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Metagenomic profiling reveals distinct signatures of pathogens, antibiotic-resistance genes and human viruses in urban river mouths of the north-western Adriatic coast

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Coastal ecosystems are increasingly threatened by microbiological risk due to urban wastewater discharges, which might affect public health and have important economic consequences on the blue tourism. Here, we examine the changes in water and sediment microbiomes at the mouths of three urban-draining rivers (Marecchia, Marano, Rio Melo) and at the Santa Giustina wastewater treatment plant, situated in one of the most densely urbanized and touristic areas of the Adriatic Sea. During the peak summer season, water and sediment samples were analysed through 16 S rRNA metabarcoding and shotgun metagenomics to identify the presence of pathogenic bacteria, human viruses, and antibiotic resistance genes (ARGs). Results revealed that impacted river mouths hosted distinct microbial fingerprints, with seawater showing higher levels of pathogenic bacteria (including *Vibrio*, *Enterococcus*, *Escherichia-Shigella*, and *Streptococcus*) than sediments. Several human viruses of risk groups 2 and 4, such as Adenoviridae, Herpesviridae, Papillomaviridae, Poxviridae, were detected, along with 99 ARGs, 82 of which were classified by the World Health Organization (WHO) as critically important. Our data suggest the persistence of pathogens in treated effluents and reveal a specific combination of bacterial taxa, viruses, and ARGs, with site-specific profiles. Overall, our findings underscore the need for systematic genomic-based monitoring to safeguard bathing water quality and mitigate risks to human and environmental health, in line with One Health principles.

Keywords Coastal environments, One Health, Metagenomics, Pathogens, Antibiotic resistance genes, Human virus

Coastal marine ecosystems are among the most productive and fragile ecosystems on Earth, providing essential services from an ecological and socio-economic point of view[1–5]. Here, anthropogenic stressors are threatening biodiversity and ecosystem functioning, with urban wastewater discharge representing a major concern due to the massive release of pathogenic microorganisms[6–10]. Carried through riverine systems that channel municipal sewage, surface runoff, and urban drainage, these microorganisms are ultimately released into marine environments, where they pose serious risks for public health and recreational water quality, particularly in densely populated coastal areas of high relevance for blue tourism[8,9,11–14]. Microbiological

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hazards can arise from multiple, interacting risk factors, including the presence of diverse pathogenic taxa, human viruses and antibiotic-resistant genes[15,16]. These microbiological risks are particularly acute along the North-Western Adriatic Coast, which represents one of the most densely urbanized regions of the Adriatic Sea within the Mediterranean basin[8,17–21]. In particular, the coastal area of the Emilia-Romagna region in Italy is characterized by a nearly continuous urban development, with minimal natural or undeveloped areas separating the municipalities. The region experiences high demographic pressure year-round, with a resident population that is significantly amplified during the summer months by intense tourism[19,22,23]. In particular, Rimini is a major hub for domestic and international tourism, featuring hundreds of hotels, restaurants, and recreational facilities concentrated along the beachfront. During the peak season, the temporary population can exceed several times the number of permanent residents, reaching nearly 800,000[24], placing a considerable burden on local infrastructure, including wastewater management systems and coastal water quality. This combination of dense urbanization, high population density, and intense seasonal tourism makes the coastal area of province of Rimini as paradigmatic example of anthropogenic pressure on the coastal environment in the Adriatic region, particularly in term of combined microbiological risks[20,25]. In the present study we profiled metagenomes at the river mouths of 3 urban draining rivers along the coastal zone between Rimini and Riccione - a municipality in the Province of Rimini with about 30,000 inhabitants - during the summer peak tourism season. The Santa Giustina Sewage Treatment Plant (STP) drain, in Rimini, was also sampled. Metagenomes were assessed using a combined metabarcoding and shotgun metagenomics approach, which was applied to water and sediment samples. This approach allowed the identification of pathogenic bacteria, human viruses, and antibiotic resistance genes (ARGs), the latter highlighted by the World Health Organization (WHO) as a pressing emerging issue[26]. Overall, our results provide new insights into the microbiome quality of coastal waters during the tourist season and demonstrate the suitability and effectiveness of combining molecular approaches to detect pathogenic bacteria, human viruses, and ARGs. These findings should inform future systematic monitoring programs aimed at assessing marine microbiome health in recreational waters and reducing risks to both human and environmental health.

Materials and methods

Study area and samples collection

The sampling was carried out on July 4th, 2024, at 6 sites along a 25-km coastal stretch between Rimini and Riccione (Emilia-Romagna region, Italy). Four impacted sites were selected, including the outlets of three urban-draining rivers and the main municipal STP. From North to South: (i) the Marecchia River (44° 4.94′ N; 12° 34.09′ E), the main river of Rimini, whose current mouth is located north of the city; (ii) the Marano Torrent (44° 1.49′ N; 12° 38.83′ E), a stream in the municipality of Riccione discharging into the sea at Spontricciolo; (iii) the Rio Melo River (44° 0.70′ N; 12° 39.77′ E), a minor river flowing into the port of Riccione; and (iv) the outlet of the Rimini STP (44° 4.79′ N; 12° 35.41′ E). Two control sites were selected to establish reference baseline conditions. These sites were located to the north and south of the study area, beyond the direct influence of river mouths and major sewage discharges: Viserbella (Rimini) as the northern control site (44° 6.21′ N; 12° 31.56′ E), and the WMesh area, near the concrete wave-dissipation barriers at the boundary between Riccione and Misano Adriatico, as the southern control site (43° 59.32′ N; 12° 41.42′ E). The selection of control sites was guided by hydrodynamic considerations and by the absence of proximal discharge points, with the aim of characterizing background coastal microbiome conditions within the same geographic and seasonal framework as the impacted sites. An overview of the sampling sites is provided in Fig. 1. The study area is located in the Northwestern Adriatic Sea, where riverine discharge, primarily from the Po River, generates a buoyant coastal current known as the Western Coastal Layer, flowing southward along the Italian coast. During autumn and winter, the development of a coastal thermohaline front retains freshwater inputs largely within the nearshore zone. In spring, seasonal warming reduces surface water density and leads to thermocline formation. Consequently, during late spring and summer, the water column becomes strongly stratified into three main layers: a low-density surface layer influenced by river runoff, an intermediate layer, and a denser bottom layer composed of winter waters or deeper Adriatic waters. Under stratified conditions, surface waters originating from the coastal zone can extend offshore toward the central basin[27]. The environmental variables measured at sampling, including pressure, depth, oxygen % saturation, dissolved oxygen, salinity, conductivity, pH, fluorescence turbidity and temperature, are reported in Supplementary Table S1. Sampling was conducted by boat at the 4 impacted sites and at the 2 control sites during the same day. At each impacted site, duplicate seawater samples (1 L each) and one sediment sample were collected at distances of 100 m, 300 m, and 500 m from the river mouth, and from the STP outlet. At the control sites, duplicate seawater samples (1 L each) and one sediment sample were collected 500 m offshore from the coastline. Seawater samples were taken at an approximate depth of 3 m, while sediment samples were collected using a Van Veen grab. All samples were stored in cooled containers (+ 4 °C) during transport to the laboratory, where they were immediately processed as described below.

Samples preparation and DNA extraction

Seawater samples were filtered in two steps, using a 1.2-µm and 0.22-µm pore size MF-Millipore membrane filters (Merck, Darmstadt, Germany) with a vacuum pump[28–30]. Microbial DNA was extracted from the entire membrane filters using the DNeasy PowerWater extraction kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. For sediments, 250 mg of each sample were weighed, and DNA was extracted with the DNeasy PowerSoil kit (QIAGEN) according to the manufacturer's instructions with minor adjustments[27]. Specifically, all samples were homogenized using the FastPrep instrument (MP Biomedicals, Irvine, CA) at 5.5 movements/s for 1 min, repeated for three cycles, and the elution step was preceded by a 5-min incubation at 4 °C[31,32]. All the extracted DNA was then quantified using a NanoDrop ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE) and stored at − 20 °C until further processing.

Fig. 1. Geolocation of sampling sites. Impacted (Marecchia, STP, Marano and Rio Melo) and control (North, South) sites are shown in red and blue, respectively.

16S rRNA gene amplification and Illumina sequencing

For all sediment and water samples, the V3–V4 hypervariable region of the 16S rRNA gene was PCR amplified in a 50µL reaction containing 25 ng of microbial DNA, 2X KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland), and 200 nmol/L of 341F (S-D-Bact-0341-b-S-17, 5′-CCTACGGGNGGCWGCAG-3′) and 785R (S-D-Bact-0785-a-A-21, 5′-GACTACHVGGGTATCTAATCC-3') primers carrying Illumina overhang sequencing adapters[33]. The thermal cycle consisted of 3 min at 95 °C, 25 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, and a final elongation step of 5 min at 72 °C[34]. PCR products were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA). Indexed libraries were prepared using limited-cycle PCR with Nextera technology and cleaned up. Libraries were normalized to 4 nM and pooled. The sample pool was denatured with 0.2 N NaOH and diluted to a final concentration of 4.5 pM with a 20% PhiX control. Sequencing was performed on an Illumina MiSeq platform using a 2 × 250 bp paired-end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA).

Shotgun metagenomic sequencing

Seawater samples were pooled for each site, resulting in a total of 5 samples: Marecchia, Marano, Rio Melo, STP, and control. The QIAseq FX DNA library kit (QIAGEN) was used for library preparation, according to the manufacturer's instructions. Briefly, 450-bp size, end-repaired and A-tailed fragments were generated through fragmentation of 10 ng DNA of each sample using the FX enzyme mix with the following thermal cycle: 4 °C for 1 min, 32 °C for 14 min and 65 °C for 30 min. The obtained fragments were then incubated at 20 °C for 15 min to perform adapter ligation in the presence of DNA ligase and Illumina adapter barcodes. Following, a first purification step with Agencourt AMPure XP magnetic beads (Beckman Coulter), library amplification with a 10-cycle PCR amplification and a further step of purification were performed. Libraries were pooled at an equimolar concentration (4 nM) to obtain the final pool, which was sequenced on an Illumina NextSeq550 platform using a 2 × 150 bp paired-end protocol, following the manufacturer's instructions (Illumina).

Bioinformatics and biostatistics

For 16 S rRNA gene analysis, raw sequences were processed using a pipeline combining PANDAseq[35] and QIIME 2[36]. High-quality reads were retained using the "fastq filter" function of the Usearch11 algorithm[37], then clustered for 97% similarity into Operational Taxonomic Units (OTUs). Taxonomy was assigned using the VSEARCH classifier[38] and the SILVA database as a reference[39]. All statistical analyses were performed using the R software (R Core Team; www.r-project.org - last access: July 2025), v. 4.3.2, with the packages vegan (version 2.6-2,[40], made4 (version 1.78.0,[41]), pairwiseAdonis (version 0.4.1,[42]), RColorBrewer (version 1.1-3,[43], ggplot2[44], RcppAlgos (version 2.9.3,[45]), cluster (version 2.1.6,[46], matrixStats (version 1.5.0,[47]), pheatmap[48], xlsx (version 0.6.5,[49], dplyr[50], tibble[51] and TIDYR[52]. β-diversity was estimated by computing Unweighted and Weighted UniFrac distance, and the data separation in the principal coordinate analysis (PCoA) was tested using a permutation test with pseudo-F ratios (function "adonis" in the vegan package). α-diversity was assessed using different metrics, namely Faith's Phylogenetic Diversity (PD whole tree), number of observed OTUs, and Shannon index. Kruskal–Wallis tests followed by post-hoc Wilcoxon rank-sum tests were used to assess significant differences in α-diversity. P-values were corrected for multiple testing with the "p.adjust" function in R, with a false discovery rate (FDR) ≤ 0.05 considered statistically significant. The exclusive OTUs to the

control samples with respect to the impacts and conversely were obtained. The number of reads associated with these OTUs, defined as absolute abundance, was scaled on a logarithmic scale (Log10) and used to produce heatmaps by means of the heatmap.2 function in R (gplot package, v. 3.2.0,[53]). OTUs networks were created using Cytoscape (v3.10.3, https://cytoscape.org/). OTUs were subjected to statistical testing to observe the correlation with sampling distance (100, 300, or 500 m). Pearson's correlation test was performed on the variables distance and taxonomic composition to verify any positive or negative linear relationship. For shotgun metagenomic analysis, raw reads were processed with *fastp* version 0.23.4 for quality control, adapter trimming, and deduplication, using the following parameters: --detect_adapter_for_pe --cut_tail -c -D. The resulting reads were then used to identify ARGs within each sample using the Resistance Gene Identifier (RGI, version 6.0.2) with the 'bwt' module for read mapping against the CARD database (version 4.0.0). ARG hits with a mapping quality (MAPQ) score lower than 30 or supported by only a single mapped read were discarded. The resulting aligned reads of the selected ARGs were normalized to express the abundance in RPKM (reads per kilobase of gene per million mapped reads) for each sample using the following formula:

$$\frac{Total\ reads\ mapped\ to\ gene}{Total\ reads * Mean\ gene\ length} * 10^9$$

RPKM values were computed for each ARGs in each sample and used to create graphical representations. A heatmap representing the abundance of the ARGs was generated using heatmap.2 function of the gplots package (v. 3.2.0)[53] to highlight their distribution patterns. Logaritmic scale was chosen to visualize evenly the small differences.

Metagenomes were also processed using the ViromeScan tool[54], which allows the user to taxonomically characterize the virome directly from metagenomic reads. In particular, we selected the option '-d human_DNA' to detect only DNA viruses for which humans are the natural host. To standardize viral abundance across samples with varying sequencing depths, a multi-step normalization and validation pipeline was implemented, ensuring that the reported viral diversity is corrected for library size and filtered against stochastic background noise. Raw viral counts (Counts Per Sample, CPS, see Supplementary Table S3) were normalized against the total number of raw metagenomic reads. For each library, the analytical sensitivity threshold was defined as the CPM (Counts Per Million) equivalent of a single sequencing read, representing the sample-specific limit of detection. Finally, a binary presence/absence matrix was generated; a viral species was scored as present (1) only if its normalized abundance met or exceeded the specific analytical sensitivity of the sample.

Heatmaps of the presence/absence of viral DNA were created using the heatmap.2 function in R (gplots package, v. 3.2.0,[53]). Bacteria and viruses were categorised into three risk groups according to the *Directive 2000/54/EC* dealing with the protection of workers from risks related to exposure to biological agents at work (EC, 2000). Biological agents were subdivided into three risk groups: group (1) biological agent unlikely to cause illness in human subjects; group (2) biological agent that can cause illness in human subjects and poses a risk to workers; unlikely to spread in the community; effective prophylactic or therapeutic measures are usually available; group (3) biological agent that can cause severe disease in human subjects and poses a serious risk to workers; the biological agent may propagate in the community, but effective prophylactic or therapeutic measures are usually available. For viruses, the fourth risk group was also used, i.e., a biological agent that can cause serious illness in humans, presenting a high risk of spread in the community in the absence of effective prophylactic or therapeutic measures. For bacteria or viruses in which species with different risk groups are associated with the same genus, the intermediate risk groups indicated as 2 or 3 was used. ARGs were classified as "critically important" or "highly important" for human health following WHO revision[26].

Results

Seawater and sediment microbiomes in the study area

A total of 40 paired seawater and sediment samples were successfully sequenced using 16 S rRNA metabarcoding. From each impacted site, including riverine locations and STP drain, samples were collected at distances of 100, 300, and 500 m from the source of impact. At each distance, two replicates seawater samples and one paired sediment sample were sequenced. For each control reference site, two replicate seawater samples and one paired sediment sample were collected at 500 m and sequenced. A total of 442,952 high-quality reads were obtained (11,073 ± 3,776 per samples), binned into 753 OTUs, with rarefaction curves reaching the plateau for all the samples analysed (Supplementary Fig. S1). The α- and β- diversity analyses revealed clear differences between seawater and sediment microbiomes (Supplementary Fig. S2A and B, respectively), with seawater samples exhibiting higher α-diversity. No significant differences were detected when comparing microbiomes from impacted versus control sites, either in seawater (Supplementary Fig. S3A and B) or sediment (Supplementary Fig. S3C and D). To evaluate the degree of microbiome connectivity across paired seawater and sediment samples from the same site and between different sites, we generated shared OTU plots (Supplementary Figs. S4, S5, and S6). Notably, different OTUs were shared among seawater microbiomes across different sites, whereas the overlap between paired seawater and sediment microbiomes from the same site was considerably lower. Specifically, seawater samples shared an average of 9 OTUs between any two sites and 5 OTUs across all sites. In contrast, paired seawater and sediment samples shared a mean of only 3 OTUs. Finally, for each site, for both sediment and seawater microbiomes, OTUs showing a correlation with the sampling distance from the coast were identified (Supplementary Table S2). In the seawater microbiomes, 2 to 4 OTUs showed a positive correlation with distance from the impact source at the Marano, Rio Melo, and STP sites. At the Marecchia site, on the other hand, distance-related OTUs were 19. Conversely, in the sediment microbiomes, the number of OTUs that correlated with distance ranged from 0 to 3, depending on the site.

Specificities of the seawater and sediment microbiomes at impacted and control sites

To identify the specificities of the seawater and sediment microbiomes at the impacted and control sites, we first evaluated the correspondent γ-diversity of OTUs and then identified those that were exclusive to the impacted or control conditions, as well as those shared between the two (core OTUs). Each identified OTU was assigned to the corresponding phylogeny and the respective risk group, according to the Directive 2000/54/EC[55]. The site-specific distribution of OTUs exclusive to either impacted or control conditions - stratified by risk group - is shown as heatmap in Figs. 2 and 3 for seawater and sediment microbiomes, respectively. Interestingly, for the seawater microbiome, each riverine impacted site displayed a distinct pattern of exclusive OTUs belonging to risk groups 2 or 3, whereas no OTUs from these risk groups were exclusively detected in the control sites (Fig. 2A–C). Specifically, for the Marecchia site, the detected OTUs in the seawater microbiome belonging to risk groups 2 or 3 were: *Vibrio*, *Legionella*, *Prevotella*, *Streptococcus*, *Mycobacterium*, *Coxiella*, *Bacteroides*, *Escherichia-Shigella*; for Rio Melo: *Vibrio*, *Streptococcus*, *Francisella*, *Prevotella*, *Enterococcus*, *Coxiella*, *Chlamydia*, *Bacteroides*; and for Marano: *Vibrio* and *Coxiella*. Strikingly, the STP site harboured only a single OTU in the seawater microbiome assigned to risk group 2: *Vibrio*. Conversely, for the sediment microbiome, no OTUs assigned to the risk group 3 were detected in any site (Fig. 3A–C). Concerning the risk group 2, 3 different OTUs assigned to *Flavobacterium* and *Vibrio* were detected at Marecchia and at Marano and Rio Melo sites. Finally, among the core OTUs – i.e., those shared between impacted and control sites – none of them belonged to risk group 3. Only 4 OTUs assigned to *Vibrio* and *Flavobacterium* (risk group 2) were observed overall within the core seawater and sediment microbiomes (Fig. 4A and B). Moreover, no OTUs assigned to risk groups 2 or 3 showed any correlation with the distance from the impact sources (Supplementary Table S2).

Distribution of human viral DNA and antibiotic-resistance genes at the impacted and control sites

To investigate the presence of human viruses and ARGs, shotgun metagenomic sequencing was performed on pooled seawater samples from each impacted and control site. While pooling enables the generation of robust composite site-level metagenomic profiles, it precludes assessment of within-site variability and limits the analysis to descriptive, rather than statistically comparative, interpretations. Overall, 239.9 million high-

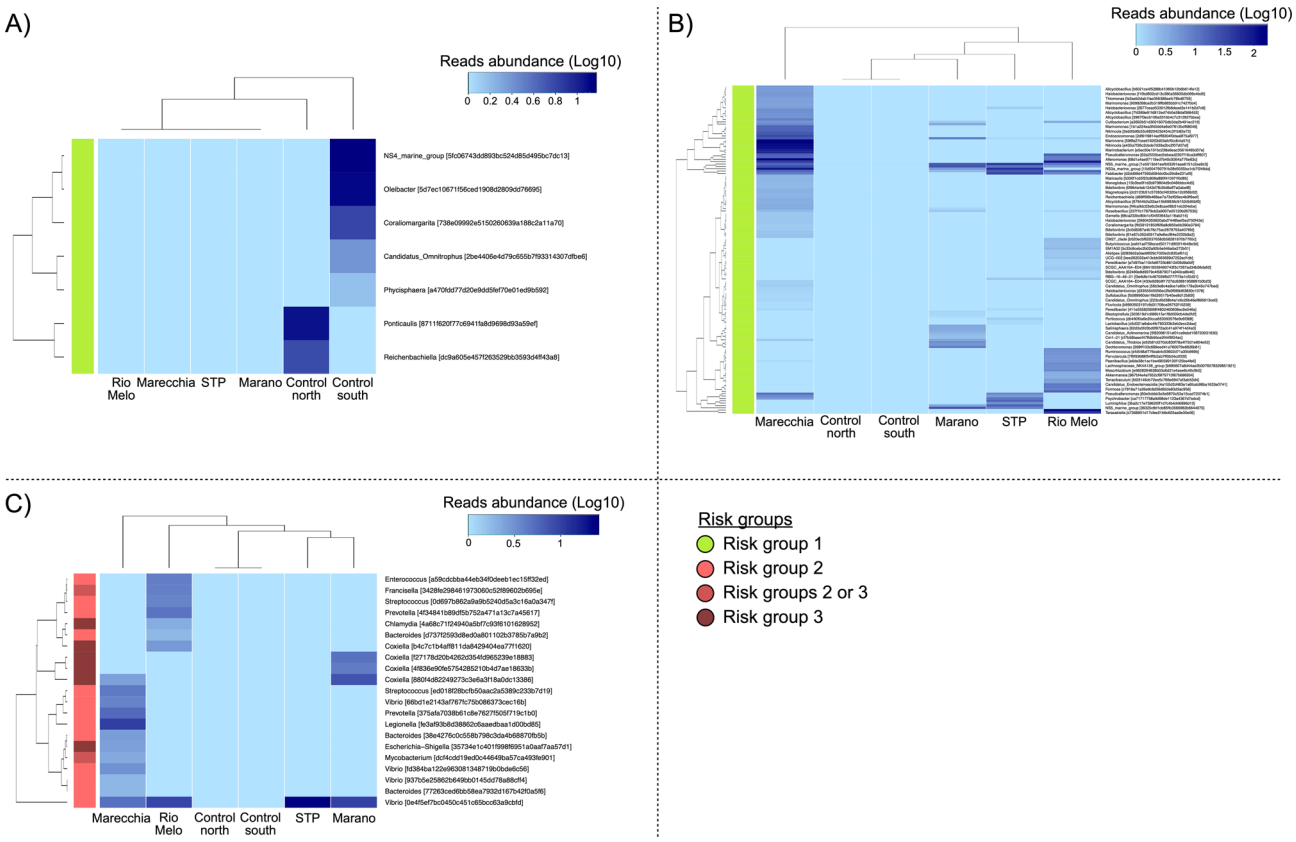

Fig. 2. Exclusive taxa to impacted or control conditions for seawater samples, divided by risk group. Heatmaps showing the taxa of seawater samples that were exclusive to control conditions (**A**), or to impacted conditions for risk group 1 (**B**) and risk groups 2–3 (**C**). On the rows, unique code OTUs are reported, correlated by the assigned taxa at genus level. The colour scale is based on the Log10 of absolute abundance of the reads assigned for a specific OTU in the sample, represented in the columns. Clustering in both in rows and columns is based on Euclidean distance. The sidebar identifies the risk group[26], as per the legend at the bottom right. Interestingly, no taxa of 2 or 3 risk group were found exclusively in control sites.

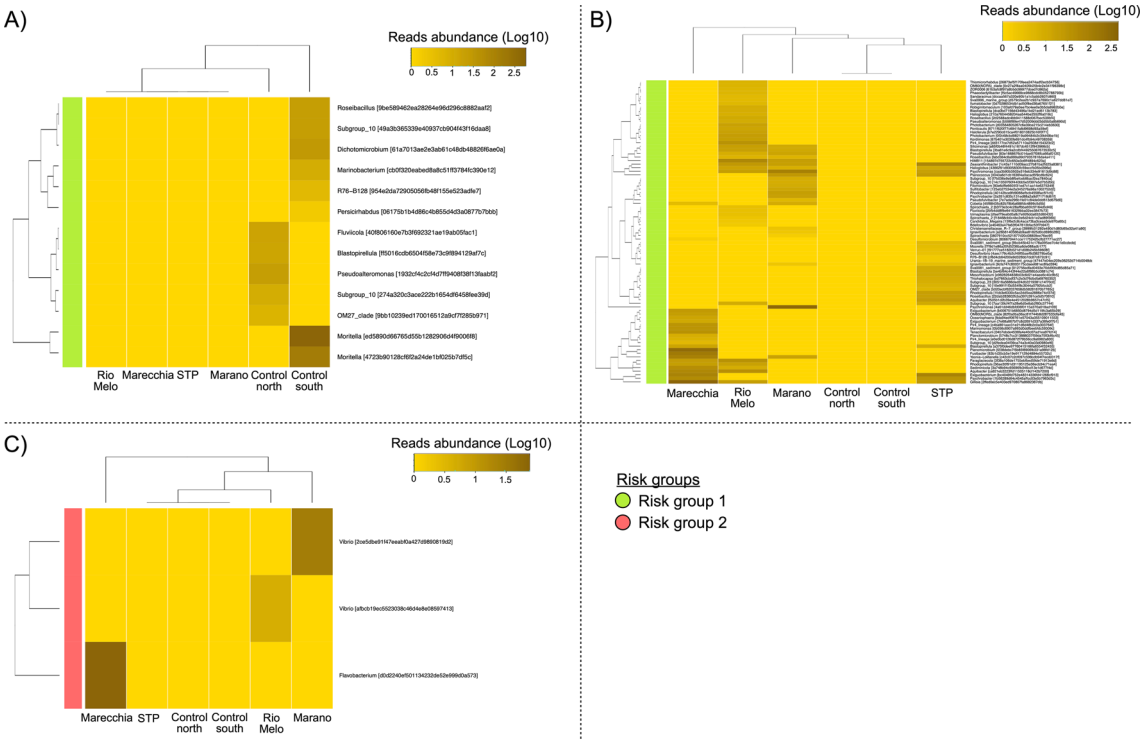

Fig. 3. Exclusive taxa to impacted or control condition for sediment samples, divided by risk group. Heatmap showing the taxa of sediment samples that were exclusive to control conditions (**A**), or to impacted conditions for risk group 1 (**B**) and risk group 2 (**C**). On the rows, unique code OTUs are reported, correlated by the assigned taxa at genus level. The colour scale is based on the Log10 of absolute abundance of the reads assigned for a specific OTU in the sample, represented in the columns. Clustering in both in rows and columns is based on Euclidean distance. The sidebar identifies the risk group[26], as per the legend at the bottom right. Interestingly, no taxa of 2 or 3 risk categories were found exclusively in control sites and no taxa of 2/3 or 3 risk group were found in sediments from impact sites.

quality reads were generated, with 47.9 ± 20.5 million per samples. A total of 20 human DNA virus was detected (Supplementary Table S3) using ViromeScan tool[54]. Viruses exclusively detected in samples from impacted or control conditions and those detected in both (i.e., core viruses) were identified and their respective distribution at the different sites is provided in Fig. 5, along with their corresponding risk group (EC, 2000). Among the human viruses specifically associated with the impacted condition, *Torque teno virus 2* was detected at all impacted sites. *Betapapillomavirus* was shared among the Rio Melo, Marecchia, and STP sites, whereas *Human herpesvirus* 5 was detected at Rio Melo and STP and Human *herpesvirus 4* at Rio Melo and Marano. With the exception of *Torque teno virus 2*, all the above mentioned viruses were belonging to risk group 2. Core human viruses include the *Human mastadenovirus* and different members of the family *Herpesviridae*, *Papillomaviridae* and *Poxviridae*, the latter being significant since the detected taxa included the genus *Orthopoxvirus*, which is considered high risk (risk group 4) for human health. No viruses were found exclusively at the control sites.

Regarding ARGs, a total of 99 genes were detected (Supplementary Tables S4 and S5). Their distribution at the impacted and control sites is reported in Fig. 6, along with the corresponding relevance for human health, as per WHO revision[26]. Notably, 82 ARGs were assigned to distinct Anti-Microbial Resistance (AMR) classes deemed as "critically important" for WHO, including aminoglycosides (9 ARGs), carbapenems (7 ARGs), cephalosporins (2 ARGs), fluoroquinolones (3 ARGs), glycopeptides (2 ARGs), macrolides (4 ARGs), penicillin β-lactams (11 ARGs), peptide antibiotic (1 ARGs), phosponic acid antibiotic (2 ARGs) and rifamycins (2 ARGs). Finally, among the critically important class, 39 ARGs were assigned to multidrug resistance. Regarding the site distribution, 33 critically important ARGs out of 39 were detected in the Marecchia river. At the Marano site, 21 ARGs (19 critically important) were identified, while at the Rio Melo site, 23 ARGs (19 critically important) were found. At the STP site, 46 ARGs (36 critically important) were detected. Finally, at the control sites, 24 ARGs (20 critically important) were identified.

Discussion

Through an approach combining 16 S rDNA metabarcoding and shotgun metagenomics, we profiled changes in the water and sediment microbiomes along the densely populated coastal zone of the Emilia-Romagna region in the northwestern Adriatic Sea. Our data indicate that the mouths of the urban-draining rivers show multiple factors of possible anthropogenic origin, including: (i) pathogenic bacteria, (ii) human viruses, and (iii) ARGs. These microbiological risk factors were predominantly detected in seawater samples, whereas

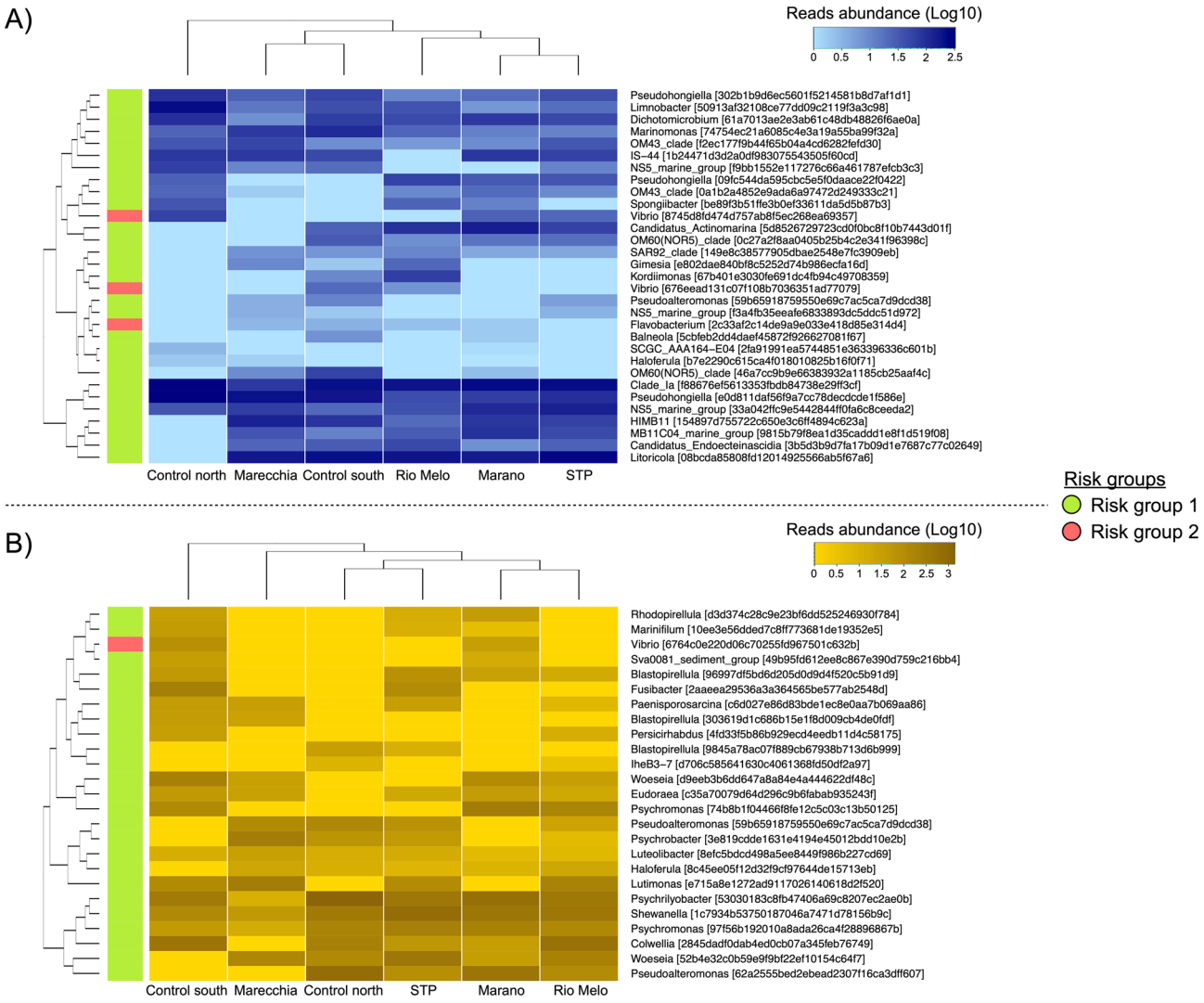

Fig. 4. Core taxa, found both in impacted and control sites, for seawater and sediment samples, divided by risk group. Heatmaps showing the core taxa of seawater (**A**) and sediment (**B**) samples, divided by risk group. On the rows, unique code OTUs are reported, correlated by the assigned taxa at genus level. The colour scale is based on the Log10 of absolute abundance of the reads assigned for a specific OTU in the sample, represented in the columns. Clustering in both rows and columns is based on Euclidean distance. The sidebar identifies the risk group[26], as per the legend on the right. No taxa of 2/3 or 3 risk group were found in the core.

sediments exhibited only limited evidence of contamination. This pattern suggests that, under the conditions of the present study, sediments at the river mouths do not appear to function as major long-term reservoirs of pathogenic signatures of possible anthropogenic origin. Instead, the anthropogenic microbial signal was primarily confined to the water column. Among the pathogenic bacteria detected in seawaters at the estuaries of the Marecchia, Marano and Rio Melo, 21 OTUs – belonging to 11 bacterial taxa – can be classified as part of the risk group 2 or 3 according to the Directive 2000/54/EC[55]. These taxa include well-known faecal indicators of human and animal origin, such as *Enterococcus*, *Streptococcus*, *Escherichia-Shigella*, *Prevotella* and *Bacteroides*, which are frequently present in urban sewage and wastewaters systems[56–67]. Specifically, *Enterococcus* is one of the most common nosocomial pathogens, which can cause urinary tract infections, wound infections and bacteremia[68,69]. Other human pathogens were detected, including *Vibrio*, *Chlamydia*, *Mycobacterium*, *Coxiella*, *Francisella*, and *Legionella*. These microorganisms – which can spread through water, aerosols, or human waste – may pose significant risks to human health, particularly in immunocompromised individuals[70–84]. Notably, the genus *Francisella* includes the zoonotic and highly pathogenic species *F. tularensis*, as well as other biofilm-producing and highly persistent species that cause infection in free-living and farmed fish[85,86]. Within ARGs, 82 genes classified as "critically important" by WHO[26] were detected in the estuarine water samples. These ARGs spanned 11 different AMR classes, targeting various antibiotics. These include inhibitors of protein synthesis (targeting aminoglycosides, macrolides, rifamycins), inhibitors of cell wall synthesis (targeting carbapenems, cephalosporins, glycopeptides, penicillin β-lactams, phosphonic acid antibiotics), and inhibitors of nucleic acid synthesis (targeting fluoroquinolones)[87–89]. Most of these critically important ARGs are characteristic of

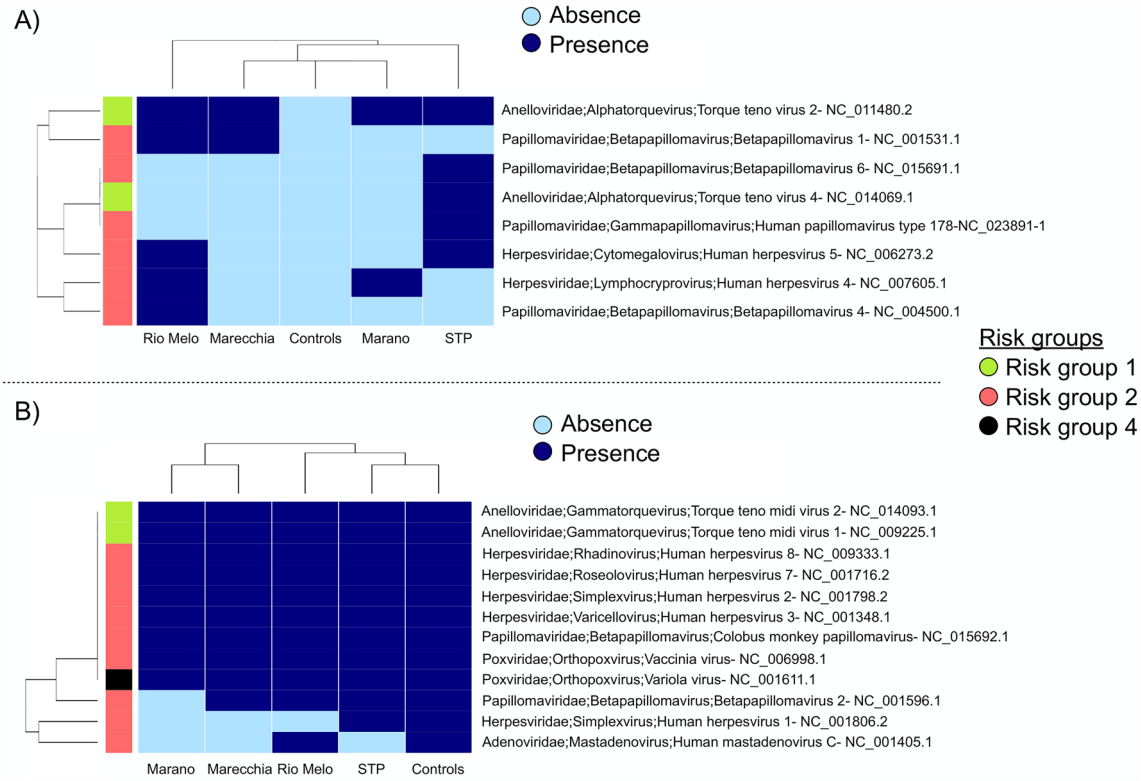

Fig. 5. Human viruses identified in seawater samples, divided by risk group. The complete taxonomy, reaching down to strain level and assigned to viral DNA is shown in the rows. The colour scale, in the top-right legend, is based on the presence/absence of normalized reads assigned to a specific OTU in the sample, represented in the columns. Clustering in both rows and columns is based on Euclidean distance. Sidebar identifies the risk group, as per the legend at the bottom. (**A**) Viral presence unique to impact sites, risk group 1 or 2. No viral presence was unique to the control sites. (**B**) Viral core presence, found either in control or impact sites, risk group 1, 2, 4.

nosocomial pathogens, likely entering municipal sewage and wastewater systems via hospital effluents[90–97]. Amongst these, we found ARGs conferring resistance to aminoglycoside antibiotics, such as *AAC(3)-VIIIa*, *AAC(6')-Ib7*, *aadA5*, *aadA6*, *ANT(6)-Ib*, *ANT(9)-Ib*, *APH(2")-If*, *APH(3')-Ia*, and *APH(3')-VIIIb*, that have been frequently detected in hospital isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterococcus spp*.[93,96,98,99]. Similarly, *vanL* and *vanO*, which confer resistance to vancomycin, have been previously identified in vancomycin-resistant *Enterococcus* strains of nosocomial origin, isolated from hospital effluents (Cetinkaya et al.[90,97,100]. The carbapenem resistance genes detected, *crxA*, *IND-17*, *IND-4*, *IND-5*, *PEDO-2*, *SGM-6*, and *SGM-7*, have been found to be prevalent in *Enterobacterales* isolated from hospital wastewater, activated sludge, and treated effluents in STP[91,92,94]. Similarly, the cephalosporin resistance genes *CSP-1* and *RASA-1* are both associated with human pathogenic species of nosocomial origin, such as *E. coli*, *Neisseria gonorrhoeae*, and *K. pneumoniae*[101]. Among ARGs conferring resistance to β-lactam antibiotics, the following genes were detected: *FRI-10*, *MecC-type methicillin resistance repressor (MecI)*, *mecI*, *OXA-1101*, *OXA-448*, *OXA-464*, *OXA-605*, *OXA-786*, *OXA-981*, *TEM-141*, and *TLA-2*. In particular, *OXA-48*, and its related variants, have been assigned to nearly all *K. pneumoniae* and *E. coli* isolates, recognized as globally prevalent nosocomial pathogens[102–105]. Notably, β-lactam ARGs have also been previously identified in coastal marine environments heavily impacted by human activity, including the Adriatic Sea[106,107]. Finally, at the impacted sites, we also detected the rifamycin resistance gene *rpoB* – previously found in multidrug resistant *Mycobacterium tuberculosis*[108]. Interestingly, among the critically important ARGs found in water samples at the riverine sites, some of putative farm origin were also detected. These include genes conferring resistance to phosphonic acid antibiotics, specifically *FosA3* and *FosB2* detected in effluents from animal farming operations[109–111], as well as macrolide, such as *mphA*, *mphG*, *Mrx*, and *srmB*, which are common in animal farm effluents[112,113]. *mphG* has been also reported in aquaculture[114,115] and European river sediments[116]. In addition, 39 ARGs associated with multidrug resistance were identified, including *acrB*, *marA*, *OXA*-type β-lactamases, *mex*-type efflux pumps and *erm* genes. The detection of these ARGs raises significant concerns about the emergence and spread of multidrug resistance in aquatic environments, posing a serious threat to both environmental and human health. According to Amin et al.[117], the high prevalence of multidrug resistant genes in such environments may be linked to the extensive use of antibiotics in the poultry and aquaculture industries, which exerts strong selection pressures for antimicrobial resistance in water bodies.

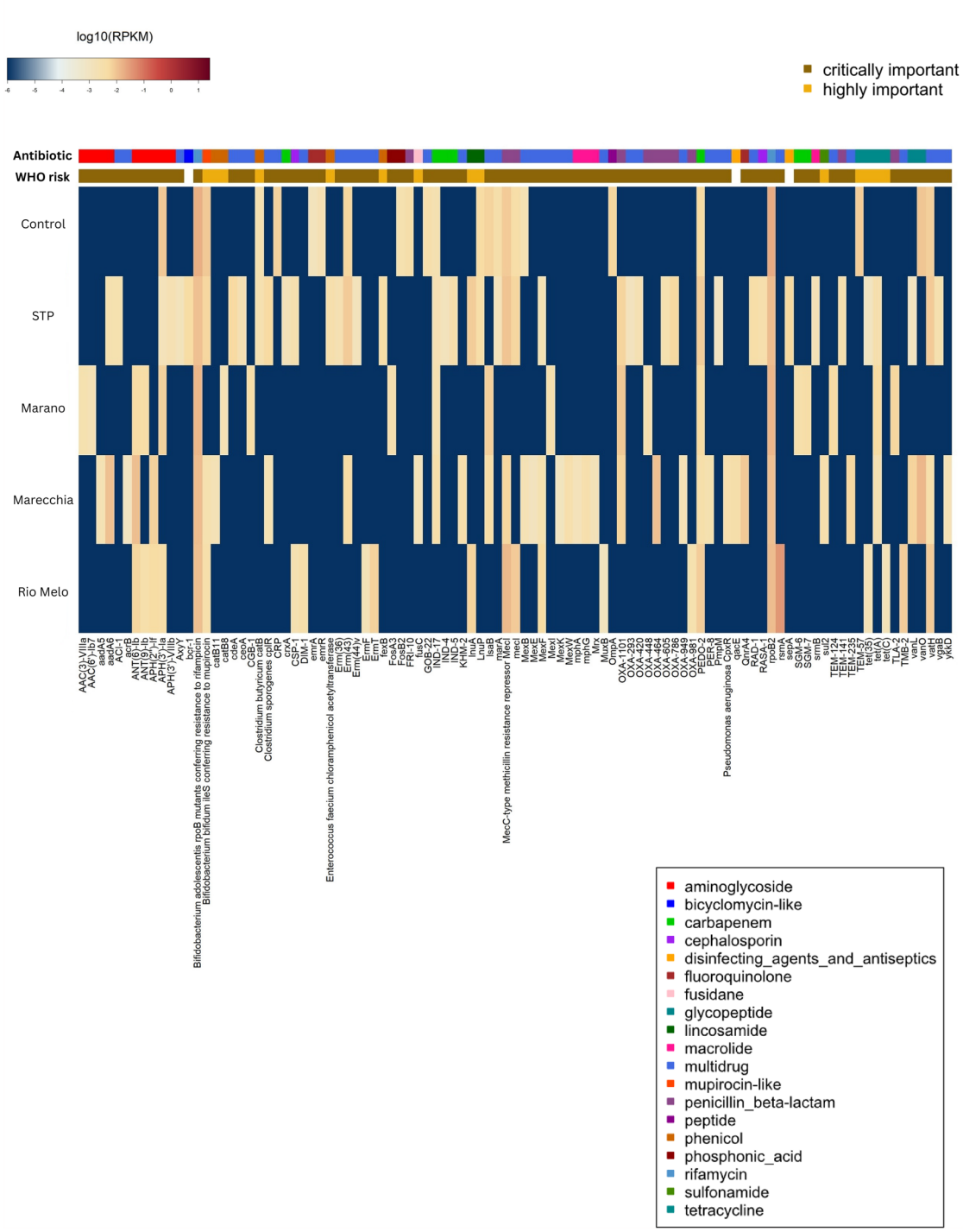

Fig. 6. Antibiotic resistance genes identified in seawater samples, divided by antibiotic class and risk group ("critically important" or "highly important") according to the World Health Organization revision. The genes are expressed in reads per kilobase of million mapped reads (RPKM) and to highlight the pattern of the abundance distribution a logarithmic scale was used. The first bar on the top of the graph indicates the risk class ("critically important" or "highly important") according to the World Health Organization revision[26]. The bar on the upper part of the graph indicates the classes of antibiotics to which the gene is resistant and is indicated in the side legend.

Among human pathogenic viruses, species belonging to the risk groups 2 (Directive 2000/54/EC) were detected in estuarine water samples. These include *Human Mastadenovirus C*, *Torque teno virus* (TTV), 7 species of *Human herpesviruses* – HHV-1, HHV-2, HHV-3 (i.e., *Varicella zoster virus*, VZV), HHV-4 (i.e., Epstein-Barr Virus, EBV), HHV-5 (i.e., Cytomegalovirus, CMV), HHV-7 and HHV-8 – and 6 species of *Betapapillomaviridae*.

Considering the genus *Orthopoxvirus* found as core, among *Poxviridae* family, most of the species are host-specific in animals and do not cause disease in humans; despite that, some can be very harmful to human health (e.g., variola virus, risk group 4)[118–120].

The great majority of these viruses have already been reported as contaminants of urban wastewaters[121–,129], and have been found in coastal seawater directly impacted by discharges[130–132]. Although information on the infectivity and persistence of viruses in seawater is limited and dependent upon environmental variables, waterborne viruses pose an important potential risk to human health, mostly considering that STP purification mechanisms are not fully effective in removing and inactivating them[133–135].

Interestingly, each river mouth displayed a specific microbiome signature, due to a specific combination of pathogenic bacterial taxa, human viruses, and ARGs. Although the discrimination of the different impact sources underlying this heterogeneity is beyond the scope of the present study, the observed patterns may reflect intrinsic differences among the estuaries[136]. Indeed, the 3 estuaries exhibit clear differences in scale, hydrology, and anthropogenic pressure. The Marecchia is a large river draining a wide Apennine catchment, including the industrialized Valmarecchia, and is characterized by substantial freshwater discharge, high sediment load, and a strong influence on coastal morphology and ecosystems. By contrast, the Marano is a small, highly seasonal torrent that functions primarily as a drainage channel and is heavily affected by urban and agricultural runoff, wastewater, and tourism, with limited flushing capacity and ecological role. The Rio Melo represents an intermediate system: a torrent originating from the hills, with moderate but variable flow and susceptibility to flash floods, less urbanized than the Marano basin but still influenced by agriculture, and with restricted estuarine dynamics due to its small water volume.

Bacterial pathogens, human viruses, and ARGs exhibited different distribution patterns across the coastal area. Unlike bacterial pathogens, which were detected exclusively at the impacted sites and displayed site-specific profiles, most ARGs and viruses appeared less spatially restricted, as they were also identified at the control sites. This broader distribution across the coastal waters is likely attributable to the greater environmental persistence of ARGs and viral particles, as previously reported by Cacace et al.[137] and Girón-Guzmán et al.[138]. The spread of antibiotic resistance is further complicated by horizontal gene transfer, which allows bacteria to acquire ARGs from their surroundings[112,139], with viruses acting as gene vectors and facilitating their dissemination among microorganisms[128]. Finally, according to our findings, the urban STP of Santa Giustina — improved in 2015 to face sewage system overflows and resulting water contamination and bathing bans, following the Optimized Seawater Protection Plan of the Municipality of Rimini[140]— proved do not release of pathogenic bacteria from urban discharges. In contrast, ARGs and viruses were observed, further supporting the notion that conventional wastewater treatment processes are less effective in eliminating these more resilient microbial determinants[128,133,137]. Taken together, our findings suggest that there is a potential microbiological risk for human health, which should be investigated further.

Conclusions

Although sampling was conducted at a single time point (July the 4th, 2024), corresponding to the peak tourism season, and thus represents a temporal snapshot that may not capture seasonal variability, this study demonstrates that metagenomics analysis of marine samples provides a comprehensive overview of the health of marine microbiomes on coastal waters and sediments, particularly regarding pathogens of nosocomial origin. Our findings underscore the importance of using such an analytical approach to implement monitoring strategies that ensure the bathing suitability of coastal marine waters and protect public and environmental health. Although metagenomics cannot distinguish between viable and non-viable organisms, its high sensitivity and ability to detect a wide range of microbial targets – beyond the limitations of traditional culturing methods – make it a powerful tool for future monitoring studies. This approach represents a strategic solution for comprehensively monitoring coastal water quality from both public health and environmental perspectives, in line with the principles of the One Health approach.

Data availability

High-quality reads from the samples sequenced in this study were deposited in the European Nucleotide Archive under the project accession number ENA: PRJEB104705.

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Author contributions

R.D., C.C. and M.C.: conceptualization. L.F., E.R., D.L., G.P., D.S. and F.M.: data curation. L.F., E.R., D.L. and D.S.: formal analysis. C.C. and M.C.: funding acquisition and project administration. G.P., D.S. and S.B.: visualization. L.F., R.D., C.C. and M.C.: writing – original draft. G.P., D.S., S.B., S.T., S.R., C.S., A.P. and F.M.: writing – review and editing.

Declarations

Competing interests

The authors declare no competing interests.

Ethical statement

This study did not involve human participants, vertebrate animals, or any other organisms requiring ethical approval. All samples consisted solely of non-living environmental materials (i.e., seawater and sediment). Therefore, ethical review and approval were not required for the collection or analysis of these inorganic biological specimens.

Additional information

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