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Abstract

Antimicrobial resistance (AMR) has emerged as a global problem, making the treatment of infectious diseases increasingly difficult and costly. Water-borne microbial taxa (including wastewater-borne ones) are linked to diseases in humans and to infections and mass mortalities of ecologically and economically important species. Natural aquatic ecosystems. Natural aquatic environments are recognized as playing a pivotal role in the propagation and dissemination of AMR and opportunistic pathogens. As final receiving bodies of microbial pollutants from multiple sources (e.g., wastewater discharges, agricultural runoff and stormwater), coastal marine environments in particular also have the potential to promote the dissemination of opportunistic pathogens and AMR across connected ecosystems and, ultimately, to humans and other organisms.

Next-generation sequencing (NGS) of bulk DNA in environmental samples (i.e. Metagenomics) can provide a unified perspective on the diversity of complex natural microbial communities and of the genetic material pool carried within an environment, giving unprecedented opportunities of studying microbiological phenomena at community scale.

In Task 3.5, the Gulf of Trieste was under study to assess the presence, prevalence and distribution of microbial pollutants (i.e. genetic materials involved in AMR, pathogenicity genes and opportunistic pathogens) through the application of multiple metagenomic approaches. The present report provides a description of the activities carried out in the Task with a focus on antimicrobial resistance phenomenon. A sampling campaign was devised to collect water and sediment samples from multiple locations in the Gulf of Trieste, here considered as a proof-of-concept of a coastal marine environment. The sampling campaign, as well as the analytical strategies and protocols, have been defined to investigate the prevalence and distribution of ARGs (resistome) and potentially pathogenic bacteria (pathobiome). To investigate land-sea interactions in the coastal zone, the stable isotope composition of organic carbon ($\delta^{13}C$) and the total organic carbon to nitrogen ratio (C/N) of sediments and particulate matter were also assessed.

Metagenomic Shotgun sequencing was implemented to perform a comprehensive analysis of the microbial community and to identify the full repertoire of Antimicrobial Resistance Genes (ARGs) present in the samples. This approach allows for the direct detection of functional genes, offering significant advantages over marker gene sequencing.

A Lagrangian model was implemented to predict the spatial and temporal distribution of ARGs originating from various point sources under different oceanographic conditions, including the time of the first samplings of this project (October 2023).

A wastewater treatment plant (WWTP) in Northern Italy was investigated as a potential source of AMR and microbial pollution in connected aquatic systems. The study focused on antibiotic resistance genes (ARGs) specific to selected antibiotic classes and examined their occurrence both in the plant's effluent and in the receiving water body. To assess the broader ecological impact, microbial community structure was reconstructed upstream and downstream of the discharge point, and a source tracking approach was applied to evaluate the contribution of the WWTP to the downstream community. These analyses are particularly relevant in the context of water reuse for agricultural purposes, where understanding the spread of ARGs and microbial contaminants is essential for effective risk assessment and management.

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4. Assessing the spread and distribution of antimicrobial resistance and potentially pathogenic bacteria in marine coastal environment

4.1 Introduction

Antimicrobial resistance (AMR) has emerged as a global problem, making the treatment of infectious diseases increasingly difficult and costly (de Kraker et al., 2016). Increased levels of AMR are an intrinsic consequence of the widespread use of antimicrobials in a variety of human activities (e.g., healthcare, livestock farming, intensive aquaculture). However, the environment, acting as both a receptor and then a source of transmission, is now widely recognized as playing a pivotal role in the propagation and dissemination of AMR (Harbarth et al., 2015).

Marine coastal environments, due to the anthropogenic pressure and their direct connection to land, are receiving bodies of microbial pollutants, such as antibiotic-resistant bacteria, genetic material conferring resistance to antimicrobials (e.g., antibiotic resistance genes, or ARGs) and opportunistic pathogenic bacteria, which can enter the marine environment through a variety of pathways (e.g., rivers, agricultural runoff, and wastewater). The presence of multiple contaminants at subminimal inhibitory concentrations, combined with the interaction between autochthonous and allochthonous microbial communities, may facilitate the acquisition of resistance by opportunistic pathogens or the emergence of novel AMR pathways through genetic material exchange (Lupo et al., 2012). At the same time, marine coastal environments have the potential to expose millions of people to AMR and disease through food consumption and, as recent studies suggest, recreational activities (Leonard et al., 2018; Stanton et al., 2022).

In recent years, there has been a notable surge in scientific studies examining the spread and distribution of AMR and opportunistic pathogens in non-clinical environments. Despite this increasing research effort, studies on AMR's prevalence, evolution, and dynamics in marine environments remain fragmented. Many studies focus on bacterial isolates, often limited to a single or a few strains, rather than considering the entire microbial community. Molecular techniques, especially next-generation sequencing (NGS) of bulk DNA in environmental samples (metagenomics), are now widely employed to identify taxa and potential ARGs. However, there are still several knowledge gaps, including a need for a more comprehensive understanding of environmental dissemination pathways, as well as the role played by natural microbial communities in either promoting or hindering the establishment of ARGs and pathogenic elements in natural settings. Numerical models can offer a valuable support in understanding the spreading of ARGs from point sources driven by different meteo-climatic and oceanographic conditions, thus permitting to elucidate the dynamics of physical transport, also taking into account the degradation with time of the biological material.

The metagenomic approach can provide a comprehensive view of natural microbial communities in their complexity. Diverging from culture-based methods, NGS enables the characterization of entire communities, which are predominantly composed of unculturable taxa. Amplicon-based sequencing strategies targeting taxonomic marker genes (metabarcoding) facilitate a swift assessment of microbiome composition and structure. In contrast, shotgun sequencing, unlike targeted approaches, provides a comprehensive snapshot of the genetic material within a sample. This allows for the exploration of microbial diversity at a higher resolution, shedding light on the functional potential of the community. Its ability to capture the full genomic diversity makes it a powerful tool in environmental microbiology and it is increasingly applied to scrutinize the natural communities in harboring ARGs and other genes involved in the dissemination of AMR.

This project leverages ‘omics approaches to elucidate the presence, prevalence, and distribution of microbial pollutants (i.e., antimicrobial resistance and potentially pathogenic bacteria) in coastal marine environments, and the ecological processes involved in their dissemination. To this end, a new sampling design was developed for the Gulf of Trieste (GoT), serving as a proof of concept of a marine coastal ecosystem. The sampling campaign is aimed at obtaining bulk DNA from seawater and sediment samples from sites chosen to represent archetypical descriptors of the main sources of mesoscale variability in the Gulf. A Lagrangian model tracking the transport and degradation of ARGs in the GoT was implemented to support the interpretation of field data.

4.2 Study area

The Gulf of Trieste (GoT) is a shallow (<25 m), river-influenced embayment in the northernmost sector of the Adriatic Sea. The convergence of freshwater inputs and intricate marine currents results in distinct hydrographic features and a heterogeneous array of habitats. The main freshwater inputs come from the Isonzo and the Timavo rivers in the northern area, while freshwater sources from the southern coast are of torrential nature (Cozzi et al., 2012). Atmospheric processes, discharge of continental waters and the oceanographic circulation cause large oscillations of salinity (29-38.5) and temperature (4-29.2 °C). The water column is stratified in summer and fully mixed in winter (Malačić et al., 2006). As the main source of organic and inorganic nutrients in the area is given by freshwater inputs, their broad temporal variability induces changes in the rates of organic matter production, processing, and mineralization (Manna et al., 2021).

The water column exhibits pronounced stratification in summer and sometimes, in specific locations, also in winter. These stratified layers play a crucial role in shaping the distribution of nutrients, dissolved oxygen, and phytoplankton, thereby influencing the entire marine food web. Nevertheless, the Gulf of Trieste confronts notable challenges, including anthropogenic impacts such as pollution, habitat degradation, and the introduction of non-native species.

4.3 Methodologies

4.3.1 Sampling design

Our sampling strategy was designed to collect water and sediment samples from 7 locations in GoT (Fig. 1) over a period of one year in order to capture the seasonal variability at each site. The selected sites are listed in Tab.1 and have been chosen to serve as archetypical descriptors of the main sources of mesoscale variability in the Gulf. Sampling was performed any two months between October 2023 and August 2024, for a total of six sampling deployments at each site.

Table 1 - Sampling sites in the Gulf of Trieste.

Code	Site	Interest	Latitude (N)	Longitude (E)	Depth (m)	Coastal distance (m)
GT01	Isonzo River mouth	River plume	45.7341200	13.577900	5.6	~1000
GT02	Monfalcone port mouth	Industrial port outflow current	45.7749600	13.555790	6.6	~1300
GT03	LTER-C1	Marine Protected Area	45.7008306	13.710000	17	~230
GT04	Trieste port	Inside the port, backflow water	45.6526400	13.767668	7.1	~90
GT05	0D	WWTP effluent	45.6434167	13.681028	23	~5000
GT06	MA2_11	Offshore water	45.6683000	13.626500	22	~9000
GTxtra	California	Bathing beach	45.6931390	13.732245	1	0

In consideration of the hydrographic characteristics in the Gulf – which lead to seasonal or local stratification despite the low bathymetry – we collected seawater samples at two depths with the only exception of the GTxtra point: half a meter below the water surface and half a meter above the seafloor. Sampling was performed using a rosette mounting 5L Niskin bottles and a CTD probe recording the vertical profiles of temperature, salinity, and conductivity (Sea-Bird Electronics SBE 19plus SeaCAT profiler). Sediment was collected using a van Veen grab (0.1 m^2).

Seawater samples were prefiltered on-board using a 200 µm mesh to remove zooplankton and large particles. Tubings, filter holders, meshes and vessels used onboard had been previously cleaned with RNase Away solution and/or bleach. Sediment aliquots were transferred into sterile PCR-grade polypropylene tubes. All samples were transported to the laboratories in the dark and at cold within 2 h for further processing.

Figure 1: Sampling points in the Gulf of Trieste.

4.3.2 Molecular biology methods

4.3.2.1 DNA extraction and library preparation

Seawater samples were filtered onto 47 mm diameter, 0.2 µm pore size polyethersulfone sterile membranes (Pall Corporation, Michigan, USA) to collect the bacterial fraction. Filtration volumes were defined by membrane clogging and ranged between 1 and 3 L. The membranes, as well as the sediment samples aliquots, were stored at -80°C until subsequent processing for the isolation of bulk DNA.

Genomic DNA was isolated from the filters using the DNeasy PowerWater Kit (Qiagen) and from the sediment aliquots using the Qiagen DNeasy PowerSoil Pro kit, with a few modifications to the manufacturer's protocol aimed at maximizing the extraction yield. After adding the lysing buffer the filters underwent three cycles of incubation at 70°C for 5 min followed by 2 min horizontal vortexing at maximum speed. Sediment samples underwent three cycles of incubation at 70°C for 5 min followed by 3 min horizontal vortexing at maximum speed. An additional 1 minute of horizontal vortexing was also performed. We then proceeded as described in the kit handbook. Elution was performed twice to maximize the nucleic acid recovery from the silica buffer. The DNA concentration was then measured using a Qubit Fluorometer (ThermoFisher). Quality was inspected both visually by gel agarose electrophoresis and by Nanodrop spectroscopy. DNA extracts stored at -80 C until further processing.

Identification of potentially pathogenic bacteria was performed by amplification and NGS sequencing of the 16S rRNA marker gene (metabarcoding). A subset of 4 sediment samples were used in 3 replicates to compare the short-read sequencing strategy with long-read NGS strategies. The DNA extracts were diluted with nuclease-free water to a concentration of 5ng/µl and amplified with KAPA HiFi HotStart ReadyMix (Roche) using the universal primer pair 341F-805R (Klindworth et al., 2013) following the official Illumina protocol. The PCR products were then sent to the sequencing facilities (i.e. IGA Technology Services srl) for the subsequent steps of the library preparation protocol, pooled in equal molar ratios, and sequenced on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) with a 2×250 bp strategy. In particular, the metagenetic libraries were prepared by transposase-based tagmentation, with simultaneous ligation of Nextera adapters and DNA fragmentation (Adey et al., 2010).

The full-length 16S rRNA gene was amplified using a degenerated 27f and 1493r primer pair to ensure broad-range bacterial coverage. PCR was executed using KAPA HiFi HotStart ReadyMix (Roche) to maintain high-fidelity amplification. The PCR thermal profile involved an initial denaturation step, followed by 25 cycles of denaturation, annealing at 57°C, and extension, followed by a final extension step. Amplicons of approximately 1500 bp were then subjected to purification to eliminate primer-dimers and unincorporated reagents and prepared for Oxford Nanopore sequencing. This involved an end-prep and A-tailing reaction, followed by the ligation of sequencing adapters containing the motor protein. The final libraries were quantified and assessed for quality before being loaded onto an R10.4.1 flow cell and sequenced on the MinION platform (Oxford Nanopore Technologies). Data acquisition was performed for 36 hours. All steps where performed locally at the OGS laboratory facilities.

ARGs detection was performed performed after high throughput sequencing of samples' whole community DNA ("shotgun" sequencing) using the Illumina platform. Extracted DNA was sent to the sequencing service provided by IGA Technology Services srl for shotgun library preparation and subsequent paired-end (2 x 150 bp) sequencing on the Illumina Novaseq platform.

4.3.2.2 Bioinformatic elaborations and High-Performance Computing (HPC)

The elaboration of metagenetic and metagenomic data required the selection of up-to-date bioinformatic pipelines and the optimization of the bioinformatic workflows. Due to the high computational demand for such elaborations, we applied to the Italian SuperComputing Resource Allocation grant to obtain computational power and data storage capacity in the Galileo100 and Leonardo supercomputers (CINECA), for a total of about 86,506 standards hour.

Raw data from the complete sequencing of the whole genetic pool in samples' microbial communities (Shotgun Metagenomics) were inspected for the detection of ARGs and genes conferring resistance to other antimicrobials (e.g: disinfectants). ARGs can be searched: 1) directly in the short reads obtained from sequencing, or 2) after assembling the reads into contigs or scaffolds. Both methods were taken into consideration as they allow the detection of ARG classes. Bioinformatic pipelines screened in this work included i) reference-based methods (AMRFinderPlus, RGI, Abricate), well suited at identifying known ARGs, and ii) deep learning (DeepARG, fARGene) or structural homology modeling (PCM) approaches, that can potentially identify unknown ARG candidates. With the exception of PCM, all pipelines for post-assembly screening are included in funcscan (https://nf-co.re/funcscan/1.1.4), a portable, integrated pipeline built with the Nextflow scripting language that manages the different tools and their environments, packaged in Docker or Singularity images.

The RGI (the Resistance Gene Identifier) is based on a sophisticated hit-calling methodology, designed to minimize both false positives and false negatives inherent in complex environmental data. RGI can operate in two principal identification strategies (i.e. Strict Hits and Loose Hits), both using the Comprehensive Antibiotic Resistance Database (CARD, v3.2.9 as reference database, an extensive and meticulously curated collection resistance gene that links genetic determinants to resistance mechanisms and drug classes (Alcock et al., 2023). Perfect Hits methodology requires 100% identity over 100% of the sequence length against a curated reference allele, providing maximum confidence for previously characterized variants. The Loose Hits mode, on the contrary, is designed for detecting emerging or divergent alleles, identifying sequences with high similarity to known ARGs but that may contain single nucleotide variations (SNVs), truncated ends, or minor structural deviations, preventing their loss due to simple identity cutoffs.

Sequence quality was checked using FastQC v0.12.1 (Andrews, 2010). Trimmomatic v0.36 (Bolger et al., 2014) was used to remove low quality sequence and to remove Illumina adapters. About 92% of the raw read passed this quality filtering step. For the reconstruction of the genetic pool conferring resistance to antibiotics, disinfectants and other antimicrobials, we went for a read-based bioinformatic approach. In particular, ARG-like sequences were inferred using RGI *bwt* (v6.0.3), a version of the RGI software tool specifically designed for large-scale metagenomic dataset and capable of dealing with the typical short-read shotgun sequences. RGI *bwt* is specifically optimized for mapping short reads directly against a nucleotide reference database and operates in the RGI Loose Hits mode.

In our workflow, the DNA sequences were aligned using KMA aligner against CARD (nucleotide reference, v3.2.9). Sequences that meet the threshold were assigned to the corresponding resistance allele and automatically inherits the associated resistance ontology terms. The analysis additionally included CARD's Resistomes & Variants data contained in the WildCARD reference database v4.0.2. The abundances of ARGs were normalised to their reference sequence length and the total number of 16S rRNA gene-like reads (copy of ARG per copy of 16S-rRNA gene), according to the method described in Beltrán de Heredia et al. (2025).

4.3.3 Chemical Analytical methods

4.3.3.1 Seawater samples

For particulate organic carbon (POC) and POC stable isotope composition (δ^{13}C-POC), particulate nitrogen (PN) and total suspended matter (TSM) in seawater, subsamples were collected directly from Niskin bottles into 3 L PE carboys which were stored in a cool and dark place until laboratory processing (within 3-4 h from sampling).

For TSM, aliquots of 1000-1500 mL of seawater were filtered over Whatman GF/F glass-fiber filters (0.7 µm nominal pore size, ø 47 mm) previously weighed on a micro ultra balance (precision 1 µg). After filtration, filters were carefully rinsed with Milli-Q water to remove salt and were oven-dried at 60 °C for at least 24 hours. TSM concentration was determined gravimetrically after cooling filters to room temperature (Strickland and Parsons, 1972; APAT IRSA CNR, 2003).

For POC, δ^{13}C-POC and PN, aliquots of 500-1000 mL of seawater were filtered over pre-combusted (450°C for 4 h) Whatman GF/F glass-fiber filters (0.7 µm nominal pore size, ø 25 mm) and stored at -80 °C until further processing. Before analysis, filters were oven-dried (60°C, ~1 h) and packed into tin capsules. For POC and δ^{13}C-POC determination, filters were also pretreated with HCl 1 mol/L to remove carbonates (Nieuwenhuize et al., 1994). The analysis of POC, δ13C-POC and PN was performed using an elemental analyzer (Vario PYRO Cube, Elementar, Germany) coupled with an isotopic ratio mass spectrometer (IRMS - IsoPrime 100, Elementar, UK). Known amounts of standard Acetanilide (C_8H_9NO; Costech, purity ≥ 99.5%) were used to calibrate the instrument for the determination of carbon and nitrogen concentrations that were expressed, respectively, as µmol C/L and µmol N/L. Quality control and quality assurance of measurements were performed using internal standards and were also verified against the certified marine sediment reference material PACS-2 (National Research Council Canada). The stable isotope ratio δ^{13}C-POC was expressed in δ notation (in ‰ units) referring to the international standard VPDB (Vienna Pee Dee Belemnite):

$$\delta = \left(\frac{{}^{13}C/{}^{12}C_{sample}}{{}^{13}C/{}^{12}C_{VPDB}} - 1 \right) \times 1000$$

IRMS was calibrated against the international reference materials IAEA-600 (International Atomic Energy Agency, Vienna, Austria), USGS40 and USGS62 (US Geological Survey).

4.3.3.2 Sediment samples

Sediment samples were analyzed for grain size, total organic carbon (TOC), TOC stable isotope composition (δ^{13}C-TOC) and total nitrogen (TN). Aliquots of 10-40 mL of surface (~ 1 cm) sediment were collected using a van Veen grab (0.1 m2). The aliquots collected for the determination of grain size were stored at +4°C, those for TOC and TN analyses were stored at −20 °C.

For grain size analyses, each sample was treated with H_2O_2 10% (Sigma-Aldrich) in order to oxidize the organic matter. Gravel and shell fragments were separated from sandy and muddy fractions by wet sieving through a 1 mm mesh. The grain size distribution was determined using a BECKMAN COULTER LS 13

320 Laser Diffraction Particle Size Analyzer. Results were expressed as percentage of sand, silt and clay. Following the grain size classification proposed in Wentworth (1922), the clay fraction refers to a textural class whose particles are smaller than 4 µm. The sediments were described following Nota (1958).

For TOC, δ^{13}C-TOC and TN analyses, sediments were freeze-dried, homogenized by grinding to a fine powder in an agate planetary ball mill (Retsch PM 200) and finally sieved on a 250 µm iron steel sieve (Endecotts LTD, UK). Triplicate subsamples of about 8-12 mg were weighed directly into tin (for TN) or silver (for TOC and δ^{13}C-TOC) capsules. Before the TOC and δ^{13}C-TOC determination, subsamples were treated with increasing concentrations of HCl (0.1 mol/L and 1 mol/L) to remove the carbonatic fraction (Nieuwenhuize et al., 1994). The analysis of TOC, δ^{13}C-TOC and TN was performed using an elemental analyzer (Vario PYRO Cube, Elementar, Germany) coupled with an isotopic ratio mass spectrometer (IRMS - IsoPrime 100, Elementar, UK). Known amounts of standard Acetanilide (C_8H_9NO; Costech, purity ≥ 99.5%) were used to calibrate the instrument for the determination of carbon and nitrogen concentrations that were expressed, respectively, as mg C/g and mg N/g. Quality control and quality assurance of measurements were performed using internal standards and were also verified against the certified marine sediment reference material PACS-2 (National Research Council Canada). The stable isotope ratio δ^{13}C-TOC was expressed in δ notation (in ‰ units) referring to the international standard VPDB as described in paragraph 4.3.3.1 for seawater samples.

4.3.4 Modelling approaches

An ocean particle-tracking model (LTRANS-Zlev) driven by hydrodynamics fields from the MITgcm model was applied to simulate the spatial and temporal distribution of biological pollutants (BPs) originating from point sources. Here we analyzed the impact of key primary sources such as wastewater treatment plants (WWTPs). The case study area, the Gulf of Trieste, receives freshwater from four rivers (Dragonja, Rizana, Timavo, Soca-Isonzo), among which the Soca-Isonzo River is the most relevant. Other primary sources include 6 WWTPs insisting on the site. The modelling tool, based on a high-resolution simulation of the circulation in the Gulf of Trieste, was used to support the interpretation of field data on ARGs (i.e., taxonomic distribution matrices) obtained for each sampling station.

4.3.4.1 The hydrodynamic model MITgcm

MITgcm is a state-of-the-art global circulation model that allows for the computation of the hydrodynamic field of a sea basin. In this work, the hydrodynamic fields of the Gulf of Trieste made available by Lombardo et al. 2025 were used as forcings for the Lagrangian model. In this model implementation, a horizontal resolution of around 750 m was adopted, and an uneven vertical resolution with 20 thinner layers of 1 m at the free surface, and 18 layers of increasing thickness, up to 11 m, towards the bottom. The simulation spans over a period of 95 days, starting from August 9th 2023 until November 12th 2023. This period notably includes the storm surge event in the study area (November, 3, 2023), thus enabling also an analysis of the impact of extreme meteo-marine events on the spread of these BPs. The model provided hourly averaged variable fields of currents, wind speed, temperature and salinity that were used to force the transport of particles in the LTRANS-Zlev solver.

4.3.4.2 The Lagrangian model LTRANS-Zlev

The LTRANS-Zlev model (Laurent et al, 2020), a three-dimensional off-line Lagrangian ocean particle-tracking model can simulate passive tracers, particles with sinking, or floating behavior (Fig. 2). In this implementation, pathogens and ARGs are assumed to behave as buoyant particles with the same density as water (e.g., Kough et al., 2015), given a density range of 1.047-1.087 g/cm^3 for marine bacteria (Inowe et al., 2007) which is very close to seawater density (approximately 1.027). BPs are advected by the hydrodynamic fields and undergo a degradation process over time, as field observations indicate that the half-life for DNA and cells in the marine environments spans from <1 day to one week (Andruszkiewicz et al., 2019). However, such estimation is still debated and whilst degradation processes are very likely affected by environmental conditions such as irradiance, temperature, and possibly other factors, there is a lack of field or laboratory data to fully resolve these relationships. As a first approximation, the degradation

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rate was assumed to be constant, and both the upper and lower estimates (1 day and 1 week) were tested with the model to take into account uncertainty.

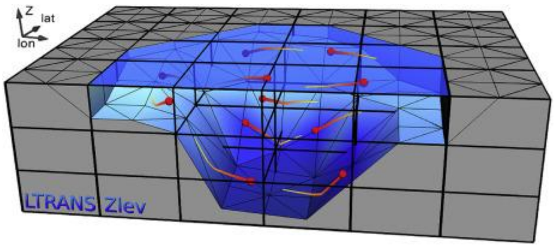

Figure 2: Illustration of particles advected by LTRANS-Zlev on a Z-coordinate vertical discretization. Source: Laurent et al, 2020.

The degradation process of the BPs was simulated through an integrity index (i) ranging from 1 to 0, computed in the post-processing phase for each particle n times after the release (t):

$$i_n(t) = e^{(-kt)}, \quad k = \frac{ln(2)}{HL}$$

where HL is the half-life time expressed in seconds, which is inversely related to the degradation constant k. Two cases with HL of 1 week and 1 day were considered. Particles with an integrity index below 20% were removed from the counting, in order to enlighten the effect of degradation.

Having in mind that the half-time life of the BPs is of the order of a few days (up to 7 days), we simulated hourly releases from each source starting from 7 to 10 days before the day under investigation, assuming that older particles will be totally degraded and not detectable anymore. The different time-frames of simulation presented in this report are synthesized in Table 2.

Table 2- Simulation run/post-processing parameters.

Simulation	Conditions	Dates	ARGs half life (d)	N. particles
run1-a1	Summer - water stratification	09-18 August '23	1	1.440.000
run1-a7	Summer - water stratification	09-18 August '23	7	1.440.000
run1-b1	Winter - storm surge	30 October - 08 November '23	1	1.440.000
run1-b7	Winter - storm surge	30 October - 08 November '23	7	1.440.000
run2-a1	First RETURN sampling	01-10 October '23	1	1.440.000
run2-a7	First RETURN sampling	01-10 October '23	7	1.440.000
run2-b1	Second RETURN sampling	07-17 November '23	1	1.440.000
run2-b2	Second RETURN sampling	07-17 November '23	7	1.440.000

Each run considered 1000 particles for each WWTP, with hourly releases. In order to take into account the different impact of each WWTP discharge we consider an index based on the mean discharge flow rate and also the population insisting on the served area of each water treatment plant, the equivalent population. The values are reported in Table 3, the results are normalized with respect to the mean equivalent index.

Table 3 – Population equivalent.

WWTP	3	4	5	6	20	21
Equivalent	26936.0	53281.0	5912.0	237377.0	105183.0	105183

4.4 Results

The Gulf of Trieste (GoT) was here under investigation as a coastal marine ecosystem subject to multiple continental hydrological inputs and exhibiting high complexity on a relatively small scale. The GoT is the receiving body for urban WWTPs and hosts two medium-sized commercially important ports. It hosts a protected marine area and several bathing sites for recreational use, as well as mussel farming sites. Being an enclosed basin with shallow water (maximum depth is about 40 m), the GoT is particularly influenced by land–coast interactions. Large fluctuation in salinity can occur due to substantial freshwater inflow from rivers and point springs along the shoreline and continuous input of salty water from the south. For these reasons, the GoT provides an opportunity to study numerous processes that can influence the dispersion of antimicrobial resistance genes (ARGs). However, a complete description of the resistome (as well as the pathobiome) in the GoT is currently lacking. Conversely, a monitoring plan for the ARGs discharged into the sea, or tracing the sources and potential AMR reservoirs, requires prior information on the types of resistance genes present in the Gulf, their distribution, and any site-specific dynamics.

The aim of the activities described here was to address this gap by implementing a survey on-field to describe the ARGs present, their association with other genetic markers, and their relationship with the specific physic-chemical characteristics of the Gulf. A sampling campaign was devised to collect water and sediment samples from multiple locations in the Gulf of Trieste, here considered as a proof-of-concept of a coastal marine environment. The sampling campaign, as well as the analytical strategies and protocols, have been defined to investigate the prevalence and distribution of ARGs (resistome) and potentially pathogenic bacteria (pathobiome). Metagenomic Shotgun sequencing was implemented to perform a comprehensive analysis of the microbial community and to identify the full repertoire of the ARGs.

The selected sites were chosen to serve as archetypical descriptors of the main sources of mesoscale variability in the Gulf. The one-year timeframe was chosen to capture the seasonal variability at each site.

A total of 78 seawater samples and 42 sediment samples were collected. Seawater samples yielded DNA from 0.3 to 3.9 ng/ml, the sediment ones from 25 to 22,393 ng/g. The lowest DNA concentrations were always obtained for station GTxtra, for both the sediment and the seawater samples.

The sampling design included the assessment of the concentration and the isotopic composition of particulate organic matter in the water column and the sediment samples, to provide insights on the land-sea interactions. The combined use of the C/N ratio and δ13C represents a widely used approach to distinguish autochthonous vs. terrestrial derived organic matter. The use of carbon isotope ratios as tracers of the origin of organic matter relies on the specific isotope composition of different organic matter sources. The total organic carbon to nitrogen ratio (C/N ratio) is generally considered a good proxy for discriminating between terrestrial and marine origins of organic matter in coastal sediments, depending mainly on the different nitrogen content between phytoplankton and terrestrial vascular plants (Rumolo et al., 2011 and references therein, Lamb et al., 2006).

4.4.1 Resistance gene occurrence in the Gulf of Trieste

A read-based bioinformatic approach was chosen to predict ARGs and ARG-like genes in the GoT metagenomic dataset, using the RGI *bwt* software tool. Despite being less precise and poorly effective in the discovery of novel genes compared to assembly-based strategies, this approach is particularly suited to broad-scale environmental ARG surveys. Assembly algorithms often fail to correctly link ARG sequences to a coherent contig, resulting in false negatives or loss of detection. On the contrary, a read based strategy can provide a comprehensive inventory of the genetic pool of interest. In contrast with the assembly-based approach, read-based investigations can also catalogue fragmented sequences and low-abundance variants, minimizing the risk of losing ARG signals. Finally, it provides a more robust and quantitative estimation of the overall resistome abundance and distribution across diverse environmental samples.

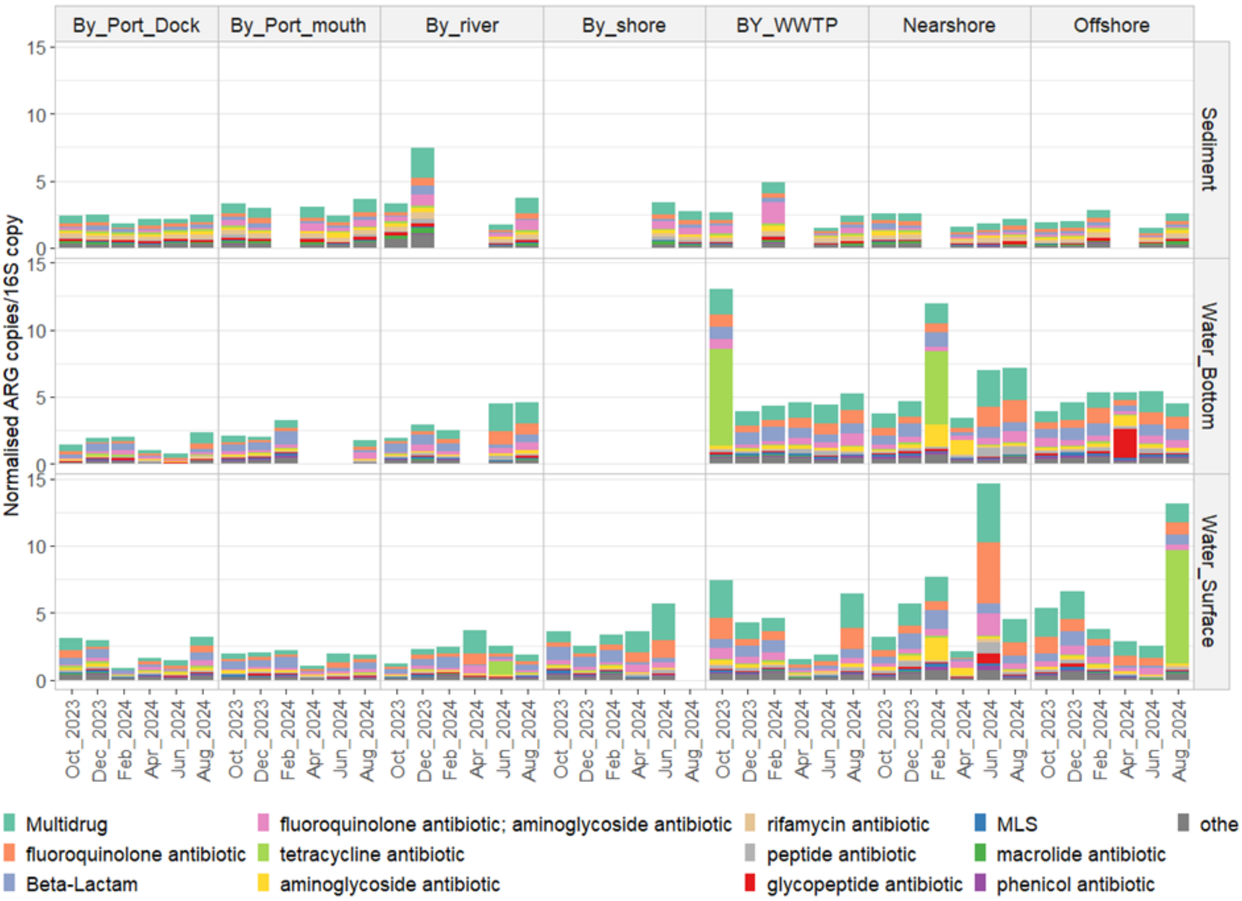

Figure 3: Resistome composition of the Gulf of Trieste in the period between October 2023 and August 2024 with respect to the antibiotic classes to which they confer resistance. The "Multidrug" class gathers all ARGs providing resistance to 3 or more antibiotic families while coding for a single biochemical mechanism. The "other" class represents the remaining antibiotic classes.

In this work, the classification of ARGs, ARG-like genes, their working mechanisms, and antibiotic/antimicrobial classes, follows the Antibiotic Resistance Ontology (ARO, Alcock et al., 2023), a formal, hierarchical vocabulary created to ensure semantic consistency in the bioinformatic outputs of the ARG prediction. ARO describes the relationships between a gene product to a given antibiotic or resistance phenotype assigning a unique accession number to every element, from broad drug categories (e.g., beta-lactam) to specific resistance mechanisms (e.g., target modification) and their precise genetic determinants.

Figure 3 shows the 16S-normalized ARG abundance in the GoT resistome with the data aggregated by the antibiotic class to which these genes confer resistance. The highest abundances were consistently observed for genes conferring resistance to several key antibiotic classes: fluoroquinolones, aminoglycosides (e.g., streptomycin), beta-lactams (including cephalosporins) and other antibiotics. Resistance genes targeting rifamycin were found at higher relative abundances in the sediment samples compared to the seawater. Tetracycline resistance genes showed occasional, yet pronounced peaks of abundance in the water column, especially at the surface level, while sediment samples had much lower abundances of genes conferring resistance to tetracyclines, yet characterized by less variation in their relative abundances.

The GoT resistome was characterized by a consistent and high presence of ARGs capable of conferring multidrug resistance (here defined as effective against three or more classes of antibiotics), a phenomenon observed both for the sediment and the water column. The main mechanism involved in multidrug resistance was antibiotic efflux mechanisms, i.e. the active, energy-dependent process where bacterial pump proteins expel antimicrobial compounds out of the cell, reducing the intracellular drug concentration below the effective level. Other relevant genes in GoT and responsible for multidrug resistance were linked to reduced i) antibiotic permeability and ii) antibiotic target protection, but these two groups of genes were considerably less abundant than genes coding for efflux mechanisms (25 to 100 times less abundant, on average). Reduced antibiotic permeability mechanism family covers a broad, often passive, alterations to membrane permeability to antibiotic molecules, usually associated to structural modifications (e.g., alteration of outer membrane porins): the drug uptake is limited, thus its cytoplasmic target is far from reaching. Antibiotic target protection is due genes coding for a factor (typically a protein), that binds or shields the target site, physically preventing the drug-target interaction and preserving the target's normal activity.

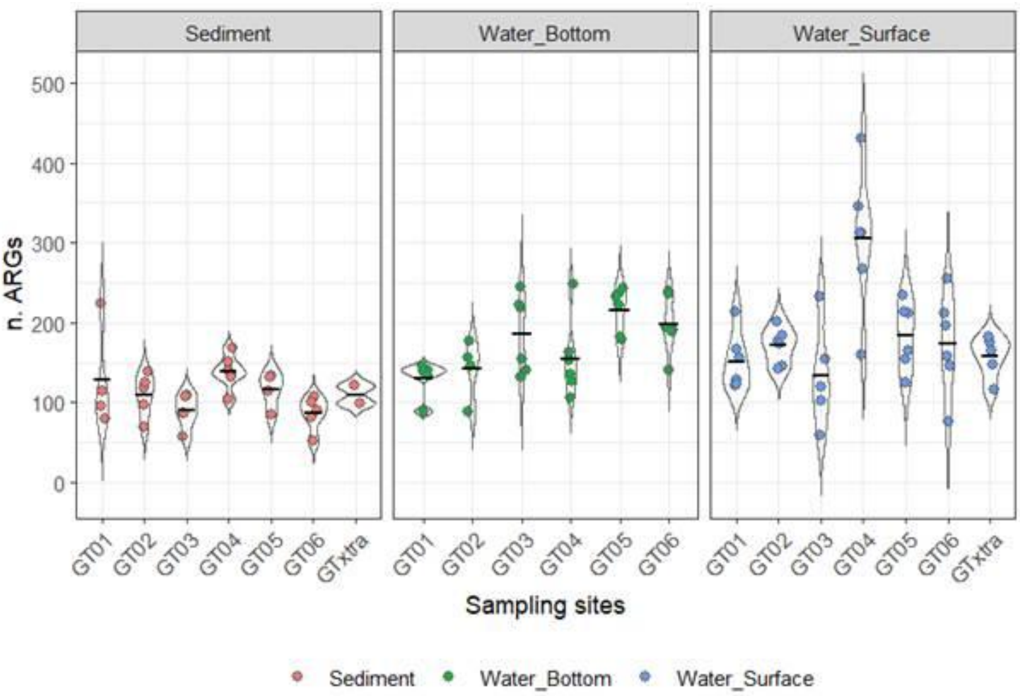

Figure 4: ARG richness (the number of unique resistance genes) in the GoT metagenomic dataset. Individual data points represent the n. unique ARGs detected at each specific sampling time.

The general distribution of ARG composition across sites and over the sampling timeframe (Figure 3) revealed a tendency to have a generalized baseline resistome all over the Gulf, with small seasonal and site-specific variations. The sediment resistome, in particular, showed a lower variability across time and the sampled sites, in terms of richness (Figure 4), diversity (Figure 5), and composition (Figure 3). This stability suggests that the benthic environment may offer a less transient condition, where factors such as temperature fluctuations and nutrient flux are less pronounced, thus allowing ARG genes to establish within the microbiome.

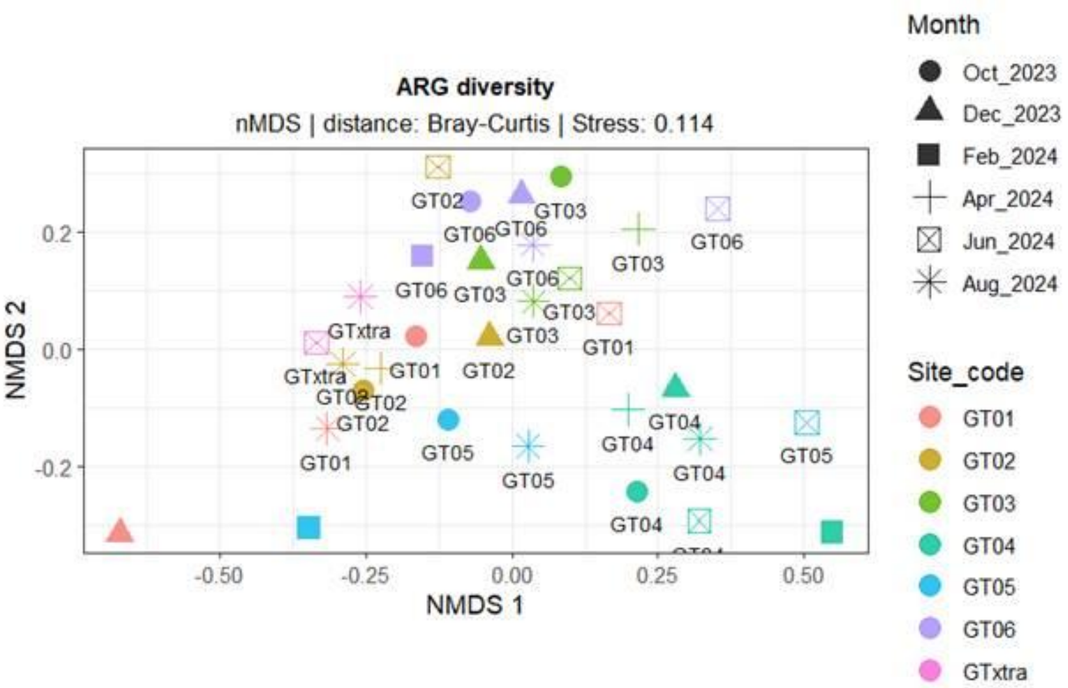

Figure 5: Non-metric Multidimensional Scaling (NMDS) ordination plot of ARG profiles in sediment samples. Dissimilarity was calculated using the Bray-Curtis distance metric.

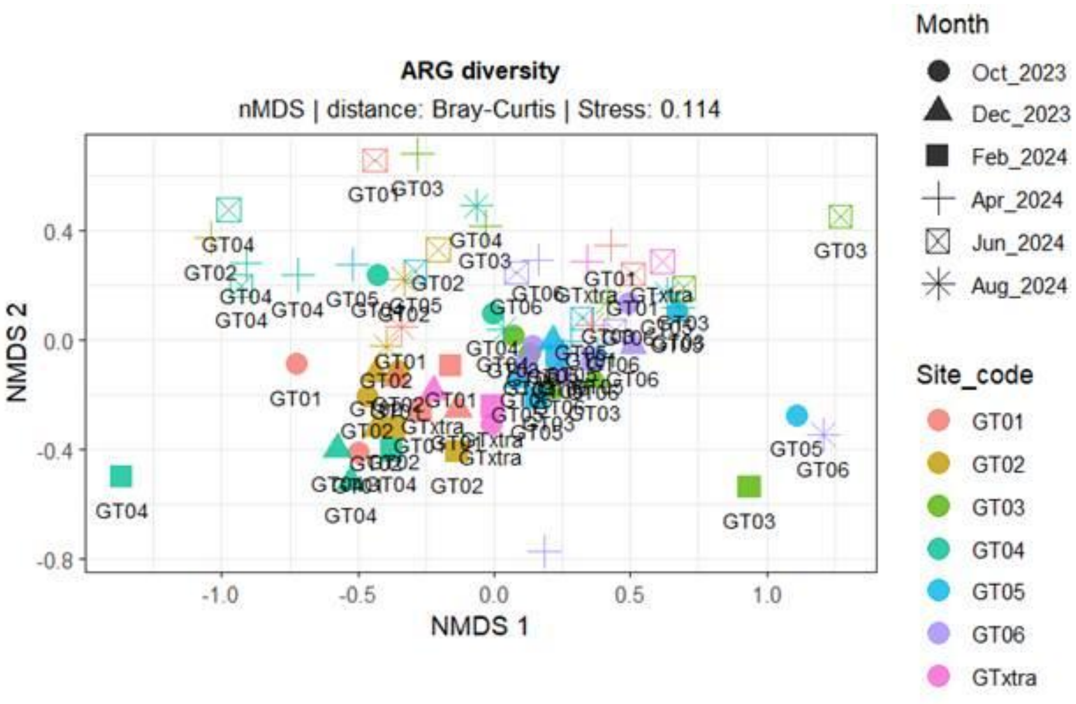

Figure 6: Non-metric Multidimensional Scaling (NMDS) ordination plot of ARG profiles in seawater samples. Dissimilarity was calculated using the Bray-Curtis distance metric.

In contrast, the water column resistome showed a tendency to seasonality, with the genetic pool of the warm months (April to August) separating from that of the cold months (October to February) in all sites but

GT05 and GT06 (Figure 6). GT05 is strongly affected by the discharge of the Trieste's municipality WWTP effluent, thus this site receives specific ARGs inputs more than other sites, that would explain the lower temporal variability. On the contrary, the GT06 point is an offshore site, where direct anthropogenic influences are clearly identifiable.

4.4.2 Organic matter dynamics

In order to infer the relative contribution of terrestrial and marine organic matter to the stations investigated in the GoT, the concentration and isotope composition of particulate and sedimentary organic matter were investigated. The GoT is a shallow basin whose hydrology, biogeochemistry and productivity are mainly influenced by rivers, the major allochthonous source of freshwater and nutrients in the area (Cozzi et al., 2012). Terrestrial organic matter delivered by rivers is generally characterized by higher C/N ratios (> 12) than marine phytoplankton (< 10; Lamb et al. 2006, Rumolo et al., 2011). The stable isotope composition of organic carbon ($\delta^{13}C$) also shows high variability. For terrestrial plants using the C3 photosynthetic pathway, $\delta^{13}C$ values usually range between −32 ‰ and −21 ‰, while $\delta^{13}C$ for C4 plants between −17 ‰ and −9 ‰. The $\delta^{13}C$ values for marine phytoplankton generally fall between −18 ‰ and −21 ‰ (Hu et al., 2006 and references therein; Lamb et al., 2006). The wide variability in both C/N ratio and organic carbon stable isotope composition among different sources of organic matter makes the combined use of these parameters a powerful tool for distinguishing autochthonous *vs.* terrestrial derived organic matter (Lamb et al., 2006; Rumolo et al., 2011).

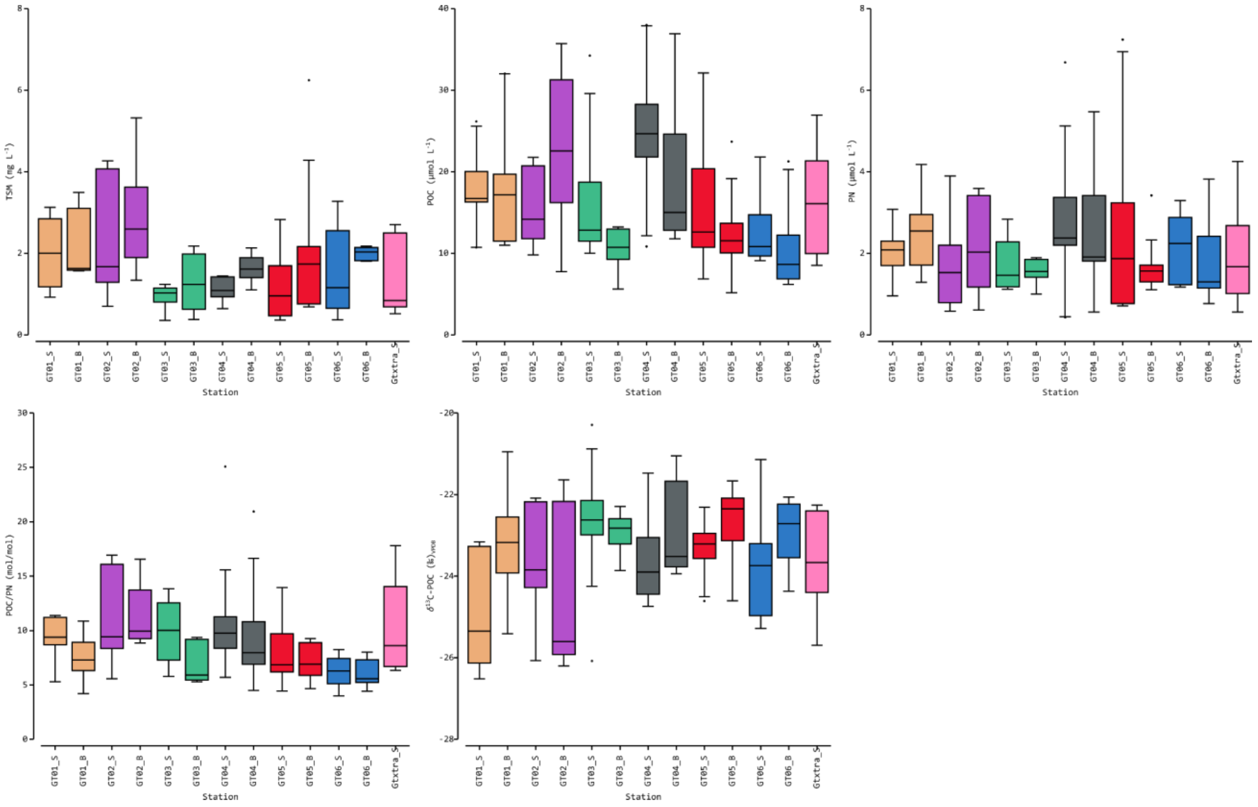

Figure 7: Boxplot describing the variability of particulate organic matter parameters for the six sampling deployments in the stations investigated (S = surface water; B = bottom water).

During the six sampling deployments, the concentration of total suspended matter (TSM; Figure 7) ranged between 0.353 mg L^{-1}, detected at station GT03 at surface in August 2024, and 6.243 mg L^{-1}, measured at station GT05 at bottom in December 2023. The stations most affected by river discharge (GT01 and GT02) were generally charachterised by higher TSM values (2.311 ± 1.196 mg L^{-1}) compared to the rest of the area investigated (1.456 ± 0.966 mg L^{-1}).

Particulate organic carbon (POC) concentration (Figure 7) ranged between 5.17 µmol L^{-1}, measured at station GT05 at bottom in February 2024, and 37.98 µmol L^{-1}, detected at station GT04 at surface in June 2024. The lowest POC concentrations were detected at station GT06, located offshore (12.14 ± 5.24 µmol L^{-1}), and at station GT03, placed within the marine protected area (13.73 ± 7.02 µmol L^{-1}). Conversely, the highest POC content was detected generally inside Trieste port (GT04: 22.02 ± 9.30 µmol L^{-1}), particularly at surface layer. POC concentration at all stations falls within the variability already observed in the Gulf of Trieste (Manna et al., 2021). The concentration of particulate nitrogen (PN) (Figure 7) was significantly correlated with TOC content ($p < 0.05$), indicating that nitrogen mainly derived from organic matter at all stations.

POC/PN ratio (Figure 7) generally fell within the typical marine range at all stations (8.9 ± 3.8). The highest variability was observed at surface layer, particularly at stations GT02 and GTxtra during October 2023 and February 2024, probably as a result of increased terrestrial inputs. The isotope composition of organic carbon (δ^{13}C-POC; Figure 7) confirms the predominantly marine origin of POC in the area investigated (average -23.44 ± 1.38 ‰). The stations most affected by river discharges (GT01 and GT02) exhibited the highest variability in δ^{13}C-POC values, as a result of the different quality of detritus and organic matter delivered by rivers in different seasons and under varying discharge regimes.

The similar characteristics of particulate organic matter in the area investigated are evident from the nMDS ordination plot (Figure 8). No specific differences were highlighted among the stations investigated by the similarity profile (SIMPROF) analysis, suggesting that the variability in concentration and quality of organic matter should be mostly related to seasonal changes in productivity and riverine inputs.

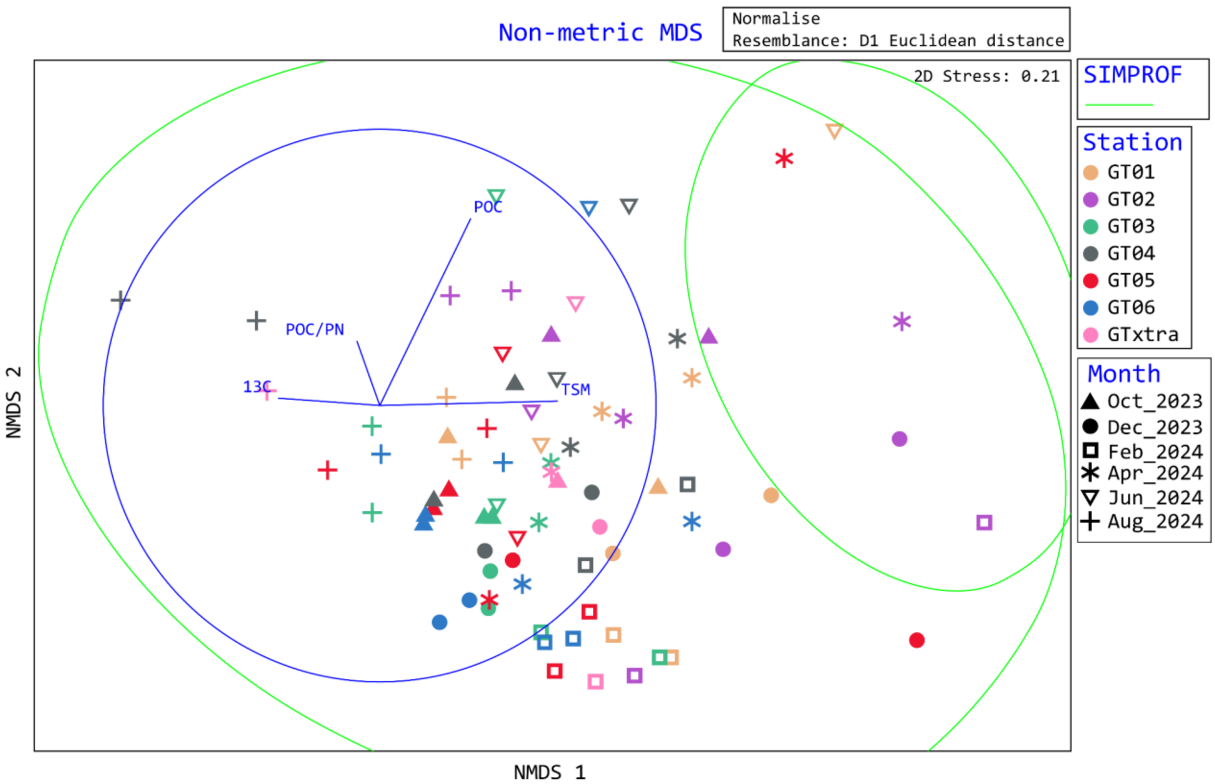

Figure 8: Non-metric Multidimensional Scaling (NMDS) ordination plot of particulate organic carbon (POC), particulate nitrogen (PN) content, C/N ratio and stable isotope composition of carbon (δ^{13}C-POC) in water column. Dissimilarity was calculated using the Euclidean distance metric. The green lines represent the groups identified by the similarity profile (SIMPROF) analysis.

Sediments in the area investigated were mainly composed of mixtures of sand and fine material, with sand content ranging between 26.0 % and 98.0 %, silt from 0.8 % to 44.0 % and clay form 0.5 % to 36.0 %. According to Nota (1958), stations GT03, GT05 and GT06 were characterized by *very sandy pelite* (30–70 % sand), while stations GT01, GT02, GT04, and GTxtra by *pelitic sand* or *sand* (>70 % sand).

High variability was observed in the concentration of total organic carbon (TOC) in the sediments of the investigated area, reflecting differences in grain size, anthropogenic pressure and river influence (Figure 9). The lowest TOC concentrations were detected, across all six sampling deployments, in station GTxtra (3.34 ± 3.50 mg C g^{-1}_{dw}), characterized by the highest content of sand. Conversely, the highest TOC concentrations were always measured inside Trieste port (GT04: 68.51 ± 10.83 mg C g^{-1}_{dw}). Stations in proximity of the WWTP pipeline (GT05) and Isonzo river mouth (GT01) showed relatively high TOC content (24.92 ± 9.90 mg C g^{-1}_{dw} and 22.56 ± 8.12 mg C g^{-1}_{dw}, respectively). TOC concentrations at the other stations (14.00 ± 5.55 mg C g^{-1}_{dw}) fell within the range of variability described for the LTER-C1 station (Franzo et al., 2019). TN concentration was significantly correlated with TOC content ($p < 0.01$), indicating that nitrogen mainly derived from organic matter at all stations.

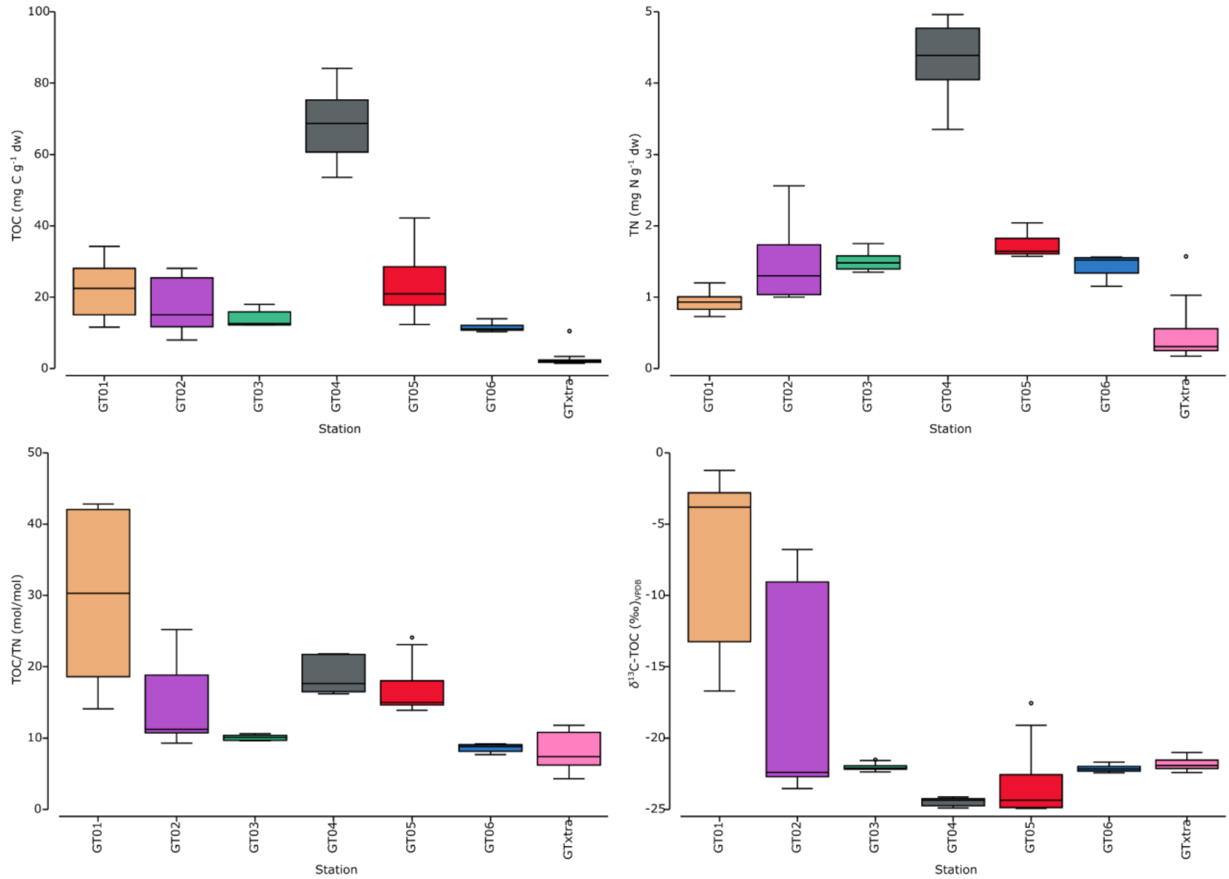

Figure 9: Boxplot describing the variability of sediment organic matter parameters for the six sampling deployments in the stations investigated.

TOC/TN ratios (Figure 9) higher than 10 characterized the stations most affected by river discharge (GT01 and GT02) and anthropogenic pressure (GT04 and GT05), whereas in the remaining area the TOC/TN ratio ranged within typical marine values. GT01 exhibited the highest TOC/TN ratios (> 20) and the greatest variability, as a consequence of fluctuations on the discharges of Isonzo river. The isotope composition of organic carbon (δ^{13}C-TOC; Figure 9) showed high variability at the stations most influenced by river discharges (GT01 and GT02), where the highest values were measured (> −10 ‰), probably related to higher content of detritus derived from C4 terrestrial plants (*e.g.* corn). Stations GT01 and GT02 also shibited the highest variability in δ^{13}C-TOC values, as a consequence of the different quality of detritus and organic matter delivered by rivers in different seasons under varying discharge regimes. The lowest values consistenlty characterized station GT04, affected by the discharge of the Trieste's municipality WWTP effluent, and station GT05, influenced by anthropogenic activities inside the port and backflow water. The δ^{13}C-TOC values detected at the other stations fall within the typical range of marine phytoplankton, highlighting the lower contribution of terrestrial organic matter.

In contrast to the results observed for particulate organic matter, the nMDS ordination plot and the similarity profile (SIMPROF) analysis (Figure 10) for sediments highlights the differences between stations in the area. Stations GT01 and GT04 exhibit significantly different characteristics than the other stations investigated, mostly related to different TOC/TN ratios, δ^{13}C-TOC values and TOC content, suggesting a higher contribution of terrestrial organic matter (GT01) and anthropogenic activities (GT04). Differences are also highlighted for station GTxtra, primarily due to the high content of sand and the low concentration of TOC. On the other hand, station GT06, located offshore, and station GT03, situated inside the marine protected area, resulted to have similar characteristics, as the sedimentary organic matter is mainly of marine origin.

The apparent contrast in the information about the origin of organic matter provided by the analysis of particulate and sedimentary organic matter depends on the different nature of these compartments. Sediments provide time-integrated information on the various sources of organic matter, whereas the particulate matter provides information specific to the moment of sampling and is subject to several processes of mixing and lateral transport that affect its distribution.

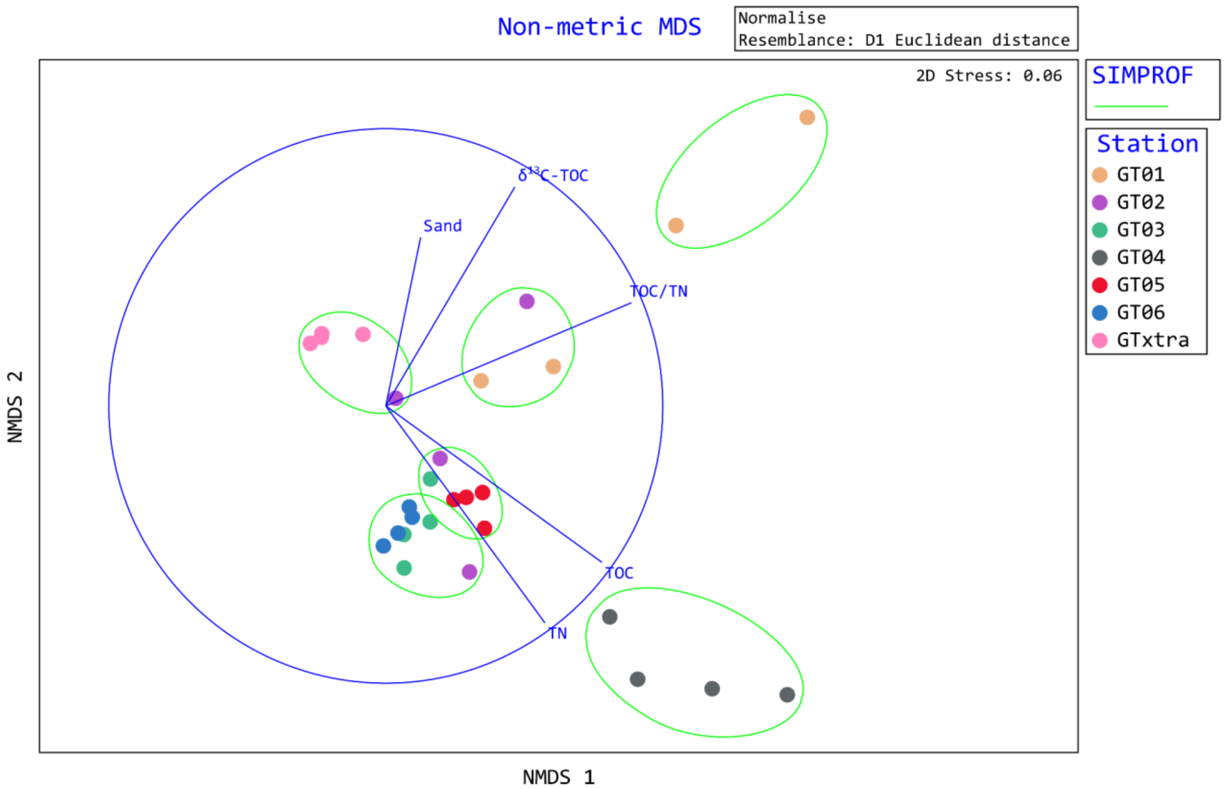

Figure 10: Non-metric Multidimensional Scaling (NMDS) ordination plot of total organic carbon (TOC), total nitrogen (TN) and sand content, C/N ratio and stable isotope composition of carbon (δ^{13}C-TOC) in sediments. Dissimilarity was calculated using the Euclidean distance metric. The green lines represent the groups identified by the similarity profile (SIMPROF) analysis.

4.4.3 Modeling the fate and transport of biological pollutants

Model results for two time frames with very different hydrodynamic conditions (run-1a and run-1b, see Table 2) are presented in in Figure 11, showing t. snapshots of model outputs for August 9th 2023 and November 3rd 2023. The first period shows the typical cyclonic circulation pattern of the Gulf, with a major counterclockwise gyre and the jet stream coming from the Isonzo river, as one can see from the particles dynamic in Figure 11a and b. The modeled particle trajectories were completely different during the second period (Figure 11c and d), which included a sequence of storm surge events (October 31, November, 1, and 3) and was characterized by peculiar oceanographic conditions that caused a deviation of the water masses toward the coast (Busetti et al., 2024). This dynamic is reflected in the higher amount of particles in the coastal areas, particularly in the scenario with longer ARGs half-life (7 days, Figure 11c). The comparison between model runs with different half-life times highlight that this parameter is critical to determine whether ARGs spread over large areas rather than remaining relatively close to their sources, pointing out the need for a better characterization of the dynamics underlying the degradation of biological pollutants in marine ecosystems.

To assess the influence of each WWTP on the 6 sampling stations, we computed the percentage of particles present in the surroundings of each sampling station at the sampling date, tracking back their source of origin. The analysis considered a 1 km^2 square area centered on each location, and the results are averaged over one day (Figure 12).

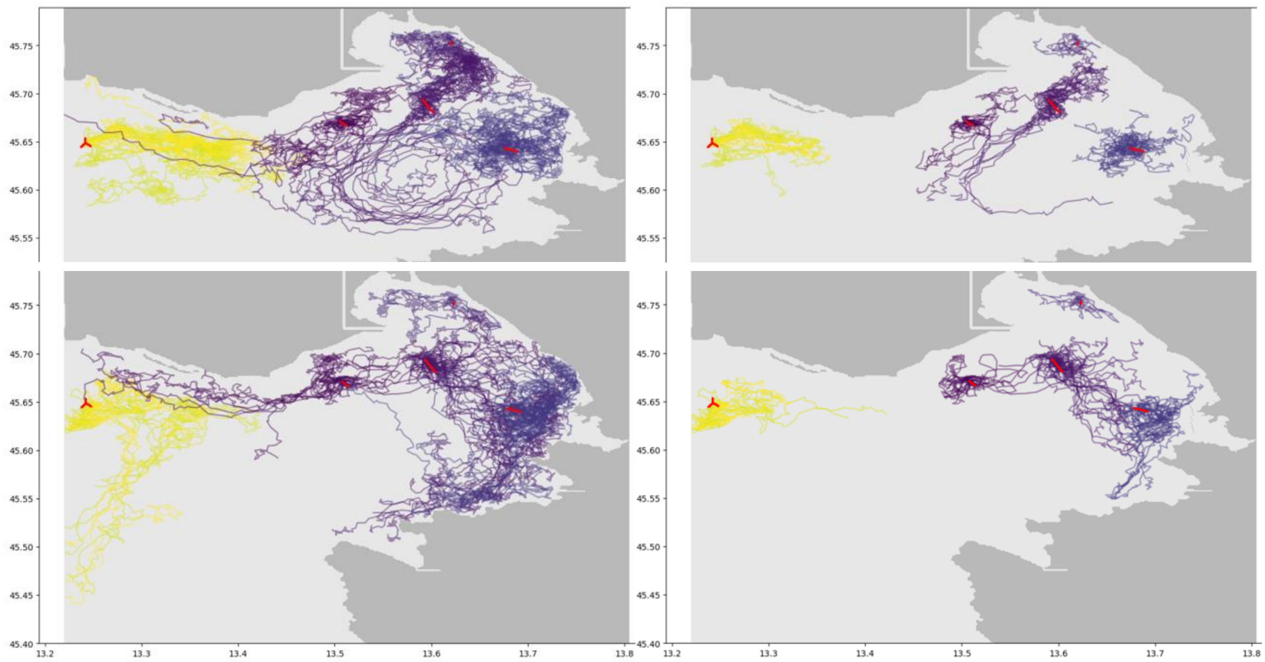

Figure 11: Trajectory of ARGs particles released from the wastewater treatment plants (WWTPs), shown as red points, in the Gulf of Trieste. Top panels show the results for August 9th 2024 (a and b), bottom panels for November 3rd, 2024 (c and d). Panels a) and c) show model results assuming particles with half-life of 7 days, while in b) and d) a faster degradation process is assumed (half-life of 1 day). Each color is associated with the WWTP of origin.

The resulting pollutant distributions are qualitatively evaluated against experimental data in order to evaluate the modelling approach and assess its reliability as a predictive tool for dispersion under present conditions as well as future scenarios. This integrated approach allows for a better data interpretation, enlightening also the connectivity among sampling sites. To this end, we run simulations targeting the days of the sampling (run2-a1 and run2-a7 for October 11th, and run2-b1 and run2-b7 for November 17th).

The barplots in Figure 13 synthesizes the results of this analysis in a quali-quantitative way for all the cases presented in Table 2: summer case (Figure 5a), winter -storm surge case- (Figure 13b), first sampling campaign of October (Figure 13c) and the second one of November (Figure 13d). During all simulated time frames, emissions from WWTPs 20 and 21, located south of the GoT, did not impact any sampling stations, likely due to particle degradation prior to reaching them combined with the circulation in the region.

No particle from WWTP 3, 20, and 21 WWTPs reached any of the sampling stations, while particles originating from WWTP 6 (Servola WWTP plant) were spread more effectively and could reach stations GT05 (100%), GT06 (2%), and at a smaller percentage also GTxtra (0.01%). Input from WWTP 4 was predicted to reach only station GT06 (4.2%). A small percentage of particles from WWTP 5 were detected at GT01 and GT02, while no particles were transported towards the coastal stations GT03 and GT04 from the WWTPs. In other words, at station GT05, ARGs and other BPs comes with highest probability from WWTP 6, which is expected due to the proximity of the station to the source, while at GT06 station, there is an equal probability for particles to originate either from WWTP 6 or 4. A non-negligible quantity of ARGs from source WWTP 5 can be detected at GT01. A small quantity reached the coastal station GTxtra from WWTP 6, while the close-by locations GT03 and GT04 seemed to be unaffected by WWTP inputs. This is most probably because they are inside local small recirculation that prevent particles from entering their region. Finally, GT02 intercepts a small number of particles from its closest source (WWTP 5).

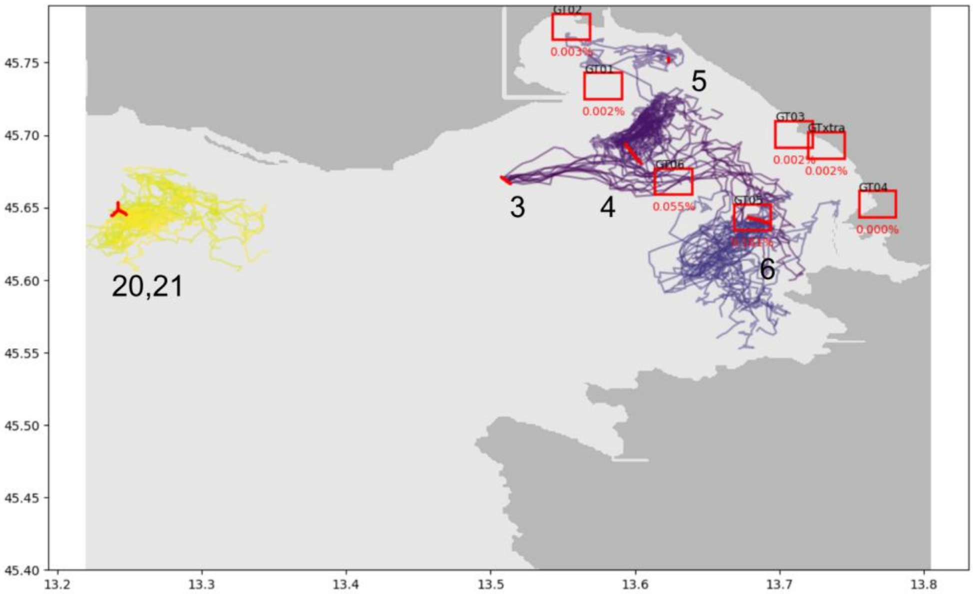

Figure 12: Example of a simulation with HL=1 day. The red boxes represent the experimental location areas, the red dots the diffuser, with the WWTP name label in black.

The same analysis is repeated for November 17th (Figure 13d). At the time of the analysis, MITgcm data were available until November 11th, and for the last few days we extrapolated results using the latest available flow field. The outcome for each station is hereafter summarized: at GT05 almost all the ARGs particles were from WWTP 6 and very few ones from WTTP4, at GT06, a large percentage of ARGs originated from source WTTP 4, but also some small quantities from WTTP 6 and 3; GT03, which was unaffected by WWTPs in the October sampling, was reached by ARGs from source WWTP 4 in November, while the results for stations GT01 and GT02, GTExtra, and GT04 were very similar to the previous sampling, showing an influence from WTTP 5 for the first two stations, no impacts on GT04, and limited effects from WTTP 6 on GTExtra.

The dynamics of ARGs at each sampling station is influenced both by the proximity to the source and the local circulation. The latter can evolve over a time scale of days or even in shorter periods, based on meteo-climatic conditions, which is the same order of the lifetime of the BPs, and could be subject to dramatic changes in the coming decades due to the increasing temperature of seawater. Numerical models can provide substantial support to the interpretation of field data, as shown in this work. This kind of integration between models and observations could also support in future studies the choice of the exact location for sampling stations depending on the predicted circulation in the week before the sampling.

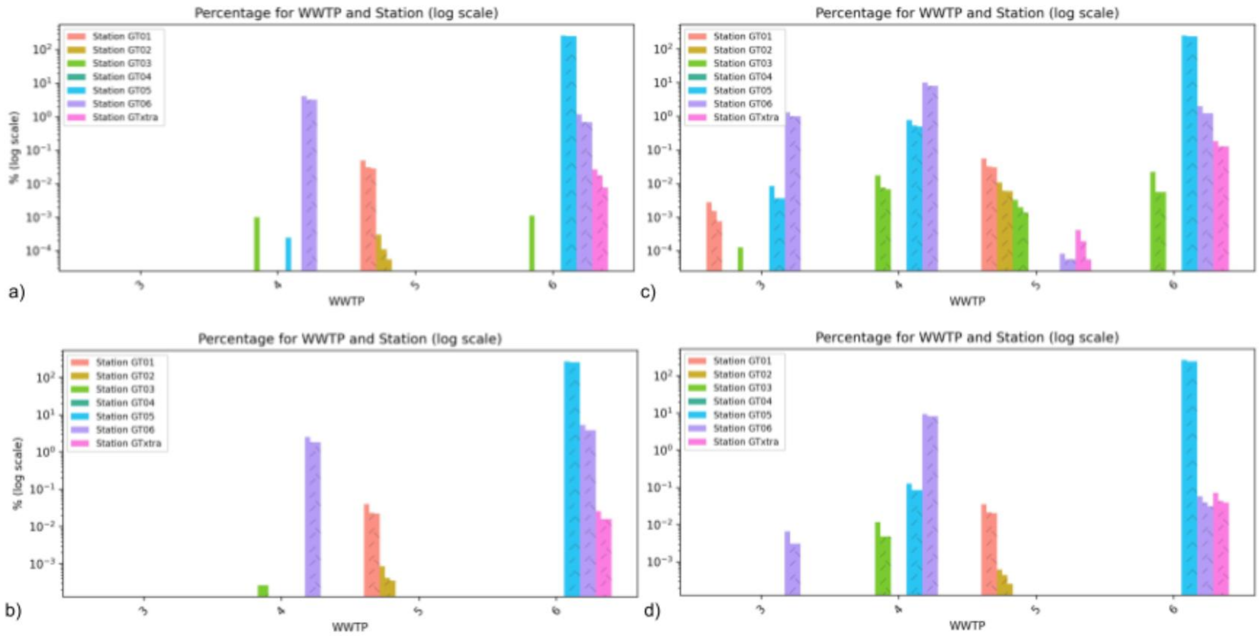

Figure 13: Histogram plot of the percentage of particles that reached each experimental station, from each WWTP (x-axis), for the summer case, panel a), for the winter storm surge case panel b), first sampling campaign c) second sampling campaign d).

5. Assessing the dissemination of AMR in wastewater reuse for agriculture

5.1 Introduction

Wastewater reuse has emerged as a crucial response to the escalating global demand for freshwater, driven by factors such as population growth, urbanization, and climate change. Especially in arid and semi-arid regions where water scarcity is acute, repurposing wastewater for irrigation offers a practical solution to alleviate this pressing issue and enhance food security. However, the increasing reliance on wastewater irrigation raises concerns about the dissemination of contaminants of emerging concern (CECs), including antibiotic-resistant bacteria and ARGs, into the environment.

While conventional wastewater treatment processes show some effectiveness in reducing ARB and ARGs loads, traces of these pollutants persist in treated wastewater effluents (Kampouris et al., 2021). The presence of ARGs in wastewater effluents, combined with the potential for horizontal gene transfer facilitated by mobile genetic elements, underscores wastewater treatment plants (WWTPs) as significant hotspots for the dissemination and accumulation of ARGs in soil, with potential pathways for antibiotic resistance transmission to humans through contaminated crops (Oliveira et al., 2020). Several studies have documented an increase in ARB and ARGs in soils subjected to wastewater irrigation. However, the impact of wastewater irrigation on ARG dissemination remains a subject of debate, influenced by various factors including climate conditions such as ambient temperatures and UV radiation, particularly in arid regions where these factors may promote ARG degradation (Shamsizadeh et al., 2021). In response to these challenges, our research focuses on investigating the prevalence and distribution of ARGs in the effluent of a WWTP and its receiving water body downstream, with a particular focus on the microbiome of the receiving water body. Moreover, the Microbial Source Tracking approach will be applied using the data obtained by the 16S rRNA gene amplicon sequencing (oligotyping). This will allow us to inspect the presence of fecal indicator taxa upstream, downstream, and in the effluent itself to assess for potential traces of fecal/microbial pollution with the final scope of elucidating its real source.

By examining the potential dissemination of ARGs in the environment, especially concerning the indirect reuse of WWTP effluents for agricultural purposes, our study aims to provide valuable insights into the environmental and human health risks associated with wastewater reuse, in a One Health perspective. First of all, the existing literature on the impact assessment related to wastewater reuse in agriculture was critically reviewed with a particular focus on the ARGs evaluation within this practice. Then, a comprehensive field monitoring campaign was designed, in which water samples were collected from three sampling locations (WWTP effluent, receiving water body upstream and downstream the effluent discharge point), during two different periods (in irrigation season and non-irrigation season) to characterize the bacterial community composition and track the prevalence and fate of ARGs. By elucidating these dynamics, our research seeks to inform decision-makers and stakeholders in the development of sustainable water management strategies, ultimately enhancing the resilience and sustainability of wastewater reuse systems in the face of evolving environmental challenges.

5.2 Study area

A full-scale WWTP located in northern Italy in a major urbanized area surrounded by fields devoted to agriculture was selected for this study. The WWTP was designed to treat the domestic wastewater of approximately 90,000 population equivalents (PE) and adopts a disinfection treatment step with ultraviolet (UV) radiation before discharging the effluent in an irrigation natural channel. The considered WWTP produces a daily volume of approximately 14,000 m^3 of effluent. The receiving canal is used to feed the irrigation channels downstream the effluent point of discharge. The landscape of the study area is characterized by agricultural fields where different types of crops are grown based on the topology of the area. In flat areas maize, barley, and wheat are the predominant crops, typically irrigated through surface irrigation, while in hilly areas, viticulture is prevalent, and drip irrigation is applied.

5.3 Experimental methods

5.3.1 Sampling design

The monitoring campaign was conducted during two different sampling periods, the first one during the irrigation season (mid-July to mid-August) and the second one after the irrigation season (mid-October to mid-November). Three sampling points were defined, two in the irrigation channel, upstream (RIV_UP) and downstream (RIV_DOWN) the WWTP point of discharge, and one in the WWTP effluent (EFF) at the outlet of the UV disinfection process. Each sampling point was sampled twice a week for four weeks straight during each sampling period. Thus, 16 water samples were collected for each sampling point in the whole monitoring campaign. On each monitoring day, samples of 5 L were simultaneously collected from the three sampling points around 9 am and stored in sterile containers. Temperature and electrical conductivity were recorded for each sample immediately after the sample collection; for the effluent samples, data regarding the WWTP operation (i.e., flowrate and UV dosage) were also collected. The samples were transported to the laboratory under refrigerated conditions and immediately processed upon arrival.

All the water samples (n = 48) were primarily pre-filtered in duplicate through a 200 µm nylon mesh fabric filter to remove large particulate matter and then filtered through a 0.22 µm pore-size polyethersulfone membranes, as detailed in § 4.1.3.1. Filtration volumes were defined by membrane clogging and ranged from 0.5 L up to 1.5 L. The membranes were stored at -23°C to be processed all together for the DNA extraction.

5.3.2 Molecular biology methods

Genomic DNA was isolated from the membranes using the DNeasy PowerWater Kit (Qiagen), with a few modifications to the manufacturer's protocol to maximise extraction yields. Half of each membrane was processed per bead tube, while reagent volumes remained unchanged. Samples underwent three cycles of 5-minute incubation at 70°C, followed by 2 minutes of horizontal vortexing at maximum speed. The procedure then continued as described in the kit handbook. Elution was performed twice to maximise nucleic acid recovery from the spin columns. DNA concentration was measured using a Qubit Fluorometer (Thermo Fisher), and quality was assessed by agarose gel electrophoresis and Nanodrop spectroscopy. DNA extracts were stored at -80°C until further processing.

DNA extracts were diluted 1:20 in nuclease-free water, based on total DNA quantification, and analysed by *real-time* PCR to quantify 7 genes conferring resistance to commonly used antibiotic families: tetracyclines (*tet*A), sulfonamides (*sul*2), macrolides (*erm*B), fluoroquinolones (*qnr*S), beta-lactams (*bla*CTX-M and *bla*OXA), and colistin (*mcr*-1). The 16S rRNA gene (specifically its V8/V9 hypervariable region) was also quantified as a housekeeping gene, to be used for normalising the gene copy number of the target ARGs.

Quantitative PCR (qPCR) assays were carried out using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) under analytical conditions previously described (Fonti et al., 2021). The primer pairs used and their annealing temperatures are shown in Table 4. Quantification was performed in two technical replicates. Absolute quantification of both the target genes was achieved by standard curve method. Standard curves were obtained by tenfold serial dilutions of a positive control with known concentration the target sequence. The initial gene copy number of each sample was then determined by interpolation from the resulting linear standard curve. In this study, qPCR assays with an amplification efficiency outside the range of 80-105% were rejected and repeated, ensuring the accuracy and reliability of the final quantification.

The specificity of the amplification reactions was verified by following qPCR assays by both melting curve analysis and visual inspection of the electrophoretic profile in an agarose gel. When multiple amplicons other than the desired one were present the sample was classified as NQ (positive but not quantifiable) for the tested gene. None of the assays performed here showed the formation of primer-dimers, while the presence of non-specific products is to be expected due to the complexity of natural environmental samples.

Table 4 - Primer sets used for qPCR.

Target	Primer name	Primer sequence (5'- 3')	Amplicon size (bp)	Annealing T (°C)
16S rRNA gene	Bact1369F Prok1492R	CGGTGAATACGTTCYCGG GGHTACCTTGTTACGACTT	142	55
ermB	ermB Fw ermB Rev	CCGAACACTAGGGTTGCTC ATCTGGAACATCTGTGGTATG	139	55
***tet*A**	tetA Fw tetA Rev	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	210	64
***sul*II**	sulII Fw sulII Rev	TCCGGTGGAGGCCGGTATCTGG CGGGAATGCCATCTGCCTTGAG	191	60
qnrS	qnrS Fw qnrS Rev	GACGTGCTAACTTGCGTGAT TGGCATTGTTGGAAACTTG	118	62
***bla*$_{CTX-M}$**	blaCTX-M Fw blaCTX-M Rev	CTATGGCACCACCAACGATA ACGGCTTTCTGCCTTAGGTT	103	60
***mcr*-1**	mcr-1 qF1 mcr-1 qR1	ACACTTATGGCACGGTCTATG GCACACCCAAACCAATGATAC	120	63

5.4 Results

Based on the literature review, it was highlighted that the presence and persistence of ARGs in wastewater treatment processes has extensively been examined, identifying WWTPs as a major source of ARGs for the environment. Instead, it remains a scarcity of studies focusing specifically on their fate and behavior in reclaimed water reuse systems. In fact, among the 165 studies selected, which primarily analyzed the impacts of wastewater reuse, only 10 studies addressed impacts related to ARGs. Most of these studies typically focus on the fate of ARGs in soil and crops (9 out of 10 studies), especially modeling how it is affected by the WWTP treatment processes (4 out of 9 studies). However, there is a conspicuous lack of comprehensive assessments regarding the potential risks posed by ARGs in reclaimed water, particularly concerning their transmission pathways and impact on the aquatic environment and human health through crop consumption. Understanding how ARGs evolve and spread through various stages of the wastewater reuse system, aiming at assessing what is the associated final risk in a One Health perspective, is crucial for developing effective mitigation strategies. Specifically, the environmental risk, intended also as the possibility of developing antibiotic resistance in aquatic ecosystems due to the presence of antibiotics, could be of significant interest, especially considering the availability of equations for assessing such risks (Bengtsson-Palme & Larsson, 2016). However, the application of these equations remains scarce in the current literature. Bridging these gaps in the literature is essential for informing evidence-based policies and practices aimed at mitigating the spread of antibiotic resistance through wastewater reuse.

Among the investigate ARGs, genes *sul*2, *erm*B, and *qnr*S were found consistently in the dataset, why genes *tet*A and *bla*CTX-M were detected sporadically. The *mcr*-1 gene was searched but never found. Due to specificity issue the gene *erm*B could not be quantifies in several river samples, both upstream and downstream the outfall point. These samples are classified as "positive but not quantifiable" (NQ). Figure 14 shows the concentration genes *sul*2, *erm*B, and *qnr*S in comparison with the concentration of the housekeeping gene.

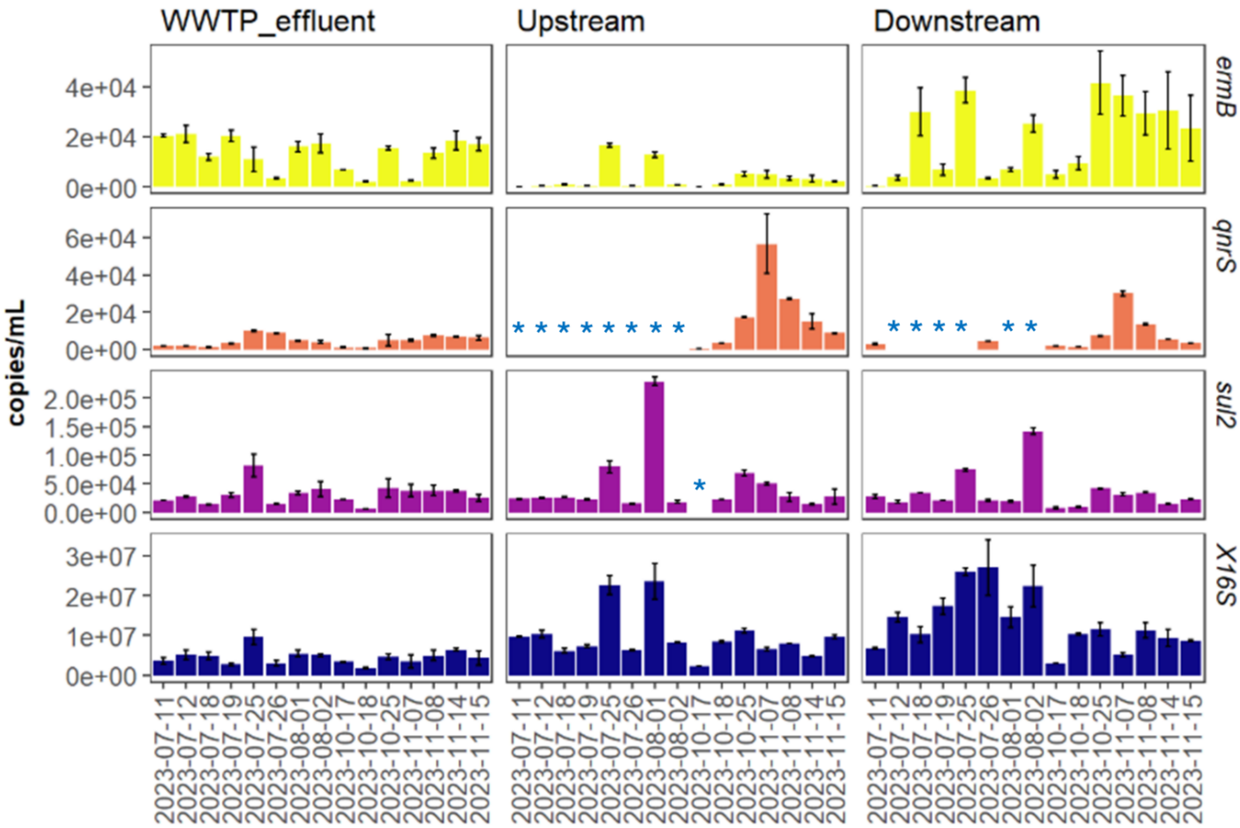

Figure 14 – Concentration of Antibiotic Resistance Genes (ARGs) and the housekeeping gene, as quantified by qPCR. Stars indicates samples that were positive to a gene but not quantifiable.

6. Conclusions

In coastal marine areas various anthropogenic pressures often converge through multiple pathways, stemming from both point and diffuse sources. These regions may exhibit hotspots of microbial contamination, including AMR, fecal pollution, and opportunistic pathogens, alongside a potential chronic background contamination. At the same time, coastal marine areas can have a role in facilitating the spread of AMR and pathogens, e.g. through the consumption of contaminated food or interaction with polluted waters during recreational activities. However, despite the widely recognized pivotal role of the environment in the establishment and dissemination of ARGs and opportunistic pathogens, information about the dynamics of such pollutants in marine environments remain fragmented. This study aims to provide an overview of the presence and distribution of ARGs and pathogens in the Gulf of Trieste, a coastal reality offering the opportunity to investigate the effects of the interplay of multiple anthropogenic and environmental factors.

WWTPs have been identified among the major sources of ARGs for the environment. Currently, the inadequate investigation of the consequences associated with the presence of ARGs wastewater reused in agriculture represents an important knowledge gap. While existing studies acknowledge the presence of ARGs in soil and crop compartments resulting from wastewater irrigation, scientific research efforts should extend beyond the mere identification of ARGs to encompass a comprehensive assessment of their potential adverse effects on both environmental and human health risks. Unraveling the dynamics of how ARGs evolve and propagate throughout the stages of wastewater reuse is now necessary for a comprehensive assessment of associated risks, towards the development of evidence-based policies and effective mitigation strategies.

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