needs to be optimised and the selection of appropriate biomarkers is one of the first aspects to be addressed. The range of suitable biomarkers for monitoring antibiotic resistance in the One Water cycle has recently been discussed (Keenum et al., 2022; Liguori et al., 2022; Manaia, 2023). In this study, the major criteria for biomarker selection were the association to humans and the high abundance in sites under strong human impacts. To test the suitability of these biomarker candidates, we analysed wastewater and receiving waters of different geographic regions and also tested blind samples to validate the reliability of the process. The genes tested in this study could have been considerably longer (Table S1), however, it was possible to demonstrate that genes associated with genetic recombination, antibiotic resistance or faecal contamination are suitable biomarkers that can be used to track anthropogenic contamination.

The candidate biomarkers investigated in this study (*intI1, sul1,*

ermB, ermF, aph(3'')-Ib, qacEΔ1, mefC, tetX, uidA and crAssphage) supported the assessment of the treatment efficiency and the impact of UWTPs discharges, while indicating highly contaminated surface waters. In general, the removal of total bacteria and ARB/ARGs occurs fairly at the same rate, which explains that estimating treatment efficacy or impacts based on prevalence (relative abundance) is poorly informative. This is an advantage of using qPCR and express the results per volume of sample to report ARGs occurrence (Manaia, 2023). However, it must be noted that although the rate of variation of the 16S rRNA gene was highly correlated with that of each biomarker, the monitoring of total bacteria alone cannot be used to predict water quality.

The persistence of ARGs after wastewater treatment, the impact of UWTPs discharges, and the influence of factors such as climate conditions or treatment types, have been extensively addressed in the literature (Ferreira et al., 2022; Pärnänen et al., 2019; Cacace et al., 2019; Rafraf et al., 2016). In this study, all UWTPs demonstrated an average log-removal for all biomarkers ranging 1.7–1.9 log-units gene copy/mL, except the Netherlands (1.0 log-units gene copy/mL). This latter result matched with the study of Calderón-Franco et al. (2022) where ARGs measured from intracellular DNA fractions decreased by 1.1 log gene units/mL during wastewater treatment. In general, the results showed that the group of 10 biomarker candidates could reliably inform about wastewater treatment or discharge impacts. The detailed analysis of data suggested that some biomarker candidates may be redundant, as provide similar responses (Table S5 in SI). Redundancy can result of common host or to genetic linkage as is known for *intI1, sul1, qacEΔ1* (Gillings 2014). However, these three genes did not show the same behaviour, as amongst the biomarkers tested, *intI1* was the most abundant in wastewater, and also the most abundant in clean waters, a behaviour that was not observed for *sul1, qacEΔ1*. This means that when other biomarkers may be below the limit of quantification, *intI1* may still be used to indicate the possible presence of low abundant ARGs. In contrast, the highest average variation between treated effluents and surface water was observed for *qacEΔ1* and *tetX*, suggesting that these may be poorly sensitive to assess impacts in surface waters. Still considering genes possibly linked, it is interesting to note that the gene *sul1* was that with the lowest average reduction between raw and treated wastewater, suggesting its suitableness to assess wastewater treatment efficacy. While the usefulness of the *intI1* gene has been noted before (Zheng et al., 2020) and was confirmed here, its pattern of variation cannot be assumed to be identical and presumably linked genes, which may occur in other sources, as is also confirmed in the metagenomes query made for this study (Table S1). In contrast, the macrolide resistance gene *mefC* was suggested as an adequate biomarker in clean waters, confirming its widespread occurrence in wastewater (Sugimoto et al., 2017). This discussion leads to the recommendation that amongst the 10 biomarkers tested, a subset can be used according to the types of water to monitor or the purpose of the monitoring.

Despite the essential role as barriers to reduce the dissemination of ARB, small UWTPs (10 000 m3/day) can discharge 13–17 log-units/day of ARGs and large UWTPs (1 000 000 m3/day) can reach 15–19 log-units/day (Manaia 2023). These discharges have important impacts in the surrounding environment and may contribute to the propagation of ARB and ARGs through distinct One Health compartments (Manaia 2023). This situation motivated the integration of antibiotic resistance monitoring as part of the EU-Urban Wastewater Treatment Directive (European Commission, 2022). Monitoring of antimicrobial resistance in wastewater and the receiving environment has been largely a research endeavour. However, efforts to transfer this knowledge into routine practice are increasingly called for, with the selection and harmonisation of methods being crucial to promote integrated and global monitoring of antimicrobial resistance (Keenum et al., 2022; Liguori et al., 2022; Nguyen et al., 2021; Rocha et al., 2020; Rocha and Manaia, 2020). It must combine easiness of implementation, cost-effectiveness, and objective interpretation. Culture-based methods have the limitations of overlooking important ARGs harbours, while are heavily laborious for full analysis (i.e., pure culture, species identification, strain typing, gene detection) (Manaia et al., 2018). The culture-independent methods that could be considered for this purpose are mainly metagenomics and qPCR, relevant and complementary (Calderón-Franco et al., 2021, 2022; Ferreira et al., 2023; Yin et al., 2023). From the high resolution provided by DNA sequencing, metagenomics provides an exhaustive overview of ARGs and mobile genetic elements, mainly expressed as relative abundance values. Besides higher sensitivity, qPCR provides results that can be expressed as gene concentrations per volume of water or mass of biomass (Ferreira et al., 2023), that can be translated as ARG loads that are important to assess treatment efficiency and impacts on the receiving water body. In addition, adequate antibiotic resistance biomarkers can be used as sentinels for water pollution. This has been suggested for detection from coastal waters (*sul1, tetX, ermF* and *intI1)* (Zhang et al., 2020), deep ocean (Mariana Trench) (*cfxA2, ermF*, and *mefA*) (Yang et al., 2021), or surface waters (crAssphage) (Chen et al., 2023). Also, *sul2, tetB, tetC*, and *tetW* on the Beijiang River (Jiang et al., 2018) and a list of ARGs found in the Subalpine area in various studies, which include amongst others, the genes *sul1, ermB, qnrS,* and bla_{OXA} (Eckert et al., 2018). Hospital effluents, considered domestic effluents that can be treated in UWTP, may contain high load of some emerging ARGs (Ferreira et al., 2022), and in such cases the list of candidate biomarkers may be complemented with genes such as those enconding carbapenemases (e.g. bla_{KPC} or bla_{NDM}) (Proia et al., 2018; Ferreira et al., 2022). In summary, the targeted nature of qPCR allows the selection of a rational set of biomarkers that can be used globally, allowing comparative and evolution analyses (Manaia 2023). This objective is demonstrated in this study, which can also be a relevant contribution to the implementation of the revised EU Urban Wastewater Treatment Directive (European Commission, 2022). Based on the implementation of standardized methods, indexed to international directives, and the definition of a group of biomarkers, it will be possible to share and compare data, and monitor and analyse water quality patterns.

5. Conclusions

The biomarker candidates selected by querying metagenomes (*ermB, aph(3'')-Ib, mefC, tetX*, and crAssphage) and/or because of the recognized association with anthropogenic impacts (*intI1, sul1, qacEΔ1, ermF* and *uidA*) were quantified by qPCR, allowing the assessment of the variation of antibiotic resistance load from raw wastewater (influent) to surface water samples ($n = 116$ + blind test DNA extracts) collected from five countries. The use of these biomarkers enabled the typing of (waste)waters based on their level of ARGs contamination, and was validated by a blind test with 15 unknown samples. This study not only showed the feasibility of globally monitoring antibiotic resistance in (waste)waters to assess treatment efficiency and environmental impacts, but also showed the importance of a regular and integrated monitoring to map the occurrence of antibiotic resistance in surface waters.

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CRediT authorship contribution statement

A. Margarida Teixeira: Data curation, Investigation, Methodology, Formal analysis, Validation, Writing – original draft, Writing – review & editing. **Ivone Vaz-Moreira:** Conceptualization, Methodology, Validation, Data curation, Writing – review & editing. **David Calderón-Franco:** Resources. **David Weissbrodt:** . **Sabina Purkrtova:** Resources. **Stanislav Gajdos:** . **Giulia Dottorini:** Resources. **Per Halkjær Nielsen:** . **Leron Khalifa:** Resources. **Eddie Cytryn:** Resources. **Jan Bartacek:** . **Célia M. Manaia:** Conceptualization, Funding acquisition, Project administration, Resources, Investigation, Supervision, Methodology, Validation, Data curation, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data not already shared in the manuscript and in the supplementary materials will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.120761.

References

Aarestrup and Woolhouse, 2020. Using sewage for surveillance of antimicrobial resistance. Science 6478, 630–632. https://doi.org/10.1126/science.aba3432, 367.

Brankatschk, R., Bodenhausen, N., Zeyer, J., Burgmann, H., 2012. Simple absolute quantification method correcting for quantitative PCR efficiency variations for microbial community samples. Appl. Environ. Microbiol. 78, 4481–4489. https://doi.org/10.1128/AEM.07878-11.

Cacace, D., Fatta-Kassinos, D., Manaia, C.M., Cytryn, E., Kreuzinger, N., Rizzo, L., Karaolia, P., Schwartz, T., Alexander, J., Merlin, C., Garelick, H., Schmitt, H., de Vries, D., Schwermer, C.U., Meric, S., Ozkal, C.B., Pons, M.N., Kneis, D., Berendonk, T.U., 2019. Antibiotic resistance genes in treated wastewater and in the receiving water bodies: a pan-European survey of urban settings. Water Res. 162, 320–330. https://doi.org/10.1016/j.watres.2019.06.039.

Calderón-Franco, D., Sarelse, R., Christou, S., Pronk, M., van Loosdrecht, M.C.M., Abeel, T., Weissbrodt, D.G., 2022. Metagenomic profiling and transfer dynamics of antibiotic resistance determinants in a full-scale granular sludge wastewater treatment plant. Water Res. 219, 118571 https://doi.org/10.1016/j.watres.2022.118571.

Calderón-Franco, D., van Loosdrecht, M.C.M., Abeel, T., Weissbrodt, D.G., 2021. Free-floating extracellular DNA: systematic profiling of mobile genetic elements and antibiotic resistance from wastewater. Water Res. 189, 116592 https://doi.org/10.1016/j.watres.2020.116592.

Chen, Z., Duan, Y., Yin, L., Chen, Y., Xue, Y., Wang, X., Mao, D., Luo, Y., 2023. Unraveling the influence of human fecal pollution on antibiotic resistance gene levels in different receiving water bodies using crAssphage indicator gene. J. Hazard. Mater. 442, 130005 https://doi.org/10.1016/j.jhazmat.2022.130005.

Chern, E.C., Brenner, K.P., Wymer, L., Haugland, R.A., 2009. Comparison of fecal indicator bacteria densities in marine recreational waters by qPCR. Water Qual. Expo. Heal. 1, 203–214. https://doi.org/10.1007/s12403-009-0019-2.

Christou, A., Agüera, A., Bayona, J.M., Cytryn, E., Fotopoulos, V., Lambropoulou, D., Manaia, C.M., Michael, C., Revitt, M., Schröder, P., Fatta-Kassinos, D., 2017. The potential implications of reclaimed wastewater reuse for irrigation on the agricultural environment: the knowns and unknowns of the fate of antibiotics and antibiotic resistant bacteria and resistance genes – A review. Water Res. 123, 448–467. https://doi.org/10.1016/j.watres.2017.07.004.

Eckert, E.M., Cesare, A.D., Coci, M., Corno, G., 2018. Persistence of antibiotic resistance genes in large subalpine lakes: the role of anthropogenic pollution and ecological interactions. Hydrobiologia 824, 93–108. https://doi.org/10.1007/s10750-017-3480-0.

European Commission, 2022. Directive of the european parliament and of the council concerning urban wastewater treatment (recast), 2022.

European Commission, 2023. https://environment.ec.europa.eu/topics/water/urban-wastewater_en, accessed in March 2023.

Ferreira, C., Abreu-Silva, J., Manaia, C.M., 2022. The balance between treatment efficiency and receptor quality determines wastewater impacts on the dissemination of antibiotic resistance. J. Hazard. Mater. 434, 128933 https://doi.org/10.1016/j.jhazmat.2022.128933.

Ferreira, C., Otani, S., Aarestrup, F.M., Manaia, C.M., 2023. Quantitative PCR versus metagenomics for monitoring antibiotic resistance genes: balancing high sensitivity and broad coverage. FEMS Microbes 4, 1–7. https://doi.org/10.1093/femsmc/xtad008/7071892.

Franklin, A.M., Brinkman, N.E., Jahne, M.A., Keely, S.P., 2021. Twenty-first century molecular methods for analyzing antimicrobial resistance in surface waters to support One Health assessments. J. Microbiol. Methods 184, 106174. https://doi.org/10.1016/j.mimet.2021.106174.

Fuschi, C., Pu, H., Negri, M., Colwell, R., Chen, J., 2021. Wastewater-based epidemiology for managing the COVID-19 pandemic. ACS ES T Water 1, 1352–1362. https://doi.org/10.1021/acsestwater.1c00050.

Gillings, M.R., 2014. Integrons: past, present, and future. Microbiol. Mol. Biol. 78, 257–277. https://doi.org/10.1128/mmbr.00056-13.

Grenni, P., 2022. Antimicrobial resistance in rivers: a review of the genes detected and new challenges. Environ. Toxicol. Chem. 41, 687–714. https://doi.org/10.1002/etc.5289.

Han, Y., Hu, Y., Wen, H., Wu, J., Liu, Y., Zhang, Y., Wu, H., 2022. Occurrence and driving mechanism of antibiotic resistance genes in marine recreational water around Qinhuangdao, China. Front. Mar. Sci. 9, 976438 https://doi.org/10.3389/fmars.2022.976438.

Harwood, V., Shanks, O., Korajkic, A., Verbyla, M., Ahmed, W., Iriarte, M., 2017. General and Host-Associated Bacterial Indicators of Faecal pollution, in: Water and Sanitation for the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management (Global Water Pathogen Project). Michigan State University. https://doi.org/10.14321/waterpathogens.6.

Hendriksen, R.S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., Röder, T., Nieuwenhuijse, D., Pedersen, S.K., Kjeldgaard, J., Kaas, R.S., Clausen, P.T. L.C., Vogt, J.K., Leekitcharoenphon, P., van de Schans, M.G.M., Zuidema, T., de Roda Husman, A.M., Rasmussen, S., Petersen, B., Bego, A., Rees, C., Cassar, S., Coventry, K., Collignon, P., Allerberger, F., Rahube, T.O., Oliveira, G., Ivanov, I., Vuthy, Y., Sopheak, T., Yost, C.K., Ke, C., Zheng, H., Baisheng, L., Jiao, X., Donado-Godoy, P., Coulibaly, K.J., Jergović, M., Hrenovic, J., Karpíšková, R., Villacis, J.E., Legesse, M., Eguale, T., Heikinheimo, A., Malania, L., Nitsche, A., Brinkmann, A., Saba, C.K.S., Kocsis, B., Solymosi, N., Thorsteinsdottir, T.R., Hatha, A.M., Alebouyeh, M., Morris, D., Cormican, M., O'Connor, L., Moran-Gilad, J., Alba, P., Battisti, A., Shakenova, Z., Kiiyukia, C., Ng'eno, E., Raka, L., Avsejenko, J., Bērziņš, A., Bartkevics, V., Penny, C., Rajandas, H., Parimannan, S., Haber, M.V., Pal, P., Jeunen, G.J., Gemmell, N., Fashae, K., Holmstad, R., Hasan, R., Shakoor, S., Rojas, M.L.Z., Wasyl, D., Bosevska, G., Kochubovski, M., Radu, C., Gassama, A., Radosavljevic, V., Wuertz, S., Zuniga-Montanez, R., Tay, M.Y.F., Gavačová, D., Pastuchova, K., Truska, P., Ṭrkov, M., Esterhuyse, K., Keddy, K., Cerdà-Cuéllar, M., Pathirage, S., Norrgren, L., Örn, S., Larsson, D.G.J., Heijden, T.van der, Kumburu, H. H., Sanneh, B., Bidjada, P., Njanpop-Lafourcade, B.M., Nikiema-Pessinaba, S.C., Levent, B., Meschke, J.S., Beck, N.K., Van, C.D., Phuc, N.do, Tran, D.M.N., Kwenda, G., Tabo, D.adjim, Wester, A.L., Cuadros-Orellana, S., Amid, C., Cochrane, G., Sicheritz-Ponten, T., Schmitt, H., Alvarez, J.R.M., Aidara-Kane, A., Pamp, S.J., Lund, O., Hald, T., Woolhouse, M., Koopmans, M.P., Vigre, H., Petersen, T.N., Aarestrup, F.M., 2019. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. Nat. Commun. 10, 1124. https://doi.org/10.1038/s41467-019-08853-3.

Jiang, H.Y., Zhou, R.J., Yang, Y., Chen, B.W., Cheng, Z.N., Zhang, M.D., Li, J., Zhang, G., Zou, S.C., 2018. Characterizing the antibiotic resistance genes in a river catchment: influence of anthropogenic activities. J. Environ. Sci. 69, 125–132. https://doi.org/10.1016/j.jes.2017.08.009.

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

Keenum, I., Liguori, K., Calarco, J., Davis, B.C., Milligan, E., Harwood, V.J., Pruden, A., 2022. A framework for standardized qPCR-targets and protocols for quantifying antibiotic resistance in surface water, recycled water and wastewater. Crit. Rev. Environ. Sci. Technol. 52, 4395–4419. https://doi.org/10.1080/10643389.2021.2024739.

Laborda, P., Sanz-García, F., Ochoa-Sánchez, L.E., Gil-Gil, T., Hernando-Amado, S., Martínez, J.L., 2022. Wildlife and antibiotic resistance. Front. Cell. Infect. Microbiol. 12, 873989 https://doi.org/10.3389/fcimb.2022.873989.

Leonard, A.F.C., Zhang, L., Balfour, A.J., Garside, R., Hawkey, P.M., Murray, A.K., Ukoumunne, O.C., Gaze, W.H., 2018. Exposure to and colonisation by antibiotic-resistant E. coli in UK coastal water users: environmental surveillance, exposure assessment, and epidemiological study (Beach Bum Survey). Environ. Int. 114, 326–333. https://doi.org/10.1016/j.envint.2017.11.003.

Liguori, K., Keenum, I., Davis, B.C., Calarco, J., Milligan, E., Harwood, V.J., Pruden, A., 2022. Antimicrobial resistance monitoring of water environments: a framework for standardized methods and quality control. Environ. Sci. Technol. 56, 9149–9160. https://doi.org/10.1021/acs.est.1c08918.

Manaia, C.M., 2023. Framework for establishing regulatory guidelines to control antibiotic resistance in treated effluents. Crit. Rev. Environ. Sci. Technol. 53, 754–779. https://doi.org/10.1080/10643389.2022.2085956.

Manaia, C.M., 2017. Assessing the risk of antibiotic resistance transmission from the environment to humans: non-direct proportionality between abundance and risk. Trends Microbiol. 25, 173–181. https://doi.org/10.1016/j.tim.2016.11.014.

Manaia, C.M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Cerqueira, F., Fortunato, G., Iakovides, I.C., Zammit, I., Kampouris, I., Vaz-Moreira, I., Nunes, O.C., 2018. Antibiotic resistance in wastewater treatment plants: tackling the black box. Environ. Int. 115, 312–324. https://doi.org/10.1016/j.envint.2018.03.044.

Miłobedzka, A., Ferreira, C., Vaz-Moreira, I., Calderón-Franco, D., Gorecki, A., Purkrtova, S., Bartacek, Jan, Dziewit, L., Singleton, C.M., Nielsen, P.H., Weissbrodt, D.G., Manaia, C.M., 2022. Monitoring antibiotic resistance genes in wastewater environments: the challenges of filling a gap in the One-Health cycle. J. Hazard. Mater. 424, 127407 https://doi.org/10.1016/j.jhazmat.2021.127407.

Monteiro, S., Rente, D., Cunha, M.v., Gomes, M.C., Marques, T.A., Lourenço, A.B., Cardoso, E., Álvaro, P., Silva, M., Coelho, N., Vilaça, J., Meireles, F., Brôco, N., Carvalho, M., Santos, R., 2022. A wastewater-based epidemiology tool for COVID-19 surveillance in Portugal. Sci. Total Environ. 804, 150264 https://doi.org/10.1016/j.scitotenv.2021.150264.

Mukherjee, M., Laird, E., Gentry, T.J., Brooks, J.P., Karthikeyan, R., 2021. Increased antimicrobial and multidrug resistance downstream of wastewater treatment plants in an urban watershed. Front. Microbiol. 12, 657353 https://doi.org/10.3389/fmicb.2021.657353.

Munk, P., Brinch, C., Møller, F.D., Petersen, T.N., Hendriksen, R.S., Seyfarth, A.M., Kjeldgaard, J.S., Svendsen, C.A., van Bunnik, B., Berglund, F., Bego, A., Power, P., Rees, C., Lambrinidis, D., Neilson, E.H.J., Gibb, K., Coventry, K., Collignon, P., Cassar, S., Allerberger, F., Begum, A., Hossain, Z.Z., Worrell, C., Vandenberg, O., Pieters, I., Victorien, D.T., Gutierrez, A.D.S., Soria, F., Grujić, V.R., Mazalica, N., Rahube, T.O., Tagliati, C.A., Rodrigues, D., Oliveira, G., de Souza, L.C.R., Ivanov, I., Juste, B.I., Oumar, T., Sopheak, T., Vuthy, Y., Ngandijo, A., Nzouankeu, A., Olivier, Z.A.A.J., Yost, C.K., Kumar, P., Brar, S.K., Tabo, D.A., Adell, A.D., Paredes-Osses, E., Martinez, M.C., Cuadros-Orellana, S., Ke, C., Zheng, H., Baisheng, L., Lau, L.T., Chung, T., Jiao, X., Yu, Y., JiaYong, Z., Morales, J.F.B., Valencia, M.F., Donado-Godoy, P., Coulibaly, K.J., Hrenovic, J., Jergović, M., Karpíšková, R., Deogratias, Z.N., Elsborg, B., Hansen, L.T., Jensen, P.E., Abouelnaga, M., Salem, M. F., Koolmeister, M., Legesse, M., Eguale, T., Heikinheimo, A., le Guyader, S., Schaeffer, J., Villacis, J.E., Sanneh, B., Malania, L., Nitsche, A., Brinkmann, A., Schubert, S., Hesse, S., Berendonk, T.U., Saba, C.K.S., Mohammed, J., Feglo, P.K., Banu, R.A., Kotzamanidis, C., Lytras, E., Lickes, S.A., Kocsis, B., Solymosi, N., Thorsteinsdottir, T.R., Hatha, A.M., Ballal, M., Bangera, S.R., Fani, F., Alebouyeh, M., Morris, D., O'Connor, L., Cormican, M., Moran-Gilad, J., Battisti, A., Diaconu, E.L., Corno, G., di Cesare, A., Alba, P., Hisatsune, J., Yu, L., Kuroda, M., Sugai, M., Kayama, S., Shakenova, Z., Kiiyukia, C., Ng'eno, E., Raka, L., Jamil, K., Fakhraldeen, S.A., Alaati, T., Bērziņš, A., Avsejenko, J., Kokina, K., Streikisa, M., Bartkevics, V., Matar, G.M., Daoud, Z., Pereckienė, A., Butrimaite-Ambrozeviciene, C., Penny, C., Bastaraud, A., Rasolofoarison, T., Collard, J.M., Samison, L.H., Andrianarivelo, M.R., Banda, D.L., Amin, A., Rajandas, H., Parimannan, S., Spiteri, D., Haber, M.V., Santchurn, S.J., Vujacic, A., Djurovic, D., Bouchrif, B., Karraouan, B., Vubil, D.C., Pal, P., Schmitt, H., van Passel, M., Jeunen, G.J., Gemmell, N., Chambers, S.T., Mendoza, F.P., Huete-Pırez, J., Vilchez, S., Ahmed, A.O., Adisa, I.R., Odetokun, I.A., Fashae, K., Sørgaard, A.M., Wester, A.L., Ryrfors, P., Holmstad, R., Mohsin, M., Hasan, R., Shakoor, S., Gustafson, N.W., Schill, C.H., Rojas, M.L.Z., Velasquez, J.E., Magtibay, B.B., Catangcatang, K., Sibulo, R., Yauce, F.C., Wasyl, D., Manaia, C., Rocha, J., Martins, J., Álvaro, P., di Yoong Wen, D., Shin, H., Hur, H.G., Yoon, S., Bosevska, G., Kochubovski, M., Cojocaru, R., Burduniuc, O., Hong, P.Y., Perry, M.R., Gassama, A., Radosavljevic, V., Tay, M.Y.F., Zuniga-Montanez, R., Wuertz, S., Gavačová, D., Pastuchová, K., Truska, P., Trkov, M., Keddy, K., Esterhuyse, K., Song, M.J., Quintela-Baluja, M., Lopez, M.G., Cerdà-Cuéllar, M., Perera, R.R.D.P., Bandara, N.K. B.K.R.G.W., Premasiri, H.I., Pathirage, S., Charlemagne, K., Rutgersson, C., Norrgren, L., Örn, S., Boss, R., van der Heijden, T., Hong, Y.P., Kumburu, H.H., Mdegela, R.H., Hounmanou, Y.M.G., Chonsin, K., Suthienkul, O., Thamlikitkul, V., de Roda Husman, A.M., Bidjada, B., Njanpop-Lafourcade, B.M., Nikiema-Pessinaba, S.C., Levent, B., Kurekci, C., Ejobi, F., Kalule, J.B., Thomsen, J., Obaidi, O., Jassim, L.M., Moore, A., Leonard, A., Graham, D.W., Bunce, J.T., Zhang, L., Gaze, W.H., Lefor, B., Capone, D., Sozzi, E., Brown, J., Meschke, J.S., Sobsey, M.D., Davis, M., Beck, N.K., Sukapanpatharam, P., Truong, P., Lilienthal, R., Kang, S., Wittum, T.E., Rigamonti, N., Baklayan, P., Van, C.D., Tran, D.M.N., do Phuc, N., Kwenda, G., Larsson, D.G.J., Koopmans, M., Woolhouse, M., Aarestrup, F. M., 2022. Genomic analysis of sewage from 101 countries reveals global landscape of antimicrobial resistance. Nat. Commun. 13, 7251. https://doi.org/10.1038/s41467-022-34312-7.

Nguyen, A.Q., Vu, H.P., Nguyen, L.N., Wang, Q., Djordjevic, S.P., Donner, E., Yin, H., Nghiem, L.D., 2021. Monitoring antibiotic resistance genes in wastewater treatment: current strategies and future challenges. Sci. Total Environ. 783, 146964 https://doi.org/10.1016/j.scitotenv.2021.146964.

Numberger, D., Ganzert, L., Zoccarato, L., Mühldorfer, K., Sauer, S., Grossart, H.P., Greenwood, A.D., 2019. Characterization of bacterial communities in wastewater with enhanced taxonomic resolution by full-length 16S rRNA sequencing. Sci. Rep. 9, 9673. https://doi.org/10.1038/s41598-019-46015-z.

Park, G.W., Ng, T.F.F., Freeland, A.L., Marconi, V.C., Boom, J.A., Staat, M.A., Montmayeur, A.M., Browne, H., Narayanan, J., Payne, D.C., Cardemil, C.V., Treffiletti, A., Vinje, J., 2020. CrAssphage as a novel tool to detect human fecal contamination on environmental surfaces and hands. Emerg. Infect. Dis. 26, 1731–1739. https://doi.org/10.3201/eid2608.200346.

Pärnänen, K.M., Narciso-da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Thuy Do, T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Luis Martinez, J., Michael, S.G., Michael-Kordatou, I., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sørum, H., Stedtfeld, R.D., Tiedje, J.M., Varela Della Giustina, S., Walsh, F., Vaz-Moreira, I., Virta, M., Manaia, C.M., 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. Sci. Adv. 27, 9124. https://doi.org/10.1126/sciadv.aau9124. PMID: 30944853; PMCID: PMC6436925.

Proia, L., Anzil, A., Borrego, C., Farrè, M., Llorca, M., Sanchis, J., Bogaerts, P., Balcázar, J.L., Servais, P., 2018. Occurrence and persistence of carbapenemases genes in hospital and wastewater treatment plants and propagation in the receiving river. J. Hazard. Mater. 15, 33–43. https://doi.org/10.1016/j.jhazmat.2018.06.058.

Rafraf, I.D., Lekunberri, I., Sànchez-Melsió, A., Aouni, M., Borrego, C.M., Balcázar, J.L., 2016. Abundance of antibiotic resistance genes in five municipal wastewater treatment plants in the Monastir Governorate, Tunisia. Environ. Pollution 219, 353–358. https://doi.org/10.1016/j.envpol.2016.10.062.

Richardson, L.J., Allen, B., Baldi, G., Beracochea, M., Bileschi, M., Burdett, T., Burgin, J., Caballero-Pérez, J., Cochrane, G., Colwell, L., Curtis, T., Escobar-Zepeda, A., Gurbich, T., Kale, V., Korobeynikov, A., Raj, S., Rogers, A.B., Sakharova, E., Sanchez, S., Wilkinson, D., Finn, R.D., 2023. MGnify: the microbiome sequence data analysis resource in 2023. Nucleic Acids Res. 51, 753–759. https://doi.org/10.1093/nar/gkac1080.

Riquelme, M.V., Garner, E., Gupta, S., Metch, J., Zhu, N., Blair, M.F., Arango-Argoty, G., Maile-Moskowitz, A., Li, A.D., Flach, C.F., Aga, D.S., Nambi, I.M., Larsson, D.G.J., Bürgmann, H., Zhang, T., Pruden, A., Vikesland, P.J., 2022. Demonstrating a comprehensive wastewater-based surveillance approach that differentiates globally sourced resistomes. Environ. Sci. Technol. 56, 14982–14993. https://doi.org/10.1021/acs.est.1c08673.

Rocha, J., Cacace, D., Kampouris, I., Guilloteau, H., Jäger, T., Marano, R.B.M., Karaolia, P., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Berendonk, T. U., Schwartz, T., 2020. Inter-laboratory calibration of quantitative analyses of antibiotic resistance genes. J. Environ. Chem. Eng. 8, 102214 https://doi.org/10.1016/j.jece.2018.02.022.

Rocha, J., Manaia, C.M., 2020. Cell-based internal standard for qPCR determinations of antibiotic resistance indicators in environmental water samples. Ecol. Indic. 113, 106194 https://doi.org/10.1016/j.ecolind.2020.106194.

Sugimoto, Y., Suzuki, S., Nonaka, L., Boonla, C., Sukpanyatham, N., Chou, H.Y., Wu, J. H., 2017. The novel mef(C)–mph(G) macrolide resistance genes are conveyed in the environment on various vectors. J. Glob. Antimicrob. Resist. 10, 47–53. https://doi.org/10.1016/j.jgar.2017.03.015.

UN-Water, 2020: Summary Progress Update 2021 – SDG 6 – water and sanitation for all. Version: 1 March 2021. Geneva, Switzerland.

Vredenburg, J., Varela, A.R., Hasan, B., Bertilsson, S., Olsen, B., Narciso-da-Rocha, C., Bonnedahl, J., Stedt, J., da Costa, P.M., Manaia, C.M., 2014. Quinolone-resistant Escherichia coli isolated from birds of prey in Portugal are genetically distinct from those isolated from water environments and gulls in Portugal, Spain and Sweden. Environ. Microbiol. 16, 995–1004. https://doi.org/10.1111/1462-2920.12231.

WHO pipeline: antibacterial agents in clinical development: an analysis of the antibacterial clinical development pipeline. Geneva: world Health Organization; 2019. Licence: CC BY-NC-SA 3.0 IGO.

Yang, H., Liu, R., Liu, H., Wang, C., Yin, X., Zhang, M., Fang, J., Zhang, T., Ma, L., 2021. Evidence for long-term anthropogenic pollution: the hadal trench as a depository and indicator for dissemination of antibiotic resistance genes. Environ. Sci. Technol. Lett. 55, 15136–15148. https://doi.org/10.1021/acs.est.1c03444.

Yao, H., Yiu, S.M., 2019. Deep analysis and optimization of CARD antibiotic resistance gene discovery models. BMC Genomics 20, 914. https://doi.org/10.1186/s12864-019-6318-5.

Yin, X., Chen, X., Jiang, X.T., Yang, Y., Li, B., Shum, M.H., Lam, T.T.Y., Leung, G.M., Rose, J., Sanchez-Cid, C., Vogel, T.M., Walsh, F., Berendonk, T.U., Midega, J., Uchea, C., Frigon, D., Wright, G.D., Bezuidenhout, C., Picão, R.C., Ahammad, S.Z., Nielsen, P.H., Hugenholtz, P., Ashbolt, N.J., Corno, G., Fatta-Kassinos, D., Bürgmann, H., Schmitt, H., Cha, C.J., Pruden, A., Smalla, K., Cytryn, E., Zhang, Y., Yang, M., Zhu, Y.G., Dechesne, A., Smets, B.F., Graham, D.W., Gillings, M.R., Gaze, W.H., Manaia, C.M., van Loosdrecht, M.C.M., Alvarez, P.J.J., Blaser, M.J., Tiedje, J.M., Topp, E., Zhang, T., 2023. Toward a universal unit for quantification of antibiotic resistance genes in environmental samples. Environ. Sci. Technol. 26, 9713–9721. https://doi.org/10.1021/acs.est.3c00159.

Zhang, A.N., Gaston, J.M., Dai, C.L., Zhao, S., Poyet, M., Groussin, M., Yin, X., Li, L.G., van Loosdrecht, M.C.M., Topp, E., Gillings, M.R., Hanage, W.P., Tiedje, J.M., Moniz, K., Alm, E.J., Zhang, T., 2021. An omics-based framework for assessing the health risk of antimicrobial resistance genes. Nat. Commun. 12, 4765. https://doi.org/10.1038/s41467-021-25096-3.

Zhang, Y., Wang, J., Lu, J., Wu, J., 2020. Antibiotic resistance genes might serve as new indicators for wastewater contamination of coastal waters: spatial distribution and source apportionment of antibiotic resistance genes in a coastal bay. Ecol. Indic. 114, 106299 https://doi.org/10.1016/j.ecolind.2020.106299.

Zheng, W., Huyan, J., Tian, Z., Zhang, Y., Wen, X., 2020. Clinical class 1 integron-integrase gene – A promising indicator to monitor the abundance and elimination of antibiotic resistance genes in an urban wastewater treatment plant. Environ. Int. 135, 105372 https://doi.org/10.1016/j.envint.2019.105372.