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Research article

Sources and dynamics of fecal pollution and antibiotic resistance across a Mediterranean land–sea continuum

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ABSTRACT

A comprehensive understanding of fecal pollution dynamics and associated antibiotic resistance is critical for effective water quality management in coastal watersheds exposed to strong hydrological variability. This study aimed to identify the relative contribution of human and animal fecal sources, assess the spatial and temporal distribution of contamination, and evaluate the occurrence of selected antibiotic resistance genes (ARGs) across a Mediterranean land–sea continuum (central Adriatic Sea, Italy) under contrasting rainfall conditions.

Surface water and sediment samples were collected during the bathing season and analyzed using host-associated microbial source tracking (MST) markers and selected ARGs. Human fecal contamination was the dominant source, as indicated by the consistent detection of HF183, which significantly increased during moderate and extreme rainfall events, indicating inputs from runoff and sewer overflows. The presence of human adenovirus (HAdV) further supported sewage-derived contamination, whereas animal-associated markers showed lower detection frequencies and more heterogeneous spatial patterns.

ARGs, including tetracycline resistance genes *tetM*, were widely detected, with generally higher concentrations at freshwater sites. While ARG distribution was influenced by hydrological variability, *tetM* showed lower temporal variability and remained relatively abundant under drought conditions, suggesting longer-term environmental persistence.

Rainfall emerged as a major driver shaping marker distribution, with water showing high temporal variability and sediments acting as secondary reservoirs enriched during heavy rainfall events. These findings provide relevant insights for improving water quality strategies and support targeted management actions to mitigate fecal pollution in Mediterranean coastal systems.

1. Introduction

Fecal pollution in aquatic ecosystems remains a pervasive global challenge, posing significant risks to both human and animal health, particularly in coastal waters where direct exposure during recreational activities is high (Garbossa et al., 2017). Fecal inputs from human and animal sources introduce a wide range of pathogenic microorganisms, including bacteria, viruses, and protozoa, associated with gastrointestinal, respiratory, dermatological, and ocular infections (Delahoy et al., 2018; Penakalapati et al., 2017). These risks are amplified in densely populated coastal areas, where urban runoff, wastewater discharges, and agricultural activities interact with hydrological variability, enhancing the mobilization and transport of contaminants.

Water quality monitoring has traditionally relied on fecal indicator bacteria (FIB), such as *Escherichia coli* and enterococci, as proxies for

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fecal contamination (USEPA, 2013). However, FIB-based approaches present important limitations, including the inability to discriminate among pollution sources and uncertainties related to their environmental persistence, regrowth, and association with sediments (Anderson et al., 2005; Badgley et al., 2011). These constraints reduce their effectiveness in identifying contamination pathways and supporting targeted management actions, particularly in complex environments such as river–coast interfaces, where multiple sources and dynamic environmental drivers interact (Ahmed et al., 2024; Harwood et al., 2014).

Microbial source tracking (MST) has emerged as a robust approach to overcome these limitations by enabling the identification of fecal pollution sources through host-associated molecular markers (Ahmed et al., 2019; Bernhard and Field, 2000; Li et al., 2022; Stoeckel and Harwood, 2007) MST tools, typically based on quantitative PCR (qPCR), allow the detection and quantification of fecal inputs from humans and a range of animal hosts, providing valuable information for source-specific management and improved public health risk assessment, and supporting the design of targeted mitigation strategies in coastal systems. Their application is particularly relevant in coastal catchments, where rainfall-driven runoff and hydrological connectivity enhance the transport of fecal contaminants from terrestrial to marine environments (Hassard et al., 2023; Ishii and Sadowsky, 2008; Sabater et al., 2011).

In parallel, the spread of antibiotic resistance in the environment has become a major global health concern, with antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) recognized among the top public health threats worldwide (Aslam et al., 2018; Larsson and Flach, 2022; World Health Organization, 2022). Aquatic environments, particularly coastal systems, act as important reservoirs and transmission pathways for antibiotic resistance genes (ARGs) and pathogenic bacteria (e.g., *Vibrio* spp.), which are introduced through wastewater discharges, hospital effluents, and agricultural activities (Ba-Alawi et al., 2022; Bengtsson-Palme and Larsson, 2016; Vezzulli et al., 2016; Zhang et al., 2022). The co-occurrence of ARGs with fecal pollution highlights the need to integrate resistance indicators into water quality assessments, linking contamination sources to potential public health risks (Garner et al., 2021).

The Arzilla stream catchment, located along the central Adriatic coast (Italy), represents a typical Mediterranean watershed characterized by mixed urban, agricultural, and livestock land uses. Despite its relatively small size, the catchment discharges into coastal waters near the city of Fano, a highly frequented recreational area. The system is subject to pronounced seasonal variability, including intense rainfall events that influence runoff dynamics and pollutant transport along the land–sea continuum. These features, combined with multiple point and diffuse pollution sources, make the Arzilla catchment an ideal case study for investigating fecal pollution dynamics in transitional environments and their implications for coastal water quality.

The objectives of this study were to: (i) characterize the spatial and event-driven temporal variability of fecal pollution along the Arzilla stream and its adjacent coastal waters using a suite of human- and animal-associated MST markers applied to water and sediment samples under dry and wet weather conditions; (ii) assess the relative contributions of major fecal pollution sources; and (iii) evaluate the occurrence of selected ARGs and their implications for public health. By integrating MST and ARG analyses, this study provides new insights into the sources, distribution, and transport of fecal contamination in Mediterranean coastal systems and supports evidence-based water quality management and targeted mitigation strategies in Mediterranean coastal systems.

2. Materials and methods

2.1. Study area and sampling design

Surface water samples were collected at five stations along the Arzilla stream, a small coastal watercourse in central Italy, arranged from upstream to downstream and discharging into the Adriatic Sea near the town of Fano (Fig. 1). Station codes (PT3, SMA, PT2, MT, and PT1) are arbitrary labels used to identify distinct sampling sites. Additional surface water samples were collected at three marine stations located along an offshore transect at increasing distances from the river mouth: 50 m (ST0), 100 m (ST1), and 150 m (ST2). The Arzilla stream is a torrential watercourse designated as a Site of Community Importance (SCI; IT5310008), with a watershed area of approximately 110 km^2. The river is prone to flooding during intense rainfall events because of its limited hydraulic capacity (Ferrarin et al., 2021).

The catchment exhibits a marked land-use gradient, with predominantly agricultural landscapes in the upper basin and increasing urbanization toward the municipality of Fano (approximately 62,000 inhabitants) and the adjacent coastal zone. In addition to Fano, the watershed includes several smaller urban settlements, including Mombaroccio, Montecicardo, Ginestreto, Villa Betti, and Santa Maria dell'Arzilla. The basin is served by multiple wastewater treatment plants and by a combined sewer system in the lower urban catchment, where domestic wastewater and stormwater are conveyed through the same network. During intense rainfall events, the hydraulic capacity of the sewer system may be exceeded, leading to the activation of combined sewer overflows (CSOs) that discharge diluted untreated wastewater into the Arzilla stream. Together with agricultural runoff and urban stormwater, these anthropogenic pressures represent multiple potential sources of human-associated fecal contamination, particularly during wet-weather conditions. The Arzilla watershed also includes several tributaries, among which the Bevano stream (PT2) was included in the sampling design because of its potential contribution to downstream microbial contamination during runoff events. These characteristics make the Arzilla catchment a suitable model system for investigating the transport of microbial contaminants and antibiotic resistance genes along the land–sea continuum.

The river mouth is located adjacent to a highly frequented bathing area and close to the Fano tourist marina, where bathing is temporarily prohibited during high discharge events because of the increased risk of sewage-related bacterial contamination (Penna et al., 2021). Coastal circulation is primarily driven by tides (range ~40 cm), wind forcing, and southward longshore transport (Orlic et al., 1992).

The study area exhibits a clear land-use gradient, with predominantly agricultural and rural landscapes in the upstream portion of the catchment and increasing urbanization toward the river mouth and adjacent coastal zone (Fig. 1).

Detailed information on station locations and sampling dates is provided in Freddi et al. (2026).

Sampling was conducted on multiple dates between May and September 2024 under a range of weather conditions (six sampling events). Rainfall conditions were classified based on total precipitation accumulated during the 24 h preceding sampling as: dry (0 mm), light (0.1–1 mm), moderate (>1–10 mm), heavy (10–50 mm), and extreme (>50 mm). Water samples were not collected at PT2 (Bevano stream, a tributary of the Arzilla) during the first five events due to insufficient flow.

At each site, surface water samples (both freshwater and seawater) were collected from the surface layer in triplicate using sterile 1-L polyethylene bottles. The three bottles represented independent field replicates. Samples were transported at 4 °C and processed within 6 h of collection. DNA was extracted independently from each field replicate. Sediment samples were collected exclusively at the three marine stations (ST0, ST1, and ST2) using a plexiglass corer. At each station, three independent sediment field replicates were collected, and the upper

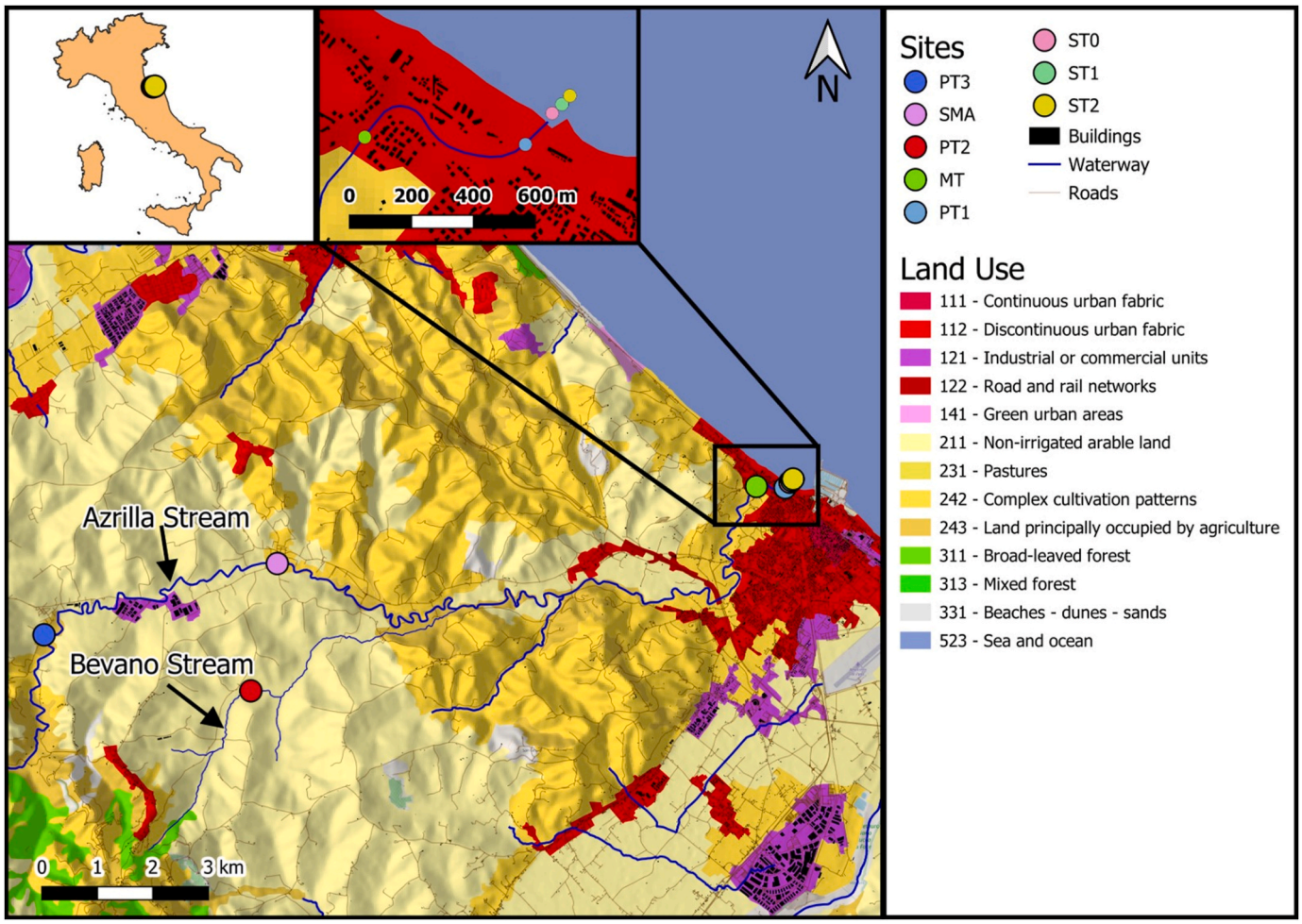

Fig. 1. Map of the Arzilla stream catchment and the adjacent coastal waters (central Adriatic Sea, Italy) showing the study area and sampling stations. Five riverine stations (PT3, SMA, PT2, MT, PT1) are located along the river continuum from upstream to the mouth (coloured circles), while three marine stations (ST0, ST1, ST2) are located along a transect offshore from the river mouth at increasing distances from the shoreline. The river network is shown in blue.

0–2 cm sediment layer was aseptically transferred into sterile 50 mL tubes, transported at 4 °C, and subsequently stored at −20 °C until DNA extraction. DNA was extracted independently from each sediment replicate.

2.2. Measurements of rainfall and physico-chemical parameters

Rainfall data were obtained from an automatic weather station located at PT1, equipped with a Campbell Scientific CLIMA VUE50 rain gauge. Precipitation values were recorded in real time and subsequently aggregated as total rainfall (mm) over the 24 h preceding each sampling event for data analysis and classification of rainfall conditions. These measurements were essential for characterizing local weather conditions and evaluating their influence on river levels and water quality dynamics. An ultrasonic river-level sensor (Siemens SITRANS LU240) was installed at the river mouth. At PT1 and at the three marine stations, in-situ measurements of temperature, salinity, dissolved oxygen, and pH were collected using a CTD probe, following the procedures described by Penna et al. (2021).

2.3. DNA extraction from water and sediment samples

Surface water samples (1 L) were filtered through 0.22 μm cellulose nitrate membrane filters (Sartorius, Göttingen, Germany) and stored at −20 °C until processing. Genomic DNA was extracted from 1 g of wet sediment and from membrane filters using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions with minor modifications. Specifically, after the addition of CD1 buffer to the PowerBead Pro tubes, samples were incubated at 70 °C with repeated vortexing (three cycles consisting of 5 min in a thermostated water bath followed by 2-min of vortexing) to enhance cell lysis (Luna et al., 2019). The resulting lysate was subsequently treated with CD2 and CD3 buffers. The clarified supernatant was transferred to MB spin columns, washed with EA and C5 solutions, and centrifuged to remove residual wash buffer. DNA was eluted with 60–100 μL of C6 elution buffer following a brief incubation at room temperature. DNA concentrations were quantified using spectrophotometry (Agilent, BioTek Synergy H1) and DNA extracts were stored at −80 °C until qPCR analyses.

2.4. PCR inhibition

The presence of potential PCR inhibitors in DNA extracted from water and sediment samples was evaluated using the Sketa22 PCR assay (Haugland et al., 2005). A defined quantity of *Oncorhynchus keta* (*O. keta*) DNA (10^4 gene copies (GC)/reaction) was added to DNase-free water to generate a reference quantification cycle (Cq). The same amount of *O. keta* DNA was then added to each water and sediment DNA extracts. Samples were considered PCR-inhibited when the Sketa22 Cq value was delayed by $\geq$ 2 cycles relative to the reference control, following previously published criteria (Ahmed et al., 2021).

2.5. qPCR analysis of microbial source tracking (MST) markers and antibiotic resistance genes (ARGs)

Quantitative PCR (qPCR) assays were used to quantify MST markers and selected ARGs in DNA extracts from water and sediment samples. MST qPCR analyses targeted sewage-associated *Bacteroides* HF183 (Bernhard and Field, 2000; Green et al., 2014); sewage-associated *Carjivirus* (Stachler et al., 2017), sewage-associated human adenovirus (HAdV), a human pathogen considered as host-specific marker of sewage pollution (Heim et al., 2003), ruminant-associated Bacteroides BacR (Reischer et al., 2006), canine-associated DogBact (Dick et al., 2005), avian-associated GFD (*Helicobacter* spp.; Green et al., 2012), and, horse-associated HoF597 (Dick et al., 2005). ARGs were quantified as indicators of antimicrobial resistance pressure in the catchment. The targeted genes included $bla_{CTX\text{-}M\text{-}1}$ (extended-spectrum β-lactamase resistance) (Colomer-Lluch et al., 2011), *sul1* (sulphonamide resistance) (Heuer and Smalla, 2007), and *tetM* (tetracycline resistance) (Ng et al., 2001). Details on qPCR primers and probe sequences, concentrations, and thermal cycling conditions are provided in Supplementary Table S1.

All qPCR reactions were performed in a final volume of 20 μL using 2× QuantiNova Probe PCR Master Mix (Qiagen), consisting of 15 μL of reaction mixture containing target-specific primer and probe concentrations (Supplementary Table S1) and 5 μL of template DNA. Each run included no-template control (NTCs) and synthetic gBlocks positive controls containing the relevant target sequence. Amplifications were carried out on a Bio-Rad CFX Opus 96 Real-Time PCR System using thermal cycling conditions reported in Supplementary Table S1. Standard curves were generated using serial dilutions of gBlocks (5×10^6 to 5 GC/reaction) to determine amplification efficiency (E), the coefficient of determination (R^2), slope and Y-intercepts (Supplementary Table S2). DNA extracted from each independent field replicate was analyzed separately. For each DNA extract, qPCR reactions were performed in technical triplicate. A sample was considered quantifiable when the target amplified in all three technical replicates and the calculated concentration was equal to or greater than the assay-specific limit of quantification (LOQ). Samples with amplification in all three technical replicates but with concentrations below the LOQ, as well as samples with amplification in only two of the three technical replicates, were classified as detected but not quantifiable (DNQ). Samples with amplification in one or none of the three technical replicates were classified as not detected (ND).

2.6. qPCR assay performance characteristics and QA/QC

The correlation coefficients (R^2) of qPCR standard curves (5×10^6–5 GC/reaction) for HF183, *Carjivirus*, HAdV, BacR, DogBact, GFD, HoF597, PMMoV, $bla_{CTX\text{-}M}$-1, *sul1*, and *tetM* ranged between 0.96 and 1.00 (Supplementary Table S2). The y-intercepts of standard curves ranged from 38.6 to 45.9. The qPCR efficiencies ranged from 91 to 102%. For each target, all positive control samples consistently yielded positive amplification, while all NTCs remained negative throughout the qPCR analysis. PCR inhibition was not observed in any water and sediment samples.

2.7. Statistical analysis

Statistical analyses were performed to assess spatial and temporal differences in environmental parameters, MST markers, and ARGs.

For statistical analysis, the limit of quantification (LOQ) for each qPCR target was defined as the lowest concentration that could be quantified with confidence (Supplementary Table S3). Samples classified as detected but not quantifiable (DNQ), including those with concentrations below the assay-specific LOQ, were excluded from quantitative statistical analyses. Consequently, only quantifiable samples were included in the analyses.

Data were $\log_{10}$-transformed prior to analysis to improve normality and homogeneity of variance. After transformation, assumptions of normality and homogeneity of variance were verified before applying parametric tests. One-way analysis of variance (ANOVA) was then used to evaluate differences among sampling events and environmental variables (temperature, salinity, pH, and dissolved oxygen), as well as differences in MST marker and ARG concentrations between riverine and marine environments. When significant differences were detected ($p < 0.05$), Tukey's post-hoc test was applied for pairwise comparisons.

Associations between MST markers, ARGs, and environmental variables were evaluated using Spearman's rank correlation coefficient (ρ), to assess the presence and strength of monotonic relationships among the variables. Correlation analyses were performed separately for river and marine samples using only quantifiable values.

Statistical significance was set at $p < 0.05$ for all analyses. All statistical analyses and graphical outputs were performed using R software (version 3.4.0), with boxplots generated using the ggplot2 package (Wickham, 2016).

3. Results

3.1. Environmental conditions

Sampling was conducted during six events under variable meteorological and hydrological conditions. Events 1–3 were characterized by moderate rainfall (0.9–3.5 mm), whereas Event 4 occurred under dry weather conditions. Event 5 followed a heavy rainfall event (54.8 mm), and Event 6 was associated with extreme precipitation exceeding 190 mm. Environmental parameters (temperature, salinity, pH, and dissolved oxygen) exhibited pronounced spatial and temporal variability throughout the study period, reflecting the influence of hydrological variability and seasonal conditions (Supplementary Table S4).

During Events 1–3, moderate rainfall resulted in relatively homogeneous temperature conditions across riverine and coastal waters (18.1–22.9 °C). Salinity was slightly reduced near the river mouth (ST0; average 30.6 PSU) and increased progressively by ~1–2 PSU along the coastal transect. Under dry conditions (Event 4), both temperature and salinity increased at all stations, particularly at coastal sites, consistent with seasonal warming during the July sampling period.

Following the heavy rainfall event (Event 5), salinity decreased near the river mouth due to increased freshwater input and surface runoff, although the reduction was less pronounced than expected given the rainfall magnitude, likely reflecting strong marine mixing. In contrast, extreme precipitation during Event 6 resulted in the lowest salinity levels (12.7–15.8 PSU) and promoted marked mixing of the water column.

pH values remained relatively stable (~7.5) during Events 1 and 6, consistent with increased freshwater influence, whereas higher values (8.0–8.2), characteristic of marine waters, were recorded at coastal stations during Events 2–5. Dissolved oxygen concentrations showed substantial variability, with lower values observed during rainfall-affected events (Events 3–5).

ANOVA results confirmed significant temporal differences in temperature ($p < 0.01$), salinity ($p < 0.001$), and dissolved oxygen ($p < 0.05$) among sampling events. Post-hoc comparisons (Tukey's test) indicated that these differences were primarily associated with rainfall-affected conditions (Events 5–6) compared to dry and moderate conditions (Events 1–4).

3.2. Quantification of MST markers and ARGs across the river-sea continuum

MST analyses revealed a clear dominance of human-associated fecal pollution along the river–sea continuum, with consistently higher concentrations in riverine waters than in marine samples (Fig. 2; values refer to mean concentrations calculated for each sampling event). Among human-associated markers, HF183 was the most prevalent and

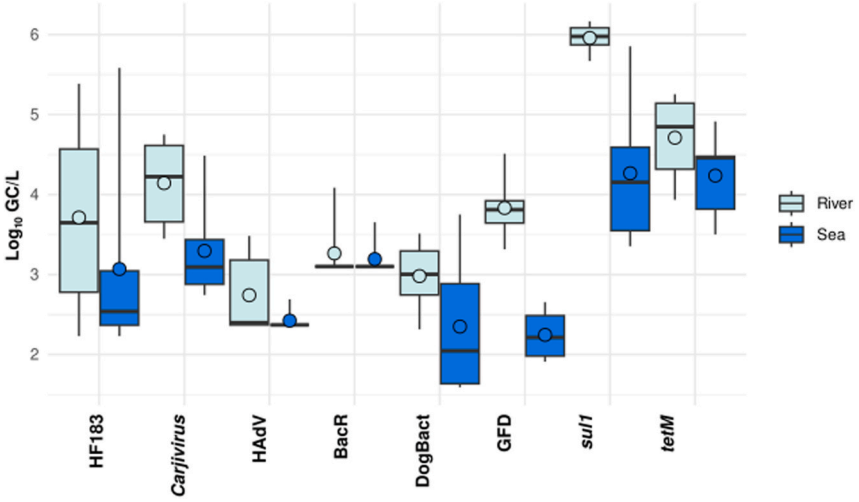

Fig. 2. Boxplots comparing the concentrations (log_{10} GC/L) of microbial source tracking (MST) markers (HF183, *Carjivirus*, HAdV, BacR, DogBact, GFD) and antibiotic resistance genes (ARGs) (*sul1, tetM)* in river and marine water samples. Boxplots represent the median and interquartile range, with dots indicating the mean. Only quantifiable data (≥LOQ) are included; assay-specific limits of quantification (LOQ) are reported in Supplementary Table S3.

abundant, with concentrations generally ranging between 2.2 and 5.4 log_{10} GC/L in riverine waters and reaching comparable levels in marine samples during extreme rainfall conditions, The highest concentrations were recorded during Event 6, when mean seawater HF183 levels approached 5.6 log_{10} GC/L. No significant differences in HF183 concentrations were observed between riverine and marine samples ($p > 0.05$; Supplementary Table S5).

Carjivirus exhibited a similar spatial pattern, with higher concentrations in river water samples (3.4–4.8 log_{10} GC/L, mean values per event) than in marine waters (2.8–4.5 log_{10} GC/L). These differences were also statistically significant ($p < 0.001$; Supplementary Table S5). Both human-associated markers were consistently detected across all riverine stations (PT3, SMA, PT2, MT and PT1; Fig. 3). HAdV was detected in both riverine and marine environments (approximately 2.3–3.5 log_{10} GC/L) and did not show significant differences between environments ($p > 0.05$).

Animal-associated MST markers showed more variable and source-specific patterns (Fig. 2). The canine marker DogBact showed mean concentrations ranging from 2.3 to 3.5 log_{10} GC/L in riverine samples and from 1.6 to 3.8 log_{10} GC/L in marine waters, with no significant differences between environments ($p > 0.05$). In contrast, the avian marker GFD showed significantly higher concentrations in riverine (mean of 3.3–4.5 log_{10} GC/L) compared to marine samples (1.9–2.7 log_{10} GC/L; $p < 0.001$). The ruminant marker BacR did not exhibit significant differences between river and marine samples ($p > 0.05$), indicating a relatively homogeneous distribution along the gradient, with occasional increases associated with specific hydrological conditions. Neither the equine marker HoF597 nor the β-lactam resistance gene $bla_{CTX\text{-}M\text{-}1}$ was detected in any sample.

Antibiotic resistance genes (*sul1* and *tetM*) were widely detected across all sampling stations and events (Fig. 2; Fig. 3). Concentrations were generally higher in riverine waters (*sul1*: approximately 5.7–6.2 log_{10} GC/L; *tetM*: 3.9–5.3 log_{10} GC/L) compared to marine samples (*sul1*: 3.3–5.9 log_{10} GC/L; *tetM*: 3.5–4.9 log_{10} GC/L). ANOVA results indicated significant differences between riverine and marine environments only for *sul1* ($p < 0.001$), whereas no significant differences were observed for *tetM* ($p > 0.05$).

Station-level comparisons showed higher ARG concentrations at upstream riverine stations, particularly PT3 and SMA, compared with marine stations ST1 and ST2 (Tukey's post-hoc test, $p < 0.05$), while no

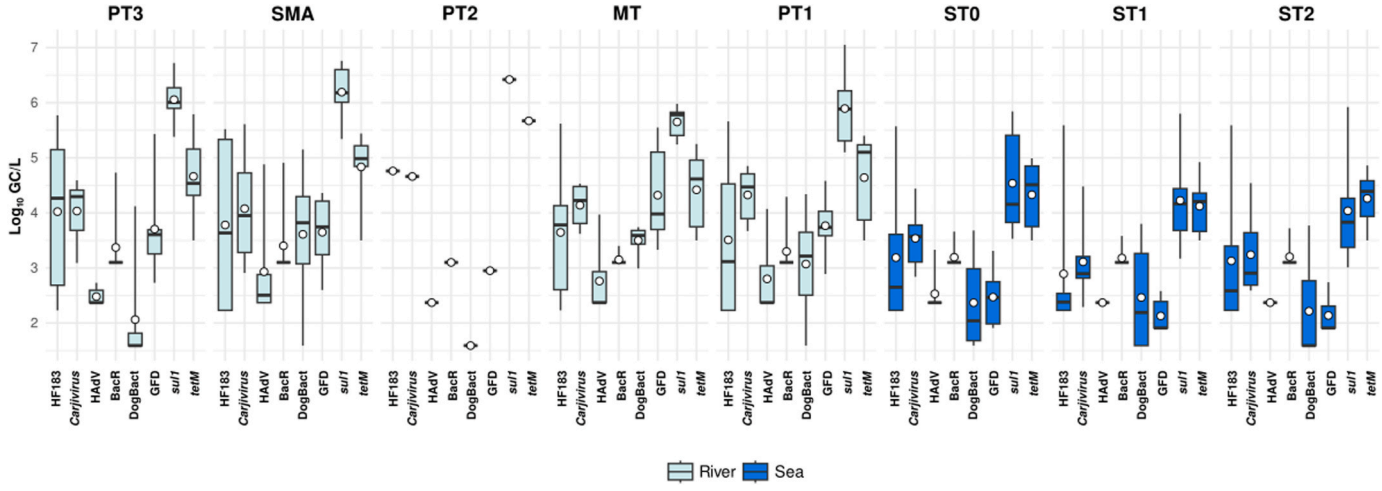

Fig. 3. Boxplots comparing the concentrations (log_{10} GC/L) of microbial source tracking (MST) markers (HF183, *Carjivirus,* HAdV, BacR, DogBact, GFD) and antibiotic resistance genes (ARGs) (*sul1, tetM*) across different sampling sites (PT3, SMA, PT2, MT, PT1, ST0, ST1, ST2) in river and marine water samples. Boxplots represent the median and interquartile range, with dots indicating the mean. Only quantifiable data (≥LOQ) are included; assay-specific limits of quantification (LOQ) are reported in Supplementary Table S3.

significant differences were observed among riverine stations. Overall, ARG concentrations were higher in riverine waters than in marine samples. During extreme rainfall events, similar concentration ranges were observed between riverine and marine samples (Fig. 3).

3.3. Distribution of MST markers and ARGs in seawater and sediment

The analysis of sediment samples showed differences compared with the overlying seawater (Fig. 4, values refer to mean concentrations calculated for each sampling event). MST markers were detected in sediments, with generally lower concentrations and reduced variability relative to seawater samples. Human-associated markers HF183 and *Carjivirus* were detected in all sediment samples, with concentrations ranging from approximately 2.2–2.9 and 2.3–3.3 $\log_{10}$ GC/g wet sediment, respectively. In seawater, both markers exhibited higher and more variable concentrations across sampling events. One-way ANOVA confirmed significantly higher concentrations in seawater compared with sediments for both markers (Supplementary Table S6). HAdV was detected in both matrices at comparable concentrations, with no significant differences between seawater and sediment samples ($p > 0.05$).

The avian marker GFD and the canine marker DogBact were detected in both matrices, with significantly higher concentrations in seawater compared to sediments ($p < 0.001$). In sediments, GFD concentrations ranged between approximately 1.9–2.0 $\log_{10}$ GC/g wet sediment), while DogBact values were generally low and showed limited variability. The ruminant marker BacR did not differ significantly between sediment and seawater samples ($p > 0.05$).

Antibiotic resistance genes displayed distinct patterns. The *sul1* gene was consistently detected in sediments (approximately 2.8–4.0 $\log_{10}$ GC/g wet sediment) and showed significantly lower concentrations compared to seawater ($p < 0.001$). Similarly, *tetM* concentrations were lower in sediments (~3.5 $\log_{10}$ GC/g wet sediment) than in seawater, with significant differences between matrices ($p < 0.001$).

3.4. Temporal variability of MST markers and ARGs across rainfall events

The temporal dynamics of MST markers and ARGs in water samples collected across six sampling events under different rainfall conditions is shown in Fig. 5. Marker concentrations varied among events, with higher variability and, increased values observed during rainfall affected events.

HF183 and *Carjivirus* were consistently detected across all sampling events (Fig. 5). HF183 concentrations ranged from approximately 2.2–5.6 $\log_{10}$ GC/L, with the highest values recorded during Event 6. Elevated HF183 concentrations were also observed during Event 1. *Carjivirus* showed a similar temporal pattern, with concentrations ranging from approximately 2.7 to 4.8 $\log_{10}$ GC/L, and higher values during Events 1 and 6. One-way ANOVA indicated significant differences among sampling events for both HF183 and *Carjivirus* ($p < 0.05$). Tukey's post-hoc test showed that Event 6 differed significantly from most other events ($p < 0.05$). In contrast, HAdV was detected in all events at moderate concentrations (~2.3–3.5 $\log_{10}$ GC/L) and showed limited variability. No significant differences among events were observed ($p > 0.05$).

Animal-associated MST markers showed variable temporal patterns. GFD marker was detected in all sampling events, with concentrations ranging from approximately 2.0 to 4.5 $\log_{10}$ GC/L. No significant differences among events were observed ($p > 0.05$). DogBact marker displayed greater variability, with elevated concentrations during Events 1 and 6. One-way ANOVA indicated significant differences among events ($p < 0.05$), and Tukey's post-hoc test identified significantly higher concentrations during Events 1 and 6 compared with other events ($p < 0.05$). BacR showed limited temporal variability, with concentrations remaining relatively stable across events and a slight increase during Event 6. No significant differences among events were detected ($p > 0.05$). The HoF597 marker and the $bla_{\text{CTX-M-1}}$ gene were not detected in any water sample.

ARGs were detected across all sampling events. The *sul1* gene showed concentrations ranging from approximately 3.3 to 6.1 $\log_{10}$ GC/L, with higher values observed during Events 2, 5, and 6. One-way ANOVA indicated significant differences among events ($p < 0.001$), and Tukey's post-hoc test showed that Event 6 differed significantly from most other events ($p < 0.05$). The *tetM* gene exhibited lower variability, with concentrations ranging from approximately 3.5 to 5.3 $\log_{10}$ GC/L. No significant differences among events were observed ($p > 0.05$).

Spearman correlation analysis showed associations among MST markers and ARGs in both river and marine samples (Supplementary Fig. S1). In river samples, a strong positive correlation was observed between HF183 and *Carjivirus* ($\rho = 0.94$, $p < 0.01$), and between *Carjivirus* and HAdV ($\rho = 0.88$, $p < 0.05$). A significant positive correlation was also detected between the ARG *tetM* and the MST marker GFD ($\rho = 0.83$, $p < 0.05$).

In marine samples, HF183 was positively correlated with DogBact ($\rho = 0.89$, $p = 0.01$), and *Carjivirus* was also positively correlated with DogBact ($\rho = 0.90$, $p < 0.01$). Positive correlations were

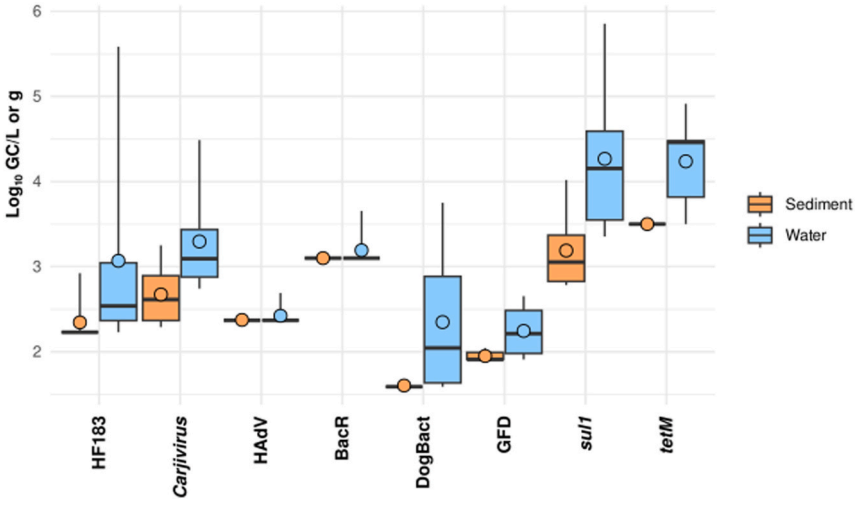

Fig. 4. Boxplots comparing the concentrations ($\log_{10}$ GC/L or g wet sediment) of microbial source tracking (MST) markers (HF183, *Carjivirus*, HAdV, BacR, DogBact, GFD) and antibiotic resistance genes (ARGs) (*sul1, tetM*) in sediment and water samples. Boxplots represent the median and interquartile range, with dots indicating the mean. Only quantifiable data (≥LOQ) are included; assay-specific limits of quantification (LOQ) are reported in Supplementary Table S3.

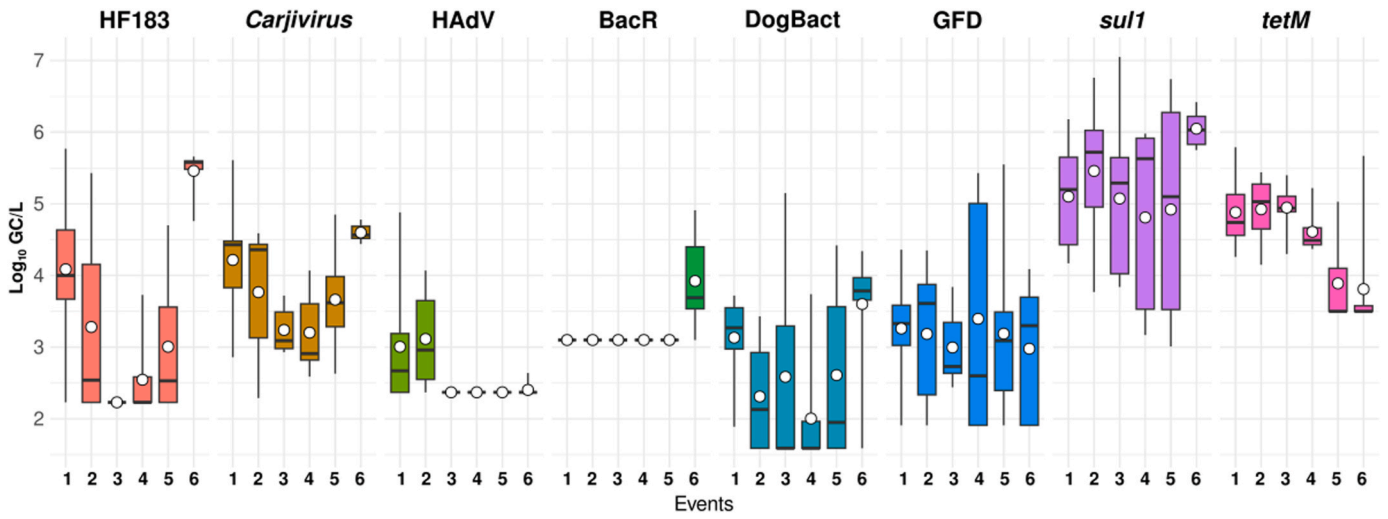

Fig. 5. Boxplots showing the concentrations (log_{10} GC/L) of microbial source tracking (MST) markers (HF183, *Carjivirus*, HAdV, BacR, DogBact, GFD) and antibiotic resistance genes (ARGs) (*sul1, tetM*) across different sampling events (1–6) in water samples. Boxplots represent the median and interquartile range, with dots indicating the mean. Sampling events were classified according to rainfall conditions as follows: Event 1 = moderate rainfall, Event 2 = light rainfall, Event 3 = moderate rainfall, Event 4 = dry weather, Event 5 = heavy rainfall, and Event 6 = extreme rainfall. Only quantifiable data (≥LOQ) are included; assay-specific limits of quantification (LOQ) are reported in Supplementary Table S3.

also observed between *sul1* and several MST markers, particularly HF183 ($\rho = 0.60$); however, these associations were not statistically significant ($p > 0.05$).

4. Discussion

Understanding the origin and dynamics of fecal pollution is essential for the effective management of coastal watersheds exposed to multiple anthropogenic and environmental pressures. In this study, an integrated approach combining host-associated MST markers and ARGs was used to identify pollution sources and to evaluate the spatial distribution and event-driven temporal variability along the river–to–sea continuum of a Mediterranean watershed under contrasting rainfall conditions. The combined analysis of water and sediment matrices allowed the identification of both short-term contamination pulses and potential longer-term environmental reservoirs. The MST approach has been increasingly applied in European and Mediterranean contexts to discriminate fecal pollution sources in rivers, lagoons, and coastal waters (Ballesté et al., 2020; Ndione et al., 2021; Toubiana et al., 2021; Valério et al., 2022). These studies have demonstrated the reliability of host-associated markers such as HF183 and GFD under the variable salinity, temperature, and rainfall regimes typical of the Mediterranean region. However, the complex hydrological connectivity between land and sea, coupled with episodic extreme rainfall, still limits our understanding of how fecal pollution and ARGs behave in small coastal catchments. The present study expands the application of MST in Mediterranean coastal watersheds represents one of the first integrated assessments combining host-associated MST and ARGs across a Mediterranean river-to-sea continuum.

4.1. Influence of rainfall and hydrological variability on MST and ARG dynamics

The present research identified human sewage as the dominant source of fecal pollution throughout the Arzilla catchment and adjacent coastal waters, with animal sources contributed locally and episodically. The consistent detection of human-associated MST markers (HF183, *Carjivirus* and HAdV) at both riverine and marine samples reflects the influence of wastewater discharges and sewer overflows typical of highly urbanized Mediterranean catchments. The Arzilla catchment is characterized by a small, highly responsive watershed draining mixed urban and agricultural areas before discharging directly into a heavily used recreational coastal zone. This strong land–sea connectivity favours the rapid transfer of fecal contamination and ARGs from inland sources to bathing waters during rainfall events.

The spatial distribution of human-associated markers revealed pronounced pollution hotspots at the upstream stations SMA and PT3, reflecting chronic sewage inputs typical of small Mediterranean catchments (Freddi et al., 2026; Rusiñol et al., 2014). Although HF183 concentrations exceeded 5 log_{10} GC/L mainly during the extreme rainfall event, elevated levels were also observed across multiple sampling events, indicating persistent human fecal inputs (Green et al., 2012; Stachler et al., 2017). The MST markers used in this study have been widely validated in Mediterranean environments and exhibit high host specificity and reliability for source discrimination (Di Cesare et al., 2017; Rusiñol et al., 2014; Yahya et al., 2017). Previous QMRA studies have proposed risk-based thresholds (RBTs) for HF183 concentrations in recreational waters. Although a formal QMRA was beyond the scope of the present study, a quantitative comparison with QMRA based thresholds reported in the literature indicates that HF183 concentrations observed during rainfall-driven events fall within ranges previously associated with elevated health risk in coastal bathing waters, underscoring the potential public health relevance of rainfall-induced fecal contamination (Ahmed et al., 2018, 2024).

In addition to human derived inputs, MST analyses indicated the presence of fecal contamination from animal sources. The avian marker GFD was consistently detected in riverine samples and at lower concentrations in marine waters, reflecting diffuse inputs likely associated with bird populations along riverbanks and coastal areas (Green et al., 2012, 2014). The canine marker DogBact was also detected across sites, with higher values at urban-influenced locations, suggesting runoff-driven mobilization of pet waste and localized inputs from domestic animals (Dick et al., 2005; Li et al., 2022). The ruminant marker (BacR) showed limited spatial variability but increased during the extreme rainfall event, indicating episodic inputs under high-flow conditions (Boehm et al., 2013; Valério et al., 2022). The equine marker HoF597 was not detected. The presence of horses in the catchment has been reported in the areas surrounding the sampling sites. However, the non-detection of the equine marker HoF597 likely reflects methodological limitations rather than a true absence of equine fecal inputs. HoF597 is one of the few currently available equine-specific molecular markers, and its sensitivity in environmental waters may be limited

under conditions of dilution, low fecal input, or DNA degradation (Gray et al., 2020). Rainfall emerged as a key driver of temporal variability, influencing both MST markers and ARG concentrations. Even moderate precipitation events (>5 mm) were associated with increased marker levels in river waters, whereas extreme rainfall (>190 mm) produced pronounced increases that were also detected in coastal samples.

The occurrence of HF183 at marine waters during high-flow conditions reflects the transfer of contamination from inland sources to the coastal zone, as previously reported in Mediterranean systems (Garbossa et al., 2017; Green et al., 2012; Stachler et al., 2017). The rapid response observed in the coastal stations reflects the short hydrological residence time of the Arzilla catchment, where contaminants mobilized from upstream urban areas can be transported to the river mouth and adjacent bathing waters within a relatively short period following intense rainfall.

ARGs showed a clear response to hydrological variability, particularly for *sul1*, which was the most abundant and persistent ARG. The similar temporal patterns observed for HF183 and *sul1* support that urban wastewater is a major contributor to ARG dissemination, in agreement with previous studies linking *sul1* to anthropogenic pollution and sewage-derived inputs (Di Cesare et al., 2017; Karkman et al., 2018). Overall, the combined behaviour of MST markers and ARGs across the river–sea continuum indicates that wastewater-derived contamination is rapidly transported during high-flow events, whereas animal-associated markers reflect more localized or diffuse sources linked to land use and runoff dynamics.

Although the positive correlation between HF183 and *sul1* in marine samples was not statistically significant, their similar temporal trends further suggest an influence of urban wastewater on ARG occurrence. Moreover, the strong associations among the human-associated MST markers HF183, *Carjivirus* and HAdV in river samples indicate a common sewage origin, further supporting their reliability for tracking human fecal contamination. The significant association between *tetM* and the avian marker GFD in river samples may indicate the simultaneous occurrence of bird-derived fecal contamination and tetracycline resistance genes under specific environmental conditions, although further investigation is needed to clarify this relationship. Likewise, the positive correlations observed between DogBact and both HF183 and *Carjivirus* in marine waters reflect the presence of multiple fecal sources in urban coastal environments rather than a direct biological relationship among these markers.

In contrast, *tetM* showed lower temporal variability and remained relatively abundant under drought conditions, suggesting a more stable environmental distribution. This pattern may indicate that *tetM* is not exclusively associated with recent fecal inputs but may also reflect longer-term environmental persistence, as previously reported in riverine environments (Brown et al., 2020; Reichert et al., 2021). In addition, the persistence of *tetM* in sediments may provide an environmental reservoir contributing to its detection under dry conditions (Brown et al., 2020). Therefore, the comparatively high *tetM* abundance observed during drought may reflect long-term retention and legacy contamination rather than rainfall-driven fecal inputs alone.

Taken together, these findings indicate that rainfall is a main hydrological driver controlling the mobilization, transport, and redistribution of fecal contamination and ARGs across the watershed. Similar responses have been reported in other coastal environments, where stormwater runoff and non-point sources substantially increase microbial contamination (Shrestha et al., 2020). Under projected climate scenarios for the Mediterranean region, characterized by more frequent and intense precipitation events, these processes are expected to further challenge coastal water quality management and public health protection (IPCC, 2021; EEA, 2022).

4.2. Water–sediment interactions and persistence of fecal pollution and ARGs

The comparison between water and sediment matrices revealed distinct but complementary patterns. Water acted as the primary transport medium for fecal pollution and ARGs, showing higher temporal variability and rapid responses to rainfall events. In contrast, sediments represented a more stable compartment, characterized by lower MST marker concentrations and reduced temporal variability. Human-associated markers were consistently detected in sediments at relatively low concentrations, indicating limited accumulation under normal hydrological conditions. Their occurrence during high-flow events suggests that rainfall may enhance the transfer of contaminated particles from the water column to the benthic compartment through resuspension and settling processes, consistent with observations reported in Mediterranean systems (Sabater et al., 2011).

Marine sediments are widely recognized as major repositories of microorganisms and extracellular genetic material, providing environmental conditions that favour the accumulation and preservation of biological material (Corinaldesi et al., 2008). In agreement with this ecological role, sediments have also been described as long-term reservoirs for fecal-associated genetic markers (Perkins et al., 2014). However, the relatively low concentrations and limited accumulation of MST markers observed in the present study, compared with ARGs, suggest a lower degree of accumulation under the investigated conditions, which may reflect marker-specific characteristics, environmental behaviour, and hydrological conditions.

Interestingly, *Carjivirus* concentrations in sediments were higher than those of HF183. Although the mechanisms underlying this pattern cannot be determined from the present study, they may reflect differences in the environmental behaviour of viral and bacterial host-associated markers, including particle association, transport and persistence. Viral genetic markers may exhibit greater retention in particle-rich matrices such as sediments, where adsorption to suspended particles and reduced exposure to environmental degradation processes may favour their persistence. Previous studies have highlighted the high environmental prevalence and persistence of crAssphage (*Carjivirus*) in wastewater and aquatic environments, supporting its suitability as a robust marker of human fecal contamination (Greaves et al., 2020; Sabar et al., 2022; Ahmed et al., 2024). Accordingly, the higher concentrations of *Carjivirus* than HF183 observed in our sediment samples are consistent with these differences in environmental persistence and particle association. By contrast, ARGs showed a different environmental behaviour. Their widespread detection and persistence of *sul1* and *tetM* in sediments in sediments, including during dry conditions, indicates greater environmental persistence and potential long-term retention than observed for MST markers.

This behavior is consistent with previous studies reporting that ARGs can persist in sediments as extracellular DNA or within resilient microbial communities, allowing their retention and potential remobilization during resuspension events (Larsson and Flach, 2022).

The absence of the *blaCTX-M-1* gene across all matrices suggested limited occurrence of extended-spectrum β-lactamase genes in the study area. By contrast, HAdV exhibited limited spatial and temporal variability and was not detected at elevated concentrations in sediments, indicating limited accumulation in the benthic compartment under the investigated conditions.

The observed differences between water and sediment matrices highlighted the importance of integrated multi-matrix approaches combining MST and ARG analyses to provide a more comprehensive characterization of fecal pollution dynamics and antibiotic resistance dissemination in coastal environments.

4.3. Implications for coastal water quality management

The results of this study have important implications for coastal

water quality management in Mediterranean catchments. The strong influence of rainfall on fecal contamination and ARG dynamics highlights the need to incorporate hydrological variability into monitoring frameworks, particularly in systems characterized by rapid land–sea connectivity.

The consistent detection of human-associated markers and their co-occurrence with ARGs suggest that urban wastewater infrastructure represents a critical control point for reducing contamination and associated health risks. In small Mediterranean catchments such as the Arzilla, characterized by short flow paths and rapid hydrological responses, even moderate rainfall events may rapidly compromise the microbiological quality of adjacent bathing waters.

In this context, integrating MST and ARG monitoring into routine bathing-water assessment could improve source identification and support targeted mitigation strategies.

The sensitivity of fecal pollution to rainfall events suggests that early-warning systems based on precipitation thresholds may enhance the management of recreational waters, particularly during high-risk periods. Under projected climate-change scenarios characterized by increasing frequency and intensity of extreme rainfall events in the Mediterranean region, contamination episodes are expected to become more frequent, posing additional challenges for water quality management and public health protection.

5. Conclusions

The integrated application of host associated MST markers and ARGs provided a comprehensive assessment of fecal pollution sources, their transport along the river–coastal continuum of the Arzilla catchment, and their association with antibiotic resistance, improving our understanding of contamination dynamics in Mediterranean land–sea systems.

Human sewage was identified as the dominant source of fecal contamination, while rainfall emerged as a major hydrological driver controlling the mobilization and transport of both fecal indicators and ARGs from inland waters to the coastal environment. The co-occurrence of MST markers and ARGs highlights the relationship between fecal pollution and antimicrobial resistance, with potential implications for recreational water quality and public health. Differences between water and sediment matrices further emphasize the importance of multi-compartment assessment approaches to distinguish short-term contamination events from longer-term environmental persistence.

Although this study was conducted in a single Mediterranean catchment and included a limited number of sampling events, it provides a robust framework for investigating fecal pollution dynamics under contrasting hydrological conditions. Future studies integrating higher temporal resolution sampling and more detailed land-use characterization, including quantitative information on livestock density and animal distribution, would further improve source attribution and support targeted management strategies.

These findings demonstrate the value of integrating MST and ARG analyses into routine water quality programs to strengthen evidence-based management of Mediterranean coastal waters under increasing anthropogenic pressure and climate-driven hydrological extremes.

CRediT authorship contribution statement

Angela Freddi: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marco Basili:** Writing – original draft, Formal analysis, Data curation. **Antonella Penna:** Writing – review & editing, Writing – original draft, Conceptualization. **Silvia Casabianca:** Writing – original draft. **Ben J. Tscharke:** Formal analysis. **Metasebia Gebrewold:** Methodology, Formal analysis, Data curation. **Stuart L. Simpson:** Writing – original draft. **Elena Manini:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Warish Ahmed:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2026.130866.

Data availability

Data will be made available on request.

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