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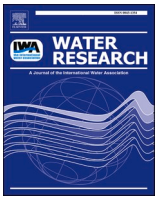

Linking antibiotic resistance gene patterns with advanced faecal pollution assessment and environmental key parameters along 2300 km of the Danube River

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ABSTRACT

The global spread of antimicrobial resistance (AMR) in the environment is a growing health threat. Large rivers are of particular concern as they are highly impacted by wastewater discharge while being vital lifelines serving various human needs. A comprehensive understanding of occurrence, spread and key drivers of AMR along whole river courses is largely lacking. We provide a holistic approach by studying spatiotemporal patterns and hotspots of antibiotic resistance genes (ARGs) along 2311 km of the navigable Danube River, combining a longitudinal and temporal monitoring campaign. The integration of advanced faecal pollution diagnostics and environmental and chemical key parameters allowed linking ARG concentrations to the major pollution sources and explaining the observed patterns. Nine AMR markers, including genes conferring resistance to five different antibiotic classes of clinical and environmental relevance, and one integrase gene were determined by probe-based qPCR. All AMR targets could be quantified in Danube River water, with *intI1* and *sul1* being ubiquitously abundant, *qnrS, tetM,* bla_{TEM} with intermediate abundance and $bla_{\text{OXA-48like}}$, $bla_{\text{CTX-M-1 group}}$, $bla_{\text{CTX-M-9 group}}$ and bla_{KPC} genes with rare occurrence. Human faecal pollution from municipal wastewater discharges was the dominant factor shaping ARG patterns along the Danube River. Other significant correlations of specific ARGs were observed with discharge, certain metals and pesticides. In contrast, *intI1* was not associated with wastewater but was already established in the water microbiome. Animal contamination was detected only sporadically and was correlated with ARGs only in the temporal sampling set. During temporal monitoring, an extraordinary hotspot was identified emphasizing the variability within natural waters. This study provides the

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first comprehensive baseline concentrations of ARGs in the Danube River and lays the foundation for monitoring future trends and evaluating potential reduction measures. The applied holistic approach proved to be a valuable methodological contribution towards a better understanding of the environmental occurrence of AMR.

1. Introduction

Bacterial antimicrobial resistance (AMR) is spreading rapidly and globally due to misuse and overuse in human and veterinary medicine causing urgent public health problems (Murray et al., 2022; O'Neill, 2016). While antibiotic resistances are ancient and originally derive from the natural microbiome (D'Costa et al., 2011), AMR can accumulate and evolve *de novo* under antibiotic selection (Orlek et al., 2023). Traditionally, most research efforts have focused on clinical sectors being hotspots for resistance evolution and transmission (Bengtsson-Palme et al., 2018). However, transfer of resistance genes between bacteria and of resistant bacteria to humans and animals can also occur through the environment (Larsson and Flach, 2022). The environmental perspective of AMR, often framed within the One Health Concept, is increasingly being considered, also in (inter-)national action plans (BMSGPK, 2021; European Commission, 2017; Interagency Coordination Group on Antimicrobial Resistance, 2019; Larsson et al., 2023; Wuijts et al., 2017). A recent Council Recommendation urges EU countries to further strengthen environmental surveillance while reducing the release of antibiotics into the environment (General Secretariat of the Council, 2023). Special attention should be paid to surface waters, as they connect large geographic areas and serve human needs on the one hand, and receive wastewater from various sources on the other hand (Amos et al., 2015; Caucci et al., 2016; Sanderson et al., 2018). Treated anthropogenic wastewater flows (from urban areas, agriculture, aquaculture, and industry) introduce antibiotics, antibiotic resistant bacteria (ARB), and antibiotic resistance genes (ARGs) into the environment, since standard technologies currently implemented at most state-of-the-art wastewater treatment plants (WWTPs) do not effectively remove them (Bürgmann et al., 2018; Rizzo et al., 2013). There is an ongoing debate on the implementation of AMR monitoring in the update of the European Urban Waste Water Treatment Directive to increase systematic understanding on the role of WWTPs introducing AMR determinants into the environment (European Commission, 2022b). Increased and systematic monitoring is urgently needed not only in wastewater but also in receiving natural waters to assess the risk of self-amplification and backflow of AMR to humans and animals (Manaia et al., 2016; Pruden et al., 2006). In this respect, there are also endeavours to add AMR to the updated watch list of water pollutants for future risk assessment efforts in ground- and surface water as soon as suitable monitoring methods have been identified (European Commission, 2022a). Improved environmental surveillance could also be useful in monitoring resistance distribution in the community, as large populations can be covered (Pärnänen et al., 2019).

Rivers form important interfaces between humans and the environment and have been increasingly studied regarding the presence and spread of AMR (Singh et al., 2019). While antibiotic concentrations in natural waters do not pose an immediate health concern (Sanseverino et al., 2018), potential transmission pathways from ARB in rivers to humans exist (e.g. via drinking water, irrigation water, recreational activities) which should be studied in more standardized and structured ways (Berendonk et al., 2015). Even though an increasing number of studies focussing on ARB and ARG in rivers exist, available studies often focus on a few highly polluted sites only (Andrade et al., 2020; Stanton et al., 2022) and in-depth investigations on entire large rivers are missing.

To fill this knowledge gap, we applied a new holistic concept to systematically assess the antibiotic resistance situation along the Danube River, Europe's second longest river. The Danube River Basin (DRB) covers approximately 801,500 km^2 and is with 19 countries the most international river basin in the world. Since 2001, the International Commission for the Protection of the Danube River (ICPDR) has been organizing whole river expeditions in 6-year intervals to assess the ecological status of the Danube. The structure of these Joint Danube Surveys (JDS) enabled us to perform a holistic and harmonized longitudinal survey of AMR which was complemented with an annual sampling campaign. Our selected AMR markers comprised a range of previously recommended genes from widespread indicators of anthropogenic pollution (e.g.: sulfonamide resistance gene *sul1* and class 1 integron-integrase gene *intI1*) to highly clinically-relevant ARGs promoting resistance to last resort antibiotics (extended spectrum β-lactamases (ESBLs) and carbapenemases) (Berendonk et al., 2015; Keenum et al., 2022). This gene selection also included relevant ARGs in *Enterobacteriaceae* to allow comparability with a concomitantly performed culture-based study of ARBs on this topic (Koller et al. in prep.). To identify the main drivers of AMR along the river, a holistic concept was applied linking ARG data with (i) advanced faecal pollution diagnostics, based on standard faecal indicator monitoring, microbial source tracking with host-associated genetic bacterial faecal markers (Demeter et al., 2023) and a novel theoretical wastewater index, as well as with (ii) a comprehensive set of environmental and chemical pollution parameters.

2. Material & methods

2.1. Study design and sampling strategy

This study was performed in the course of the Joint Danube Survey 4 (JDS4), involving scientists from various countries in the DRB. Sampling was performed between June 30th and July 19th, 2019 along a 2311 river kilometre (rkm) long stretch of the Danube River, comprising a wide range of different land-use types, agricultural, urban and (semi-) natural landscapes. A total of 36 sampling sites from Germany to Romania were included in this longitudinal monitoring campaign, including the major (Inn, Drava, Tisza, Sava) and some highly anthropogenically influenced tributaries (Moson Danube, Timok, Rusenski Lom, Arges) (Fig. 1). Sampling sites were selected according to previous surveys (Kirschner et al., 2009, 2017) with a special focus on hotspots of faecal pollution as well as some pristine reference sites. For half of the sites (n = 18), samples were collected at three positions of the river profile (left and right river bank & middle of the river). Quadruplicate 500 mL grab samples were collected from a rubber boat at 30 cm water depth, cooled immediately and brought to partner laboratories for sample processing within four hours. For all sampling sites, the four replicates were pooled prior to processing in the lab. To enable a harmonised sample analysis, unified SOPs for all investigated parameters were applied during the whole survey by the same trained team of scientists. In addition to the longitudinal survey (JDS4), a temporal monitoring (annual cycle) was performed throughout one year. Ten sampling sites in three countries (Austria, Hungary and Serbia) were sampled seven times every second month from October 2020 till October 2021. The sites were focused on the capitals of Austria (Vienna), Hungary (Budapest) and Serbia (Belgrade) including upstream low-pollution sites and on additional prior pollution hotspots in these countries. The three studied countries also show a gradient in applied wastewater treatment, ranging from fully implemented state-of-the-art treatment in Austria, via enhanced treatment (tertiary treatment covering approx. 70% of the population) in Hungary to largely absent treatment in Serbia (ICPDR, 2021). The sites in Austria were identical to the JDS4 sampling sites, in Hungary one additional site in the middle of

Budapest after the inflow of Rákos stream was added and in Serbia sites were adapted to capture the metropolitan areas of Belgrade and Smederevo. Immediate sample processing was done in the respective partner labs by the trained partner scientists already involved in JDS4 (MedUni Vienna, ELTE Budapest & IBISS, Belgrade); all molecular analyses were performed at MedUni Vienna. Further details on the sampling sites can be found in **Supplementary Material, Table S1 and S2**.

2.2. On site chemo-physical parameters, discharge data and total suspended solids

During all samplings, water temperature, pH, conductivity, and oxygen concentration were measured on site with the handheld multiparameter metre Multi 3430 (WTW, Xylem Analytics, Germany). Discharge data was retrieved from national monitoring systems for all sampling sites. Total phosphorus, total nitrogen and total organic carbon were determined for the longitudinal sample set according to international standard methods at the National reference laboratory of the Serbian Environmental Protection Agency. Total suspended solids and inorganic particulate matter were determined during the annual cycle according to Eiler et al. (2003).

2.3. Quantification of antibiotic resistance genes (ARGs)

For molecular ARG analyses, 300 mL of water samples (minimum volume 141 mL due to high turbidity during flooding, maximum 319 mL) were filtered through 0.2 μm polycarbonate filters (diameter 47 mm). Filter controls were prepared alongside. Until further processing, filters were stored at −80 °C. DNA was extracted from filters according to Linke et al. (2021) and Reischer et al. (2008) by applying a combined phenol-chloroform and bead-beating extraction method and stored in 100 μL of TRIS buffer (10 mM, pH 8.0) at −80 °C. This protocol has been successfully applied to previous JDSs (Kirschner et al., 2017; Savio et al., 2015).

We applied a molecular approach to determine nine AMR markers with quantitative real-time PCR (qPCR). This method allows highly sensitive and reproducible quantification of interpretable indicator targets and is important to estimate the spread of resistance amongst the total bacterial community and assess the complete resistance reservoir (Liguori et al., 2022). Nine antibiotic resistance markers and the 16S rRNA gene target were quantified on a qTOWER^{3G} (Analytik Jena AG, Germany). For details on all qPCR assays see Table 1 and **Supplementary Material, Table S3**. The 16S rRNA gene target assay was performed with the iQ™ SYBR® Green Supermix (Biorad, Austria). All ARGs applied probe-based chemistry using the Luna Universal Probe qPCR Master Mix (New Englands BioLabs GmbH, Germany). For the 16S rRNA gene target quantification, plasmids and for antibiotic resistance markers, synthetic gBlock DNA fragments (Eurofins, Austria) were used to prepare qPCR standards**.** Further details on the qPCR analyses are provided in the **Supplementary Material (**Section 2.3**).** Targets were expressed per 100 mL of original water sample by considering DNA sample dilution and the respective filtration and reaction volumes. The threshold of detection (TOD) concept was applied (Demeter et al., 2023) and calculated according to Reischer et al. (2011). Samples were considered to be quantifiable if the standard deviation of the Ct values of the duplicates was <1. Samples with <10 copies per reaction and standard deviations >1 and samples below the TOD were defined to be not quantifiable and therefore set to zero for statistical analyses.

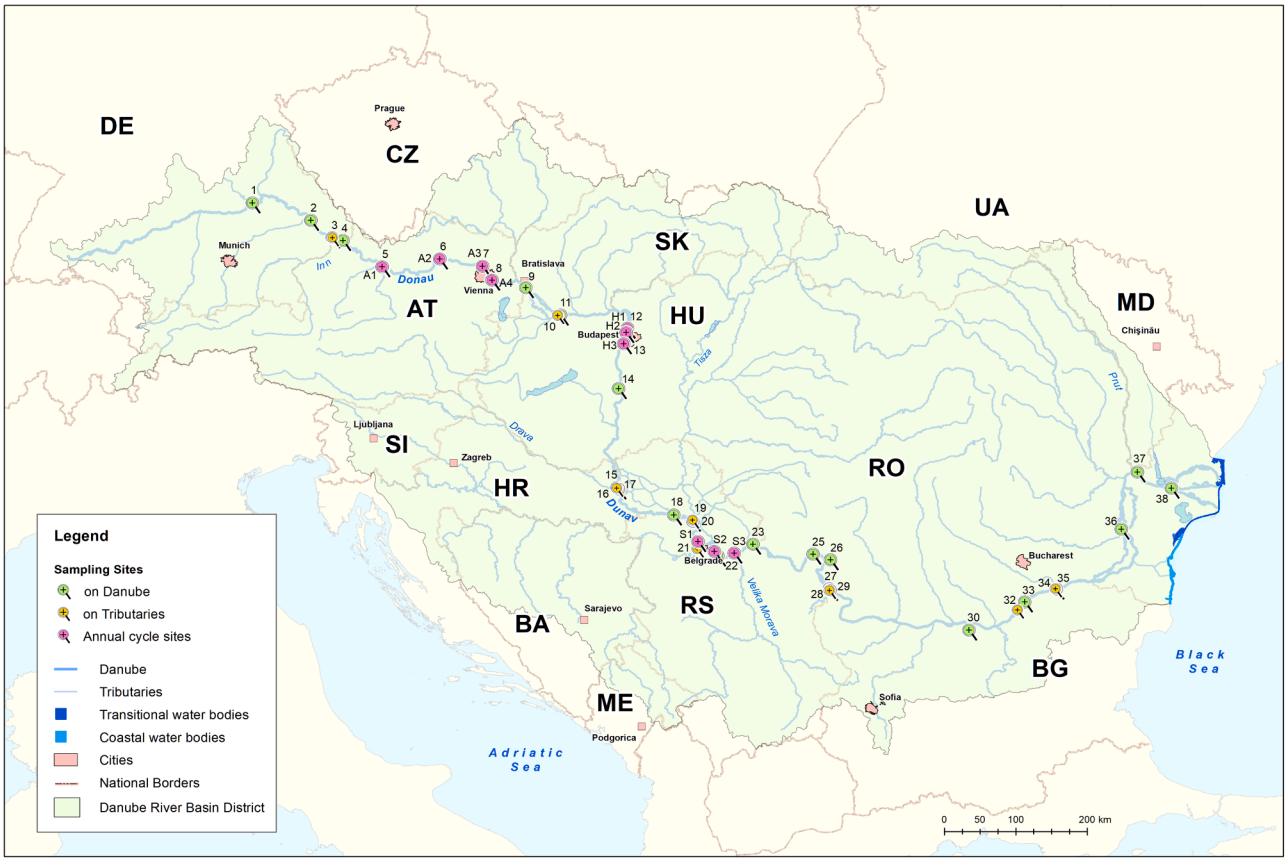

Fig. 1. Location of sampling sites analysed in this study within the Danube River Basin; sites 24 and 31 were not sampled and are therefore not shown; DE = Germany, CZ = Czech Republic, AT = Austria, SK = Slovakia, HU = Hungary, SI = Slovenia, HR = Croatia, UA = Ukraine, RO = Romania, RS = Republic of Serbia, BA = Bosnia and Herzegovina, ME = Montenegro, MD = Moldova, BG = Bulgaria; map provided by International Commission for the Protection of the Danube River (ICPDR).

Table 1
Overview on quantified genetic targets in qPCR assays; $bla_{CTX-M-1\ group}$ targets all $bla_{CTX-M-1}$ variants except for $bla_{CTX-M-12\ \&\ 30}$, $bla_{CTX-M-9\ group}$ targets the variants $bla_{CTX-M-9,\ 13,\ 14,\ 16\ to\ 19,\ 21,\ \&\ 27}$, MST = microbial source tracking.

Indicator function	Genetic target	Genetic feature	Citation
AMR markers	*intI1*	Integrase gene associated with integrons containing resistance gene cassettes	Barraud et al. (2010)
	sul1	Genes for resistance to sulfonamides	Heuer and Smalla (2007)
	qnrS	Plasmid mediated fluoroquinolone resistance genes	Colomer-Lluch et al. (2014)
	tetM	Tetracycline resistance genes	Peak et al. (2007)
	bla_{TEM}	Beta-lactamase (with ESBL variants) genes	Lachmayr et al. (2009)
	bla_{OXA-48}	Carbapenemase genes	Brown-Jaque et al. (2018)
	bla_{KPC}	Carbapenemase genes	Ellington et al. (2016)
	$bla_{CTX-M-1}$	ESBL genes	Colomer-Lluch et al. (2011b)
	$bla_{CTX-M-9}$	ESBL genes	Colomer-Lluch et al. (2011a)
Bacterial community	16S rRNA gene	V1/V2 region of 16S rRNA gene	Savio et al. (2015)
MST markers	BacHum	Targeting human associated Bacteroidetes	Kildare et al. (2007)
	BacR	Targeting ruminant associated Bacteroidetes	Reischer et al. (2006)
	Pig2Bac	Targeting pig associated Bacteroidetes	Mieszkin et al. (2010)

2.4. Advanced faecal pollution assessment

For comprehensive faecal pollution assessment, a three-level approach was applied, consisting of standard faecal indicator monitoring, host-associated microbial source tracking and a new index of the theoretical wastewater influence at each site.

2.4.1. Enumeration of the standard faecal indicator Escherichia coli (E. coli)

E. coli was selected as standard faecal indicator bacterium (SFIB) due to investigations during three prior JDS, showing high sensitivity and representativeness also when compared to other faecal indicators (Kirschner et al., 2017). *E. coli* concentrations were determined in two different volumes (100 mL & 1 mL) according to ISO 9308–2:2012 (International Organization for Standardization, 2012) using the Colilert-18 system according to the manufacturer's instructions (IDEXX, Ludwigsburg, Germany). Quantification was performed after 18–22 h of incubation at 36 ± 1 °C, through counting in a UV cabinet and comparing the obtained results with the most probable number (MPN) table provided by the manufacturer. Weighted means were calculated to report *E. coli* MPN per 100 mL of sample.

2.4.2. Genetic microbial source tracking (MST)

For genetic microbial source tracking (MST), host-associated Bacteroidetes groups were targeted. The Bacteroidetes phylum is highly abundant in the intestine of mammals and different host associated markers have been proven to be very reliable targets for MST being widely applied in aquatic ecosystems (Reischer et al., 2013). Human (BacHum), swine (Pig2Bac) and ruminant (BacR) associated markers were quantified on a Rotor-Gene Q thermocycler (Quiagen Inc.) as previously done in Kirschner et al. (2017). All MST assays applied probe-based chemistry; for quantification, plasmid standards were used. QPCR procedures were consistent with those described for ARG assays (Section 2.3.) and further details and assay specifications are shown in the **Supplementary Material (**Section 2.3 and **Table S3)**. Concentrations were provided as marker equivalents (ME) per 100 mL according to previous studies (Kirschner et al., 2017; Reischer et al., 2008, 2011).

2.4.3. Theoretical assessment of municpal wastewater impact

To estimate the impact of municipal wastewater at each sampling site, a new theoretical sum parameter (wastewater impact index, WII) was calculated. The index was calculated using formula (1) which incorporates the size, type and distance of the next WWTP impacting the respective sampling site. The size of the treatment facility was given in person equivalents (PE). The treatment type was accounted for as $\log_{10}$ reduction of the discharged wastewater. In case of tertiary treatment, PE values were multiplied by 0.003, assuming an average 2.5 $\log_{10}$ reduction (Mascher et al., 2017; Mayer et al., 2016); for all other treatment types values were multiplied by 1, as no reduction was assumed. The distance of the sampling site to the next discharge site was provided in km and a correction factor to account for the respective river side was included. Values were multiplied by 1 if the sample was taken at the same river site as the wastewater inflow, 0.1 if the sample was taken from the middle and 0.01 if the sample was taken from the other side as the inflow, assuming a gradual 10 fold linear dilution throughout the river cross section. For statistical analyses, WII was $\log_{10}$ transformed to reduce data scattering. The wastewater data was kindly provided by the ICPDR.

$$WII = \frac{person\ equivalents\ *\ treatment\ type\ factor}{distance\ to\ sampling\ site\ [km]\ *\ river\ side\ correction\ factor} \quad (1)$$

2.5. Bacterial cell counts

As recommended in a recent publication (Yin et al., 2023), concentrations of ARGs should be normalized to the number of total bacterial cells, to allow comparison between habitats and sample types. In our study, total bacterial cell counts (TCC) were assessed by epifluorescence microscopy, which is the gold-standard for cell-based TCC determination and comparable to flow cytometry and single copy gene quantification (Liang et al., 2020). One mL of water sample was fixed with sterile paraformaldehyde at a final concentration of 0.8 %, filtered through an Anodisc filter (pore size 0.2 μm, diameter 25 mm, Whatman, Germany) and stained with 1:400 diluted SYBR gold solution (Van Driezum et al., 2018). TCC were determined with a Nikon Eclipse 80i epifluorescence microscope by counting at least 200 bacterial cells in 10 microscopic fields per filter and were presented as TCC per 100 mL (Velimirov et al., 2011).

2.6. Advanced chemical analyses

For quantification of metals and other elements, 50 mL of water samples were collected in cleaned polystyrene beakers, stabilized with 0.5% HNO_3 (v/v), stored at 4 °C and analysed within six months. Metals and other elements were quantified on a double-focusing sector field high-resolution inductively coupled plasma mass spectrometer (HR-ICP-MS), ELEMENT2 from Thermo Finnigan (Bremen, Germany) equipped with a cyclonic spray chamber and a conical nebuliser (Glass Expansion, West Melbourne, Australia). For detailed information on the measurements see **Supplementary Material (**Section 2.6**)**.

For the quantification of antibiotics and pesticides, 125 mL of water sample was frozen at −80 °C and analysed within 12 months by using LC-MS/MS (liquid chromatography tandem mass spectrometry) according to the protocol for multiple contaminant classes as described and validated in Steiner et al. (2020). An overview on the methodology and the quantified targets is provided in the **Supplementary Material**

(Section 2.6 and **Table S4**).

2.7. Data analysis

Microbiological data was $\log_{10}(x+1)$ transformed. To assess relative AMR marker abundances, respective gene targets were normalised by dividing by 16S rRNA gene targets and TCCs. Statistical analyses were performed in SPSS 26 (IBM, New York, USA) and graphical representations were generated in R (v. 4.2.3). To evaluate associations between microbiological and environmental parameters, Spearman's rank correlations were calculated for all samples where quantitative molecular data could be obtained, applying $p \leq 0.001$ as significance level, due to Bonferroni correction for > 60 parameters. Those parameters showing a significant correlation with at least one tested AMR marker were included in heat maps. Simple stepwise linear regression models were calculated to estimate the effect of significantly correlated environmental parameters on the quantified ARGs. Differences between countries studied in the annual cycle were assessed with one-way ANOVA followed by Tukey's post-hoc test after confirming normal distribution of the studied ARGs. A principal component analysis (PCA) was performed in R studio using prcomp() function and visualized applying the fviz_pca_biplot() function. Network analysis was performed to detect key interactions between ARGs and other assessed parameters. Networks were visualized using the R package qgraph and node placement was optimized based on Fruchterman-Reingold algorithm (Fernandez et al., 2015).

3. Results

3.1. Longitudinal monitoring (Joint Danube Survey)

3.1.1. Absolute and relative abundances of AMR markers along the Danube River

During the longitudinal river survey, all targeted genes could be detected and quantified. Specific patterns were found for the different AMR targets. The class 1 integron-integrase gene *intI1* and the sulfonamide resistance gene *sul1* showed highest detection frequencies while the targeted ESBL genes were only detected sporadically. Overall, the concentrations could be ranked as *intI1* > *sul1* > *qnrS* > *tetM* > bla_{TEM} > $bla_{\text{OXA-48like}}$ > $bla_{\text{CTX-M-1 group}}$ > bla_{KPC} > $bla_{\text{CTX-M-9 group}}$ (Fig. 2**A**). To assess the distribution of AMR markers within the river water communities across samples, relative ratios per TCCs and per 16S rRNA gene targets were determined. Both relative abundances were highly significantly correlated with absolute abundance values (Fig. 2**B** and **C**), but TCC-normalized values ($r = 0.998$, $p < 0.001$) showed markedly less variability than values normalized to 16S rRNA gene targets ($r = 0.983$, $p<0.001$). Due to the very high correlation between absolute and TCC-normalized ARG abundances, absolute ARG abundances were assumed

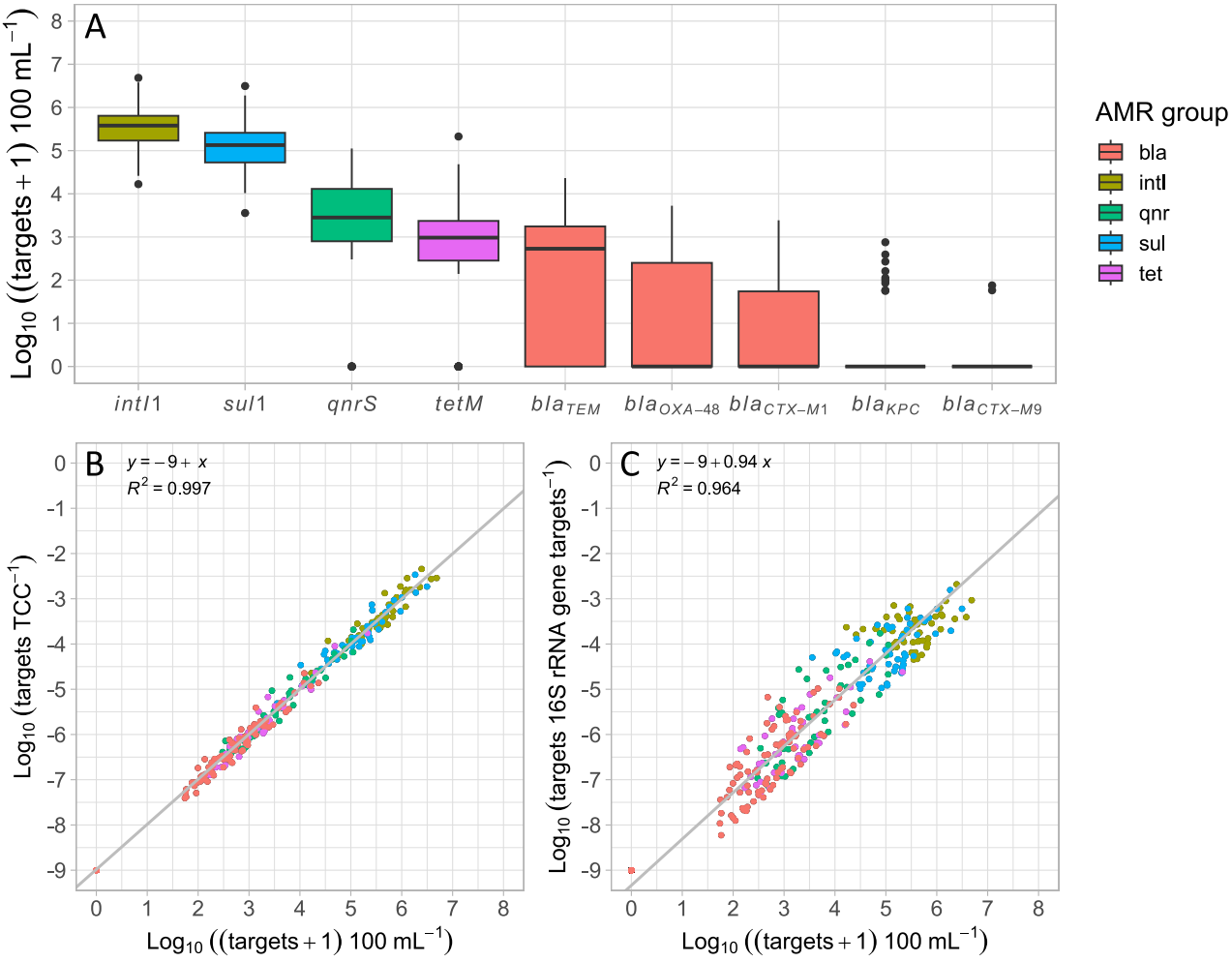

Fig. 2. Occurrence of AMR markers in all samples collected during JDS4 ($n = 53$), colours represent different ARG groups, red: β-lactamases, olive: integrase gene, green: fluoroquinolone resistance gene, blue: sulfonamide resistance gene, purple: tetracycline resistance gene; A: absolute abundances, B: relative abundance per TCC, C: relative abundance per 16S rRNA gene targets; not quantifiable samples were set to log10 (−9) in the relative abundance plots for data representation; abundance data presented as box-plots with lower and upper hinges corresponding to first & third quartile, whiskers correspond to 1.5 * IQR (Inter-quartile range) from closer hinge, dots showing outliers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to be representative in showing AMR trends and hotspots in the Danube River and in the following only absolute values are shown.

3.1.2. Patterns and hotspots of AMR markers

During the longitudinal sampling campaign, TCC ranged from 2.55×10^8 to 1.97×10^9 cells per 100 mL. 16S rRNA gene targets showed higher variability than TCC with values from 3.27×10^4 to 9.64×10^9 gene targets per 100 mL. A pronounced drop was observed after the inflow of the tributary Inn until upstream of Budapest (rkm 2225 – 1660) resulting in 3 $\log_{10}$ orders lower 16S rRNA gene targets in comparison to TCC (Fig. 3**A** and **B**). At the time of sampling, the Inn was in flood (**Supplementary Material, Fig. S1**), carrying high loads of suspended solids and chemical elements, impairing DNA extraction. From rkm 1660 (site 12, upstream Budapest), a reduction of chemical elements to low levels and increased recovery of 16S rRNA gene targets occurred. Also, Rusenski Lom showed a peak for many chemical elements leading to a similar effect (**Supplementary Material, Table S5 & Fig. S2**). As a quality criterion for DNA extraction efficiency for our sample set, only samples were considered for further molecular analysis when 16S rRNA gene targets and TCC were within one $\log_{10}$ concentration range. This resulted in the exclusion of 19 samples, marked with red boxes in Fig. 3**B** which were not included in further molecular analyses. The TOD for the qPCR assays ranged from 1.73 to 2.24 $\log_{10}$ targets per 100 mL. The integrase gene *intI1* and the sulfonamide resistance gene *sul1* were continuously present in all quantifiable samples at high concentrations ranging from 4.22 – 6.69 $\log_{10}$ (*intI1*) and from 3.55 to 6.5 $\log_{10}$ (*sul1*) (Fig. 3**, Supplementary Material, Table S6** and **S7**). The genes for fluoroquinolone resistance (*qnrS)*, tetracycline resistance (*tetM)*, and the beta-lactamase gene bla_{TEM} were detected in more than 50% of the samples with maxima of 5.85, 5.33 and 4.36 $\log_{10}$ targets per 100 mL, respectively. Both carbapenemases ($bla_{\text{OXA-48like}}$, bla_{KPC}) and two ESBL genes ($bla_{\text{CTX-M-1 group}}$, $bla_{\text{CTX-M-9 group}}$) were only slightly above TOD and only sporadically detected. These ARGs were mainly quantified in the middle and lower river sections (rkm 1869 – 104); however, $bla_{\text{OXA-48like}}$ was also present in the tributary Moson Danube (site 10, rkm 1794). The detection of low-abundance ARGs at specific river sites was particularly informative. E.g. $bla_{\text{CTX-M-9 group}}$ was detected only twice on the left river side at site 26 (rkm 926) and 35 (rkm 429). By comparing ARG abundance patterns along the river, specific hotspots (with highest AMR marker abundances) could be identified (**Supplementary Material, Table S8**). A major hotspot was the tributary Arges (site 34, rkm 432) with maximum concentrations of five ARGs (*intI1, sul1,* bla_{TEM}, $bla_{\text{CTX-M-1 group}}$, bla_{KPC}). Also, site 35 L (rkm 429) located immediately after the confluence of this tributary, showed second-highest concentration for four AMR targets. Other hotspots were identified downstream of Simijan (site 26, rkm 926) which showed overall high AMR marker concentrations and highest abundances for two markers (*qnrS,* $bla_{\text{CTX-M-9 group}}$) and after the inflow of the tributaries Drava and Tisza (sites 17 & 20, rkm 1377 & 1212). Next to sites 26 (rkm 926, bla_{KPC}) and 35 (rkm 429, *sul1*), third-highest AMR marker concentrations were detected in the lower river section downstream of the cities Ruse (site 33, rkm 488, *intI1,* bla_{TEM}) and Tulcea (site 38, rkm 104, *qnrS, tetM,* $bla_{\text{OXA-48like}}$, $bla_{\text{CTX-M-1 group}}$).

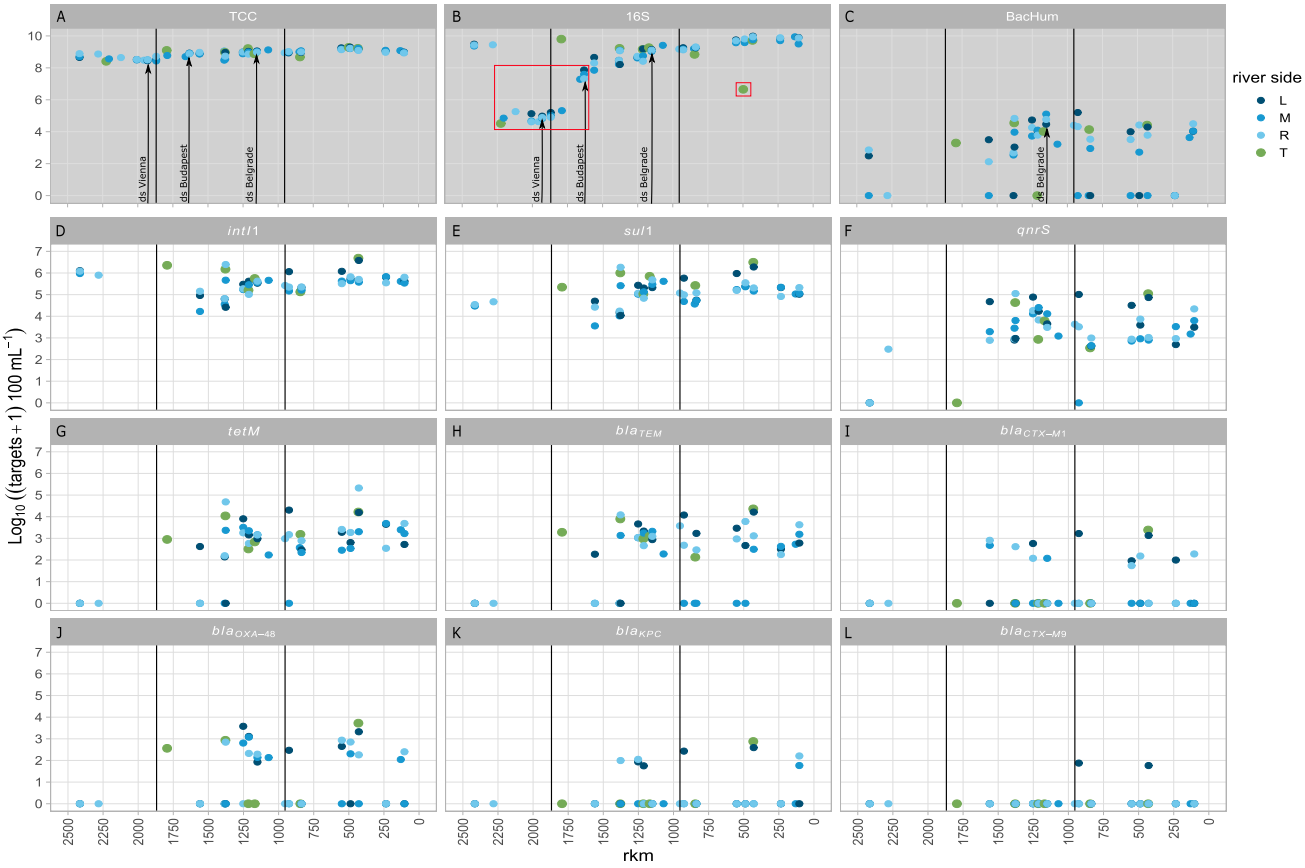

Fig. 3. Longitudinal occurrence of total cell counts (TCC) (A), 16S rRNA gene targets (B) (both $n = 72$), BacHum marker equivalents (C), *intI1* gene targets (D), and 8 ARGs (*sul1, qnrS, tetM,* bla_{TEM}, $bla_{\text{CTX-M-1 group}}$, $bla_{\text{OXA-48like}}$, bla_{KPC}, $bla_{\text{CTX-M-9 group}}$; E - L) ($n = 53$); all samples obtained during Joint Danube Survey 4. Colours and symbol sizes correspond to Danube River sampling sites (L = left, M = middle, R = right) and tributary samples (T). Black vertical lines mark the three river sections, upper (until rkm 1869), middle (until rkm 954), and lower Danube River. Red boxes indicate excluded samples due to impaired DNA extraction efficiency. Black errors mark the downstream sampling sites of the capitals Vienna, Budapest & Belgrade.

3.1.3. Faecal pollution

E.coli – Concentrations of the standard faecal indicator *E.coli* ranged from 0.61 to 4.64 $\log_{10}$ MPN per 100 mL, with hotspots in the lower river sections and a tendency to increasing concentrations from the upper to lower river stretch (**Supplementary Material, Fig. S3).** The detected patterns were largely in agreement with previous studies (Kirschner et al., 2009, 2017).

MST markers - BacHum, the genetic MST marker indicating human faecal pollution, was detectable in 74% of the quantifiable samples with a trend towards rising concentrations throughout the middle river section (rkm 1869 – 954) (Fig. 3**C, Supplementary Material, Table S6, S7**). The other two studied MST markers were only detected sporadically (Pig2Bac: $n = 8$, BacR: $n = 1$) and at concentrations very close at the threshold of detection (data not shown).

3.1.4. Correlation analysis integrating AMR markers, faecal pollution diagnostics and environmental parameters

Spearman rank correlations were calculated to elucidate the key factors responsible for the observed variations in AMR markers. For the longitudinal dataset, all chemical, environmental and microbial pollution related parameters were included. Quantified antibiotics, pesticides, metals and nutrients were included in the calculations if they were above the limit of quantification in more than 20% of the samples per dataset. Six metals, five pesticides but no antibiotics were statistically significantly positively correlated with at least one ARG. The newly developed theoretical wastewater impact index (WII) assessing local municipal wastewater influence showed strong correlation with general faecal pollution (r = 0.634) (**Supplementary Material, Table S9, Fig. S4**) and was included as an explanatory parameter to the correlation. The hierarchically clustered heat map showed that AMR targets were strongly correlated with *E. coli* and BacHum concentrations and WII (Fig. 4). Within the AMR markers, $bla_{\text{CTX–M-1 group}}$, $bla_{\text{CTX–M-9 group}}$ and bla_{KPC} formed an own cluster with the pesticide synergist piperonyl butoxid, separated from the other ARGs. *TetM* was significantly negatively correlated with river kilometre, indicating higher concentrations in downstream sections. Most other parameters were sporadically correlated with single resistance markers; only *qnrS* was correlated with

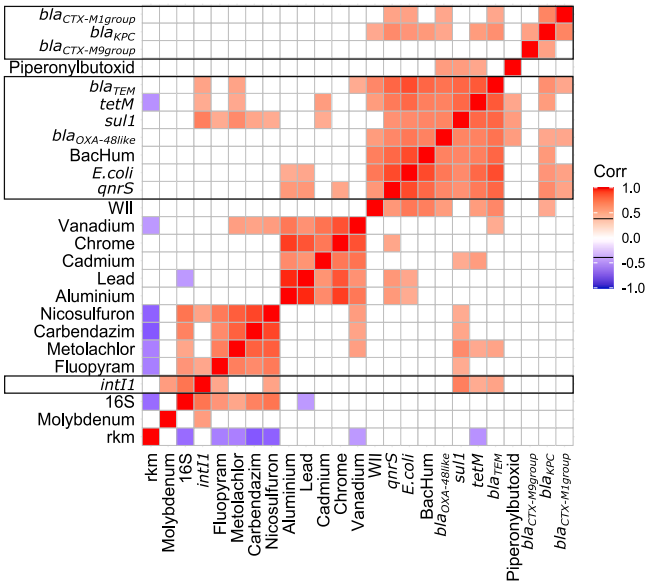

Fig. 4. Pairwise correlation heat-map of AMR, faecal pollution markers and environmental parameters for JDS4 samples. Only significantly correlated parameters are included and only significant correlations are shown. Fill colours depict Spearman correlation coefficient, samples were clustered hierarchically, boxes indicate locations of AMR markers; WII: wastewater impact index, rkm: river kilometre.

three different metals (aluminium, chromium, lead) and *sul1* with all pesticides. *IntI1* clustered elsewhere of the ARGs, it showed highest correlation with 16S rRNA gene targets.

3.2. Temporal monitoring (annual cycle)

3.2.1. Patterns and hotspots of AMR markers during temporal monitoring

During the temporal monitoring campaign over one year, a focus was put on the upper and middle section of the Danube (rkm 2121 – rkm 1107). At four sites, left and right river sides were investigated, especially those sites downstream of the three studied capitals, Vienna (A4), Budapest (H3) and Belgrade (S2). Again, the quality criterion based on the comparison of TCCs and 16S rRNA gene targets was applied which resulted in 10 samples to be excluded due to impaired DNA extraction efficiency (red circles in Fig. 5**B**). For all sampling sites, no uniform seasonal pattern across the seven sampling months could be detected, but pronounced variability was observed with some peaks only detected in specific months (**Supplementary Material, Fig. S5**). While in Austria all samples from February 2021 had highest AMR marker concentrations (significant for *intI1* & *sul1*; Kruskal-Wallis, $p<0.05$), in Hungary samples from October 2020 showed highest concentrations (significant for *sul1, qnrS, tetM,* bla_{TEM} & $bla_{\text{CTX-M-9 group}}$; Kruskal-Wallis, $p<0.05$). In Serbia, fluctuations were much smaller and no temporal hot-spot was observed. The observed patterns corresponded to those observed in the longitudinal sampling with *intI1* and *sul1* showing highest concentrations with maxima of 7.16 and 7.18 $\log_{10}$ targets per 100 mL, respectively (Fig. 5**D-L, Supplementary Material, Table S10**). Generally, detection rates were higher in comparison to JDS4, with *qnrS* quantified in 97%, *tetM* in 93% and bla_{TEM} in 85% of quantifiable samples. The rarely detectable genes bla_{KPC} and $bla_{\text{CTX–M-9 group}}$ were detected in 24% and 15% of the samples at maximum concentrations of 3.65 and 3.9 $\log_{10}$ targets per 100 mL, respectively (**Supplementary Material, Table S11**). Significant differences between countries were observed, indicating an increase in most AMR markers from Austria to Hungary and Serbia (**Supplementary Material, Fig. S6**).

Even though not all hotspots detected during JDS4 were included in the annual cycle, the detected AMR marker concentrations were higher during annual monitoring. Regarding the maximal concentrations, an extraordinary hotspot amongst all sites was detected in the centre of Budapest (site H2, rkm 1655) in October 2020 (**Supplementary Material, Table S12**). At this site, highest concentrations of all AMR markers except for bla_{KPC} were measured. Regarding other hotspots, the site upstream Belgrade (S1, rkm 1177) showed high concentrations across several sampling months. Most hotspots were found in Hungary and Serbia while for *tetM* and $bla_{\text{CTX–M-9 group}}$ second-highest concentrations were detected in Austria in Wachau (A2L-0221) and downstream of Linz (A1–0621).

3.2.3. Faecal pollution

E.coli concentrations increased from Austria to Serbia (**Supplementary Material, Fig. S7)** with concentrations ranging from 1.07 to 5.08 $\log_{10}$ MPN per 100 mL. The maximum was again detected at the exceptional Hungarian hotspot H2 in October 2020. The BacHum marker could be detected in all 88 quantifiable samples and varied between 2.34 and 6.96 $\log_{10}$ marker equivalents (ME) per 100 mL (**Supplementary Material, Table S10, S11).** Faecal markers associated with ruminants and pigs were quantified in 32 and 17 out of 88 samples with maximum concentrations of 4.56 and 3.35 $\log_{10}$ ME per 100 mL, respectively.

3.2.4. Correlation analysis integrating AMR markers, faecal pollution diagnostics and environmental parameters

The correlation heat-map for the temporal monitoring dataset showed much stronger correlations than for the longitudinal sampling due to the higher number of replicates for each site (Fig. 6). The most significant cluster of AMR markers was again observed with the faecal

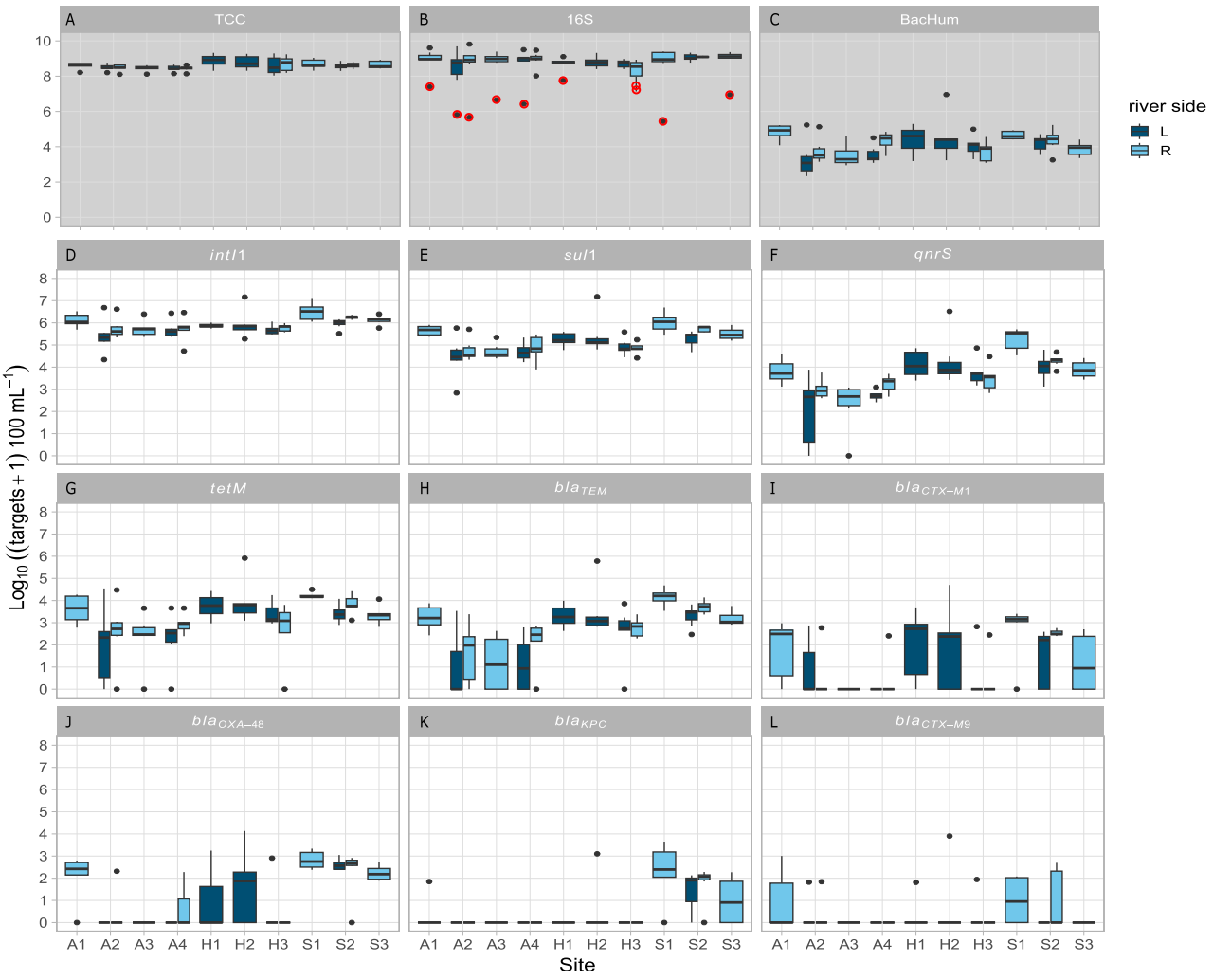

Fig. 5. Boxplots showing variability of quantified targets total cell counts (TCC) (A), 16S rRNA gene targets (B) (both $n = 98$), BacHum marker equivalents (C) and 9 AMR markers (D - L) in the annual sampling campaign ($n_{total} = 88$, $n_{each\ bar} = 7$). Sites labelled A1 – A4 were situated in Austria, H1 – H3 in Hungary & sites S1 – S3 in Serbia. Lower & upper hinges correspond to first & third quartile, whiskers correspond to 1.5 * IQR (interquartile range) from closer hinge, dots show outliers. Colours differentiate samples collected from left (L) & right river (R) side, small red circles in panel B depict excluded samples due to impaired DNA extraction efficiency.

indicators *E. coli* and BacHum, while also antimony, boron and silver were significantly correlated. For most AMR markers, a strong negative correlation existed with river kilometres. Positive correlations with discharge were detected for seven AMR markers, only $bla_{CTX-M-1\ group}$ and $bla_{CTX-M-9\ group}$ did not correlate. Animal associated faecal markers were positively correlated with most resistance markers, forming an own cluster. No significant correlations were detected with total suspended solids, temperature and conductivity.

3.3. Multivariate analyses to assess parameters influencing the patterns of AMR markers

For both, the longitudinal and the temporal sample set, linear regression analysis corroborated that faecal pollution, predominantly of human origin, was the most significant driver of AMR occurrence in the Danube River. For most AMR markers in the JDS4 dataset, *E. coli* concentrations were the main predictor with coefficients of determination (r^2) ranging from 0.283 to 0.646. *Sul1* abundance was best explained by the combination of the herbicide metolachlor and *E. coli* ($r^2 = 0.572$). In contrast, *intI1* was primarily linked to the number of 16S rRNA gene targets corroborating that this gene is already widely distributed in the bacterial populations in the Danube River. For $bla_{CTX-M-9\ group}$ no reliable regression analysis could be performed due to the small number of quantifiable samples. Further minor predictors were water river kilometre, zinc, arsenic, carbendazim and piperonyl butoxid (**Supplementary Material, Table S13**). In the annual dataset, *E. coli* or BacHum marker concentrations explained approximately 50% of the variability of six AMR markers. For *intI1,* 16S rRNA gene targets and *E. coli* explained 80%, and for $bla_{KPC,}$ river kilometre (negative) and BacHum were the strongest predictors, explaining together 50% of variation. Other identified parameters that explained a maximum of 11% of the observed ARG variations were aluminium, boron and the pig-associated MST marker (Pig2Bac) (**Supplementary Material, Table S14**).

To improve the understanding of general data variability and especially of the relations between AMR markers and other parameters throughout the observed Danube River samples, a principal component analysis (PCA, combining the samples of both datasets) was performed (Fig. 7). Component 1 primarily represented loadings of all AMR markers as well as some metals, while component 2 mainly represented chemical compounds (**Supplementary Material, Fig. S8**). All AMR markers clustered together with *E. coli* and the human faecal marker, as well as with samples characterized by heavy faecal contamination, corroborating the strong relation between human contamination and AMR. Network analysis separately performed for both data sets

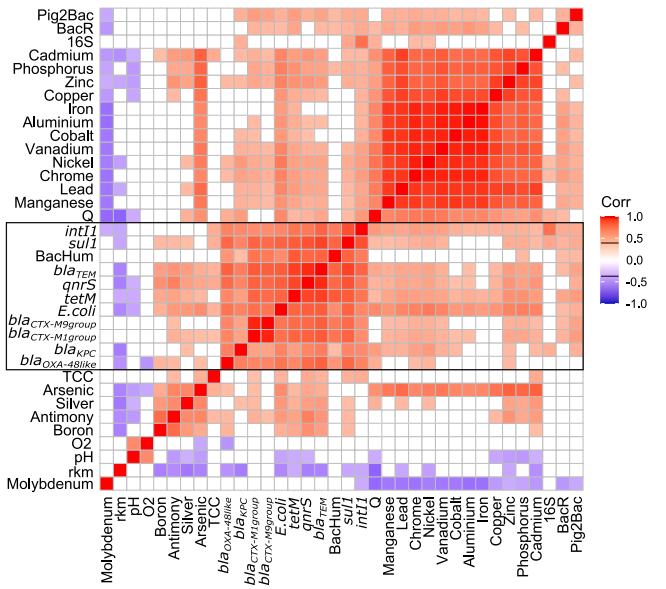

Fig. 6. Pairwise correlation heat-map of AMR, faecal pollution markers and environmental parameters for samples of the annual cycle; only significant correlations are shown. Fill colours depict Spearman correlation coefficient, samples were clustered hierarchically, the box shows the AMR marker cluster; Q: discharge, rkm: river kilometre.

corroborated the findings from correlation, regression and principal component analysis. In both networks, most AMR markers clustered with human faecal parameters, especially the ARGs of high and intermediate abundance and *intI1* showed a strong connection to 16S gene targets (**Supplementary Material, Figs. S9** and **S10**). For the longitudinal sampling campaign, AMR markers clustered with piperonyl butoxid (specifically $bla_{\text{OXA-48like}}$), *sul1* was associated with metolachlor, fluopyram and nicosulfuron, and *qnrS* with Pb and Al. For the temporal sampling campaign, AMR markers clustered also with Pig2Bac and partly with BacR as well as with many metals, most strongly *tetM* and *intI1*. Furthermore, strong associations of AMR markers were observed with river kilometre (negative) and with discharge (positive).

4. Discussion

Comprehensive investigations on AMR in rivers at continental scales have been carried out sporadically in the past, but a clear link to faecal pollution diagnostics was largely missing (Gao et al., 2023; Lee et al., 2020; Paulus et al., 2020). Here, we present a holistic study on AMR markers along 2311 km of the Danube River, which drains about 10% of continental Europe (ICPDR, 2009). We combined high spatial and temporal resolution quantification of nine resistance-related genes with advanced three-step faecal pollution monitoring and the determination of key environmental and chemical parameters to identify patterns, hot-spots and key drivers of AMR along this most international river in the world.

4.1. ARG hotspots were mostly found in the middle and lower section of the Danube River

Along the river, highest concentrations and detection rates of all resistance markers were found in the middle and lower river sections. Most identified hotspots showed peaks for several ARGs simultaneously and the high correlation between ARGs indicated comparable dynamics of the studied AMR markers. A major ARG hotspot was the tributary Arges (rkm 435), receiving wastewater from Romania's capital Bucharest, which was already one of the most polluted sites in previous JDS (Alygizakis et al., 2019; Kirschner et al., 2009, 2017). Further hotspots were detected downstream of large cities in Romania and Bulgaria and in polluted tributaries in Croatia and Serbia. Compared to the longitudinal monitoring, higher ARG detection rates were observed during the annual cycle that mainly focused on three capitals (all with over 1.3 million inhabitants). The highest concentrations of all ARGs (except bla_{KPC}) and human faecal pollution markers were detected in the middle of Budapest (site H2) in October 2020. This site was situated downstream of an urban stream (Rákos patak) which is known to be impacted by pollution from several point and non-point sources. Since downstream of Budapest Danube water is used to produce drinking water through river-bank filtration (Nagy-Kovács et al., 2019) this might be of immediate health relevance and should be further investigated. The extremely high ARG values occurring at this site only in October 2020 could be due to illegal point source pollution which has been

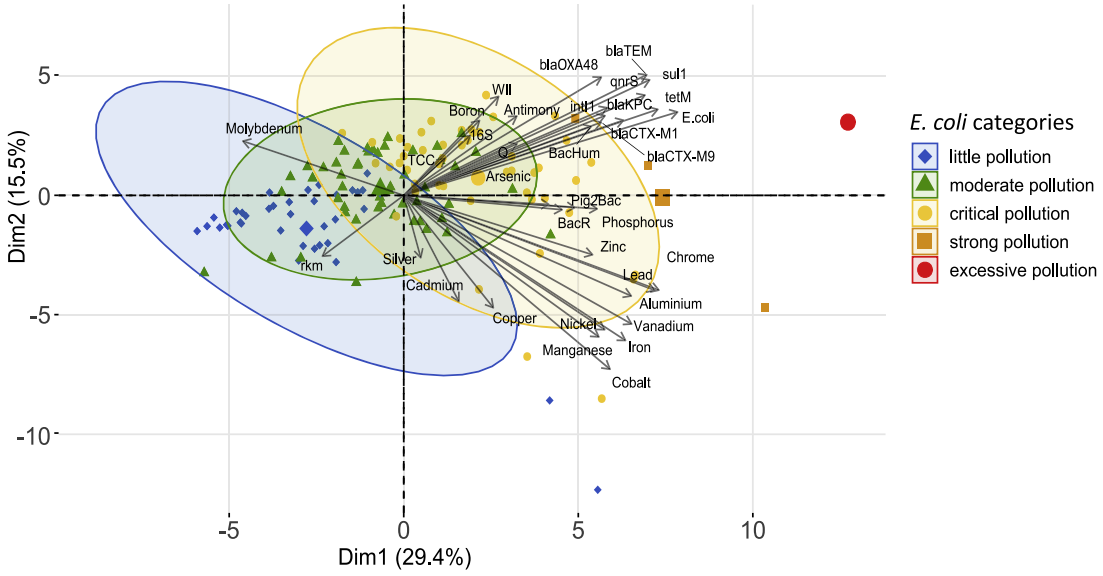

Fig. 7. PCA of both datasets (longitudinal & temporal) combined, first two components shown, points represent samples ($n = 141$) grouped by *E. coli* concentrations (clustering according to Supplementary Material, Table S15), arrows show the direction of each variable ($n = 34$), ellipses represent an approximated 95% confidence area for each *E. coli* category, for categories "strong" and "excessive pollution" not enough samples existed to calculate an ellipse; all shared parameters with no missing data were included.

detected sporadically at this stream (local news) and such temporal hotspots underline the importance of consecutive monitoring and to follow long-term trends (Ling et al., 2013). In contrast to other studies (Chen et al., 2013; Di Cesare et al., 2017; Sabri et al., 2020), we did not observe a uniform seasonal pattern across the investigated sampling sites in the three countries, indicating that other than seasonal factors are driving ARG dynamics. This is in agreement with papers focusing on strongly anthropogenically influenced rivers where spatial influences were bigger than temporal ones (Paulus et al., 2020; Reichert et al., 2021).

ARG concentrations were generally comparable to those detected by Cacace et al. (2019) in upstream and downstream river sites of 16 European WWTPs. These authors introduced a clustering of three abundance groups which was also adopted for this study with abundant (*intI1, sul1*), intermediate (*qnrS, tetM,* bla_{TEM}) and rare AMR markers ($bla_{OXA\text{-}48like}$, $bla_{CTX-M\text{-}1\ group}$, bla_{KPC}, $bla_{CTX-M\text{-}9\ group}$). *IntI1* and *sul1* were ubiquitous throughout the whole Danube River, coinciding with their predominance throughout WWTPs in the DRB (Alygizakis et al., 2019). Both are amongst the most frequently assessed qPCR targets for evaluating AMR in human, animal, and environmental samples (Gillings et al., 2015; Pruden et al., 2012). *IntI1*, the class one integron-integrase gene is commonly associated with mobile ARGs and was recommended to serve as a proxy for total AMR load (Amos et al., 2015). Tarek and Garner (2022) recommended *sul1* as one of five minimally redundant targets for wastewater monitoring. Sulfonamides are amongst the globally most widely detected antibiotics in WWTPs (Sanseverino et al., 2018). Both genes were highly inter-correlated in the Danube River, explainable by their frequent co-occurrence in polluted environments (Berglund et al., 2015; Chen et al., 2015; Di Cesare et al., 2016; Paulus et al., 2020). Bla_{TEM}, *qnrS* and *tetM* showed similar distribution patterns as the abundant AMR markers and were present in most samples at concentrations and detection rates largely congruent to other rivers (Proia et al., 2018; Reichert et al., 2021; Zhang et al., 2019). Among+ the rare markers, the $bla_{CTX-M\text{-}1\ group}$ was most widespread and present in 30% of samples during JDS4 and in 42% of samples during annual sampling. For all rare AMR markers the top three concentrations were detected exclusively in Hungary and Serbia, except for $bla_{CTX-M\text{-}9\ group}$, for which the second highest concentrations were detected in Austria in June 2021, downstream of the discharge of the WWTP Linz. Bla_{CTX-M} represent the most common gene family of clinically relevant ESBL and therefore a recommended environmental indicator, even though it is rarely found in natural environments (Keenum et al., 2022). Bla_{CTX-M} was also selected since it was the dominant ESBL family in *Enterobacteriaceae* in the Danube River in a previous study. Most isolates carried the gene for $bla_{CTX-M\text{-}1\ group}$ but also one isolate isolated close to Belgrade harboured $bla_{CTX-M\text{-}9\ group}$ (Kittinger et al., 2016). Another clinically relevant gene suggested for environmental monitoring is the carbapenemase bla_{KPC} (Berendonk et al., 2015), which was also previously detected in *Klebsiella* spp. from the Danube River (Kittinger et al., 2016). During the temporal monitoring, 19 of 21 detects were made in Serbia, one detect of bla_{KPC} was made in Austria and Hungary. During JDS4, hotspots of this gene were coinciding with hotspots of other ARGs (Arges, downstream Arges and downstream Turnu-Severin/Simijan). In wastewater effluents in the DRB in 2017, bla_{KPC} was recently not detectable (Alygizakis et al., 2019). Whether there has been an increase in the occurrence of this carbapenemase in the DRB over the past years or whether these inconsistencies were due to methodological differences cannot be determined.

4.2. Human faecal pollution is the dominant driver of ARG occurrence in the Danube River, while intI1 is widespread within the microbial community

To understand the spread of ARGs in the environment, it is important to consider faecal contamination as a major influencing factor (Demeter et al., 2023; Karkman et al., 2019). By combining nine different AMR markers with *E. coli*, genetic MST markers and the theoretical wastewater index (WII), we were able to directly link AMR occurrence with specific pollution sources. For all investigated ARGs, human faecal pollution was a dominant influencing factor during both monitoring campaigns at predominant baseflow conditions. Correlation and multivariate regression analyses demonstrated the primary influence of general (*E. coli*) and human-associated pollution (BacHum) on the observed ARG concentrations. The hotspots of faecal contamination were largely consistent with earlier JDSs (Kirschner et al., 2009, 2017) and therefore mark potential long-term AMR hotspots. Both, general and human pollution and ARG concentrations tended to rise from upper to lower sections of the Danube River. Nevertheless, contamination appears to have a local influence and does not promote a general accumulation of AMR along the run of the whole Danube River. Although a significant negative correlation was found with river km in the temporal campaign, this does not reflect general trends along the Danube, as shown by the longitudinal study. This finding may instead reflect the status of wastewater treatment in the Danube River Basin (DRB). While in the "old" EU member states in the upper DRB (Germany, Austria), state-of-the-art wastewater treatment accounts for >99% of the population, state-of-the-art wastewater infrastructure has been continuously implemented in the last two decades in the "new" EU member states in the middle (Hungary, Slovakia, Croatia) and lower DRB (Romania, Bulgaria) and non-EU member states in these sections (Serbia, Ukraine, Moldova) are still lagging behind (ICPDR, 2021). By observing a significant correlation of five ARGs with our calculated WII, we demonstrated that size, treatment type and distance of the effluent discharge shape the AMR patterns along the whole Danube River. Strong connections between WWTP size and AMR loads were also reported in an English catchment area (Elder et al., 2021).

During the annual cycle, positive correlations also with animal-associated markers were detected. Strongest correlations were detected for *tetM,* an ARG commonly associated with livestock and agricultural influence (Orlek et al., 2023). In line with this, the second highest *tetM* concentrations during the temporal monitoring were detected in Austria (A2 in February 2020), a site showing highest ruminant and pig-associated MST marker numbers throughout the whole study. In contrast to all ARGs, *intI1* followed a different trend. Regression analysis showed that 16S rRNA gene targets were its main predictor during both campaigns. As *intI1* was extensively promoted as an anthropogenic indicator in the environment (Gillings et al., 2015; Pruden et al., 2012) we doubt this applicability in the Danube River where the gene seems to be already widespread within the general microbial community.

4.3. AMR markers are also associated with environmental and chemical parameters

Only during the temporal monitoring, river discharge was significantly correlated with most AMR markers. Discharge varied during this campaign with maxima of 72% above the annual mean, whereas for JDS4, samples were collected at base-flow conditions (maximum 11% above the annual mean). Hydrological and meteorological factors can cause intrusion of diffuse pollution sources through surface run-off or combined sewer overflows causing larger effects at more pristine upstream sites (Reichert et al., 2021). This could explain the elevated ARG concentrations at site A2 (rkm 2007) in Austria in February 2020 at increased discharge. Even though discharge maxima were also detected in Hungary and Serbia during this month, no pronounced increase in ARGs was recorded. Other seasonal variations were inconsistent across the investigated sites during the temporal monitoring and no significant correlation with water temperature was observed. Presumably, the influence of faecal contamination exceeds the influence of hydrological and temporal dynamics in these heavily polluted stretches. Interestingly, Cacace et al. (2019) have detected higher concentrations of several ARGs in samples upstream of an Austrian WWTP than downstream and they suspected that these concentrations were attributable to diffuse

sources (surface run-off, combined sewer overflows), however without reporting hydrological characteristics. The influence of increased runoff due to rainfall events on ARG concentrations has been frequently reported in rivers (Di Cesare et al., 2017; Garner et al., 2017; Lee et al., 2022).

We found no positive correlation with antibiotic concentrations in the Danube River, in contrast to rivers in India and China where discharges from antibiotic production industries promote resistance development (Kristiansson et al., 2011; Li et al., 2010) and positive correlations of sulfonamides and *sul1* as well as tetracyclines and *tetM* were described (Chen et al., 2015). On a global scale, resistance spread is only affected to a minor extent by antibiotics in the environment (Karkman et al., 2019), in Europe, antibiotic production is negligible. The detected antibiotic concentrations in the Danube River were lower than 0.025 μg L^{-1} for all antibiotics that were quantifiable in more than 20% of samples. Previous studies reported no clear selective effect through environmental (sub-MIC) antibiotic concentrations (Berglund et al., 2015; Haenelt et al., 2023). The low concentrations and detection rates of quantified antibiotics in the Danube River largely coincide with results from another study in the Danube River Basin (Ng et al., 2023).

Amongst the analysed chemical parameters, the herbicide metolachlor showed significant positive correlations with *tetM, sul1,* bla_{TEM} and the insecticide antagonist piperonyl butoxide with *tetM, sul1,* $bla_{OXA\text{-}48like}$. *Sul1* was also correlated with carbendazim, fluopyram and nicosulfuron. Some of these substances (metolachlor, carbendazim) have been clearly attributed to wastewater effluents in rivers and were suggested as indicator compounds for chemicals of emerging concern (Zhang et al., 2021a, 2021b). Pesticides as well as heavy metals and ARGs and ARB have been described as co-occurring pollutants in wastewater-impacted environments which could potentially enhance co-selection mechanisms for resistances (Reddy et al., 2022). All 16 included metals showed correlations with at least some AMR markers, while at the same time all being highly positively correlated with *E. coli* concentrations indicating a common wastewater origin. Whether potential selective effects exist cannot be clarified within this study and would require in-depth field investigations and lab experiments.

4.4. Strict quality control and normalization are important for quantitative, comparable results

Although a well-established DNA extraction procedure was applied in this study (Reischer et al., 2006), that was already successfully applied in previous Joint Danube Surveys (Kirschner et al., 2017; Savio et al., 2015), DNA extraction was impaired in samples with elevated turbidity, specifically during a major flood event. Most recently, it has been reported that increased turbidity presented a challenge for DNA extraction for eDNA analysis during flood events in the Danube River (Pont et al., 2023). In future studies targeting genetic (AMR and MST) markers, when large spatial or temporal gradients in the background matrix of the water samples are expected, a universal standard spike should be included as a strict quality control along the whole chain of analysis (sampling, filtration, DNA extraction, molecular analysis) (Demeter et al., 2023).

Comparability between studies is another important aspect. ARG ratios assessing the frequency in bacterial populations allow for improved comparison between targets and across different habitats such as biofilms or sediments (Keenum et al., 2022). We applied normalization to two different reference parameters (TCC & 16S rRNA gene targets). While most studies refer to 16S rRNA gene targets for relative ratios, a recent article has recommended calculating relative concentrations per bacterial cells, implementing it as universal unit (Yin et al., 2023). Microscopic direct cell counting yields much more precise bacterial numbers since 16S rRNA genes targets are often present in multiple copies across many bacteria (Větrovský and Baldrian, 2013). Appropriately, in our dataset relative abundances per TCC (in comparison to 16S rRNA gene targets) had higher correlation with absolute concentrations, being a more precise reference parameter to assess ARG abundance within the microbial community (Liang et al., 2020). In the Danube River, general bacterial numbers increase slowly and gradually. Wastewater pollution only has a minor influence on TCC but alters the community composition (Savio et al., 2015; Velimirov et al., 2011), explaining the very high observed correlation between absolute and relative (per TCC) AMR marker concentrations.

5. Conclusions

The applied new concept combining genetic AMR markers with advanced faecal pollution diagnostics and the determination of key environmental and chemical parameters allows capturing spatio-temporal dynamics, identifying hotspots, and key drivers of AMR in rivers. This comprehensive understanding provides the basis for a targeted management to reduce the spread of AMR in river basins. Up to now, environmental baselines for ARG concentrations are largely missing. We present the first comprehensive ARG data set throughout the Danube River which will help to follow trends in the future. To increase our understanding of AMR spread and dynamics, AMR in other environmental compartments should also be investigated, such as in river biofilms or sediments that could act as long term reservoirs.

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CRediT authorship contribution statement

Iris Schachner-Groehs: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Michael Koller:** Investigation, Writing – original draft, Writing – review & editing. **Melanie Leopold:** Investigation, Methodology. **Claudia Kolm:** Methodology, Validation. **Rita B Linke:** Methodology, Validation. **Stefan Jakwerth:** Investigation. **Stoimir Kolarević:** Investigation, Conceptualization. **Margareta Kračun-Kolarević:** Investigation. **Wolfgang Kandler:** Methodology, Data curation. **Michael Sulyok:** Data curation, Methodology. **Julia Vierheilig:** Methodology. **Marwene Toumi:** Investigation. **Rózsa Farkas:** Investigation. **Erika Toth:** Investigation, Conceptualization. **Clemens Kittinger:** Conceptualization, Investigation. **Gernot Zarfel:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing. **Andreas H Farnleitner:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing. **A.K.T. Kirschner:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alexander Kirschner reports financial support was provided by Austrian Science Fund. Alexander Kirschner reports financial support was provided by International Commission for the Protection of the Danube River. Alexander Kirschner reports financial support was provided by Austrian Federal Ministry of Agriculture, Forestry, Regions & Water Management. All other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.121244.

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