# D4.5.2 Report

February 2022
Version n.1

# PROJECT AdSWiM

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|---|---|
| Work Package: | 4. Innovative solutions in analytical, microbiological controls and to treat urban wastewaters (UWW) |
| Activity: | 4.5 IAMD – Survey of emerging pathogens |
| Phase Leader: | OGS |
| Deliverable: | D4.5.2 by OGS |

| | | | |
|---|---|---|---|
| Version: | Final 1.0 | Date: | 21 February 2022 |
| Type: | Report | | |
| Availability: | Confidential | | |
| Responsible Partner: | OGS | | |
| Editor: | Viviana Fonti [OGS] | | |
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# 1 BACKGROUND

Treated and untreated urban wastewaters (i.e., sewage) represent important sources of microbial pollution for marine coastal areas (Bruschi et al., 2021; Medlicott et al., 2020; Nogales et al., 2011). Despite last decades' interventions within regional and communitarian programs, the majority of the objectives addressing the management of urban wastewater have not been achieved or partially achieved, with the Mediterranean Sea still receiving poorly treated sewage. Discharge of untreated or minimally processed wastewater is still a common practice in certain Mediterranean countries, while other regions face chronic malfunctioning of a few wastewater treatment plants (WWTPs) (Court of Justice of the European Union, n.d.; European Environment Agency, 2020). Basic mechanical treatments (i.e., "primary treatments") aim at removing solids, fats, and sand. Still, they do not substantially affect fecal pollution indicators (e.g., fecal coliforms and streptococci, fecal sterols, and potentially pathogenic bacteria – i.e., PPB) nor bacterial community structure (Paliaga et al., 2017). Most WWTPs are designed to remove nutrients and biodegradable organic compounds and reduce the pathogen load through additional aerobic biological processes (i.e., "secondary treatment") and a final disinfection step. However, conventional treatments do not target most emergent contaminants, including antibiotic-resistant genes (ARGs), antibiotic-resistance bacteria, and opportunistic pathogens, with urban WWTPs becoming a major source of microbial pollution (di Cesare et al., 2020; Hendriksen et al., 2019; Pazda et al., 2019; Wang et al., 2020). Marine coastal environments, as the WWTP effluent receiving bodies, have the potential of disseminating and promoting the establishment of ARGs and other microbial pollutants, thus facilitating the emerging of new antimicrobial resistance (AMR) pathways and the exposure of millions of people to disease and opportunistic pathogens (Forsberg et al., 2012; Lupo et al., 2012; Newton and McClary, 2019). Recent studies have shown that people's probability of contracting gastrointestinal and non-gastrointestinal illnesses increases in correlation with aquatic recreational activities because of antibiotic-resistant bacteria and other community-acquired microorganisms (Cabral-Oliveira et al., 2015; Leonard et al., 2018a). Furthermore, frequent bathing in coastal waters has been shown to be associated with gut colonization by *E. coli* harboring ARGs, including, for example, *bla*CTX-M genes, which provide resistance to clinically relevant antibiotics (cefotaxime) and which are easily mobilized via horizontal gene transfer (HGT), thus increasingly found in natural environments as well as in WWTPs (Leonard et al., 2018b).

The microbiological quality of coastal water is routinely assessed by enumerating Fecal Indicator Bacteria (FIB) (i.e., *Escherichia coli* and enterococci) through culture-based methods. However, FIB association with gastrointestinal and non-gastrointestinal diseases is currently questioned. Several bacteria occurring in natural environments and causing infectious disease are not primarily associated with human or animal fecal contamination. At the same time, studies have shown that substantial populations of fecal bacteria can persist and regrow in aquatic environments, followed by the discovery of environmentally adapted populations of *E. coli* and by retrieving fecal bacteria in the absence of obvious fecal sources. Moreover, most bacteria are or become unculturable because of specific metabolic requirements, physiological conditions, or because damaged by the disinfection process (Di Cesare et al., 2020).

It is also worth to point out that routine monitoring of coastal waters is specifically addressed to the assessment of the quality of bathing waters, whereas the control of the environmental status in areas of treated wastewater discharge is spatially scattered and mainly dependent on local/regional policies. In this respect, information about the diffusion of ARGs and of non-conventional, potentially pathogenic

bacteria in the Mediterranean is very scarce. To the best of our knowledge, no dedicated study on the presence of ARGs in Adriatic waters has been performed so far. Driven by this paucity of data, we aimed at depicting the transfer of these types of microbial pollution from WWTPs to seawater, following the hypothesis that the sewage treatment technology affects not only the spreading of classical FIB but also the abundance of other potential pathogens and ARGs.
Within Activity 4.5 we characterized the prokaryotic community in treated wastewater and in the seawater affected by the discharge of selected WWTPs and performed a survey of ARGs.

# 2 METHODS

## 2.1 SAMPLING ACTIVITIES

Six urban WWTPs with outfalls in the Adriatic Sea were object of investigation, in accordance with the decisions taken by the project PPs, as reported in D3.3.2. We collected both samples of treated wastewater (taken upstream the injection into the discharging pipeline) and seawater in the proximity of the main outfall points (Tab. 1).

**Table 1.** List of collected samples.

| Site | Sampling day at WWTP | Sampling day at sea | Marine station coordinates |
|---|---|---|---|
| Francavilla al Mare | 28 April 2020 | 28 April 2020 | 14.275°E; 42.464°N |
| | 29 May 2020 | 29 May 2020 | |
| | 17 June 2020 | 17 June 2020 | |
| | 21 July 2020 | 21 July 2020 | |
| | 28 August 2020 | 28 August 2020 | |
| | 22 September 2020 | 22 September 2020 | |
| Zadar | 30 July 2019 | 30 July 2019 | 15.236°E; 44.087°N |
| | 13 September 2019 | 13 September 2019 | |
| | 7 November 2019 | 7 November 2019 | |
| | 22 April 2020 | 22 April 2020 | |
| | 18 May 2020 | 18 May 2020 | |
| | 18 June 2020 | 18 June 2020 | |
| | 27 July 2020 | 27 July 2020 | |
| | 26 August 2020 | 26 August 2020 | |
| | 29 September 2020 | 29 September 2020 | |
| Katalinića Brig | 31 July 2019 | 31 July 2019 | 16.453°E; 43.490°N |
| | 27 February 2020 | 27 February 2020 | |
| | 23 April 2020 | 23 April 2020 | |
| | 28 May 2020 | 28 May 2020 | |
| | 26 June 2020 | 26 June 2020 | |
| | 22 July 2020 | 22 July 2020 | |
| | 21 September 2020 | 21 September 2020 | |
| Stobreč | 31 July 2019 | 31 July 2019 | 16.518°E; 43.482°N |
| | 27 February 2020 | 27 February 2020 | |
| | 23 April 2020 | 23 April 2020 | |

| | | | |
|---|---|---|---|
| | 28 May 2020 | 28 May 2020 | |
| | 26 June 2020 | 26 June 2020 | |
| | 22 July 2020 | 22 July 2020 | |
| | 21 September 2020 | 21 September 2020 | |
| Lignano Sabbiadoro | 30 April 2019 | 30 April 2019 | 13.1707°E; 45.643°N |
| | 26 May 2019 | 26 May 2019 | |
| | 27 June 2019 | 27 June 2019 | |
| | 17 July 2019 | 17 July 2019 | |
| | 29 August 2019 | 29 August 2019 | |
| | 08 October 2019 | 08 October 2019 | |
| | 27 April 2020 | 27 April 2020 | |
| | 27 May 2020 | 27 May 2020 | |
| | 24 June 2020 | 24 June 2020 | |
| | 29 July 2020 | 29 July 2020 | |
| | 02 September 2020 | 02 September 2020 | |
| | 01 October 2020 | 01 October 2020 | |
| San Giorgio di Nogaro | 30 April 2019 | 30 April 2019 | 13.2429°E; 45.6553°N |
| | 26 May 2019 | 26 May 2019 | |
| | 27 June 2019 | 27 June 2019 | |
| | 17 July 2019 | 17 July 2019 | |
| | 29 August 2019 | 29 August 2019 | |
| | 08 October 2019 | 08 October 2019 | |
| | 27 April 2020 | 27 April 2020 | |
| | 27 May 2020 | 27 May 2020 | |
| | 24 June 2020 | 24 June 2020 | |
| | 29 July 2020 | 29 July 2020 | |
| | 02 September 2020 | 02 September 2020 | |
| | 01 October 2020 | 01 October 2020 | |

The sampling activities were carried out (almost) monthly in spring and summer 2019 and 2020 (from April to September) in the northern Adriatic sites and only in 2020 in the Central Adriatic sites. We collected extra samples in the Croatian sites in July 2019 and autumn 2019 / winter 2020. Samples of treated sewage were taken before unloading, just upstream the injection into the discharging pipeline. Seawater samples were collected in the proximity of each WWTP outfall by means of 5L Niskin bottles, 1 m above the main diffusion point.

## 2.2 MICROBIAL COMMUNITY CHARACTERIZATION

Seawater and sewage samples were filtered onto 0.22 µm hydrophilic PES membrane filters (PALL Laboratory) until clogging of membrane pores and used for the isolation of genomic DNA. Filtered volumes were in the range of 1-2L for seawater samples and 40-500 mL for sewage samples, respectively. DNA extraction was performed through the DNeasy PowerWater Kit (Qiagen) following the manufacturer's protocol, with few modifications aimed at increasing the extraction yield (Celussi et al., 2018; Fonti et al., 2021).

The V4-V5 hypervariable region of the 16S rRNA gene was amplified using universal primer pair 515F-Y/926R (Parada et al., 2016). Libraries were prepared following the Illumina protocol (Illumina, San

Diego, CA, USA) at the ARGO Open Lab Platform (Area Science Park, Trieste, Italy). Samples were sequenced in multiple runs. Since the library size of the second run was up to 10× higher than the first one, we subsampled the second run reads prior to any bioinformatics elaboration. Reads processing was performed using the "DADA2" package (v 1.20.0) in R 4.1.0 (Callahan et al., 2016; R Core Team, 2021). Non-target-length sequences and any singletons arisen after reads merging and chimera removal were filtered out. Taxonomy was assigned using the naive Bayesian classifier (Wang et al., 2007) and SILVA SSU reference database version 138 (Quast et al., 2013; Yilmaz et al., 2014). The subsequent downstream analyses (i.e., inspection, visualization, and pre-processing) were performed in R 4.1.0 using "phyloseq" package (McMurdie and Holmes, 2013). ASVs with unassigned domain and those taxonomically assigned as chloroplast, mitochondria, and Eukaryota were removed. Phyla with both extremely low prevalence and read count were filtered out in preliminary inspection phases. Sequences were then imported into QIIME2 (Bolyen et al., 2019), aligned using MAFFT (Katoh and Standley, 2013) and used to infer an unrooted phylogenetic tree based of the FastTree (Price et al., 2010).
A subset of taxa was further investigated as microbial signature of fecal pollution, according to the approach proposed by Newton et al. (2013). The families Enterobacteriaceae and Enterococcaceae (which include the genera *Escherichia* and *Enterococcus*, respectively) was here used to investigate traditional fecal indicator taxa. As alternative fecal indicator taxa we used: i) five feces-associated bacterial families (i.e., Bacteroidaceae, Porphyromonadaceae, Clostridiaceae, Lachnospiraceae, and Ruminococcaceae) as indicators of human and non-human fecal contamination, and ii) three sewer infrastructure-associated bacterial genera (i.e., *Acinetobacter*, *Arcobacter*, and *Trichococcus*) as indicator of sewage contamination.

## 2.3 Quantification of Antibiotic Resistance Genes (ARGs)

DNA extracts were diluted in nuclease-free water (i.e., 10× in the case of seawater samples, 10-100× for treated sewage samples) and analyzed for the quantification of 16S rRNA gene (as housekeeping gene), the *int*I1 gene (as proxy of anthropogenic pollution), and 8 ARGs genes conferring resistance to commonly used antibiotic families, such as tetracyclines (*tet*A), sulfonamides (*sul*2), macrolides (*erm*B), fluoroquinolones (*qnr*S), beta-lactams ($bla_{TEM}$, $bla_{CTX-M}$, and $bla_{OXA}$), and colistin resistance gene (*mcr*-1). qPCR assays were performed in SYBR green chemistry (SsoAdvanced universal SYBR Green supermix, Bio-Rad), using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) in the analytic conditions previously described (Di Cesare et al., 2015). Details about the primer pairs used in this study are shown in the Supplementary Materials with the respective annealing T (Bibbal et al., 2007; Bontron et al., 2016; Di Cesare et al., 2015, 2013; Marti et al., 2013; Marti and Balcázar, 2013; Ng et al., 2001; Pei et al., 2006; Subirats et al., 2017). Quantification was performed in two technical replicates. Synthetic consensus sequences from the Comprehensive Antibiotic Resistance Database (CARD; McArthur et al., 2013) cloned into the pEX-A128 plasmid vector (Eurofins) were used to build the standard calibrations curves, after linearization as PCR products to avoid issues due to supercoiled circular configuration (Hou et al., 2010). ARG abundances were expressed as ARG copies per copy of 16S rRNA gene to prevent differences among sample cell abundances from becoming a confounding factor.

**Table 2.** Primer pairs used for qPCR.

| Target | Primer name | Primer sequence (5'- 3') | Amplicon size (bp) | Annealing T (°C) | Reference |
|---|---|---|---|---|---|
| 16SrDNA | Bact1369F | CGGTGAATACGTTCYCGG | 142 | 55 | (Di Cesare et al., 2015) |
| | Prok1492R | GGHTACCTTGTTACGACTT | | | |
| *ermB* | ermB Fw | CCGAACACTAGGGTTGCTC | 139 | 55 | (Di Cesare et al., 2013) |
| | ermB Rev | ATCTGGAACATCTGTGGTATG | | | |
| *tet*A | tetA Fw | GCTACATCCTGCTTGCCTTC | 210 | 64 | (Ng et al., 2001) |
| | tetA Rev | CATAGATCGCCGTGAAGAGG | | | |
| *sul*II | sulII Fw | TCCGGTGGAGGCCGGTATCTGG | 191 | 60 | (Pei et al., 2006) |
| | sulII Rev | CGGGAATGCCATCTGCCTTGAG | | | |
| *qnrS* | qnrS Fw | GACGTGCTAACTTGCGTGAT | 118 | 62 | (Marti and Balcázar, 2013) |
| | qnrS Rev | TGGCATTGTTGGAAACTTG | | | |
| $bla_{CTX-M}$ | blaCTX-M Fw | CTATGGCACCACCAACGATA | 103 | 60 | (Marti et al., 2013) |
| | blaCTX-M Rev | ACGGCTTTCTGCCTTAGGTT | | | |
| *mcr*-1 | mcr-1 qF1 | ACACTTATGGCACGGTCTATG | 120 | 63 | (Bontron et al., 2016) |
| | mcr-1 qR1 | GCACACCCAAACCAATGATAC | | | |
| $bla_{TEM}$ | blaTEM Fw | TTCCTGTTTTTGCTCACCCAG | 112 | 60 | (Bibbal et al., 2007) |
| | blaTEM Rev | CTCAAGGATCTTACCGCTGTTG | | | |
| $bla_{OXA}$ | Oxa-rt Fw | AGGCACGTATGAGCAAGATG | 189 | 60 | (Subirats et al., 2017) |
| | Oxa-rt Rev | TGGCTTGTTTGACAATACGC | | | |

# 3 RESULTS: CENTRAL ADRIATIC AREA

Four WWTPs were investigated in the central Adriatic area: Katalinića and Stobreč WWTPs (Split) were designed for basic mechanical treatment, while the Zadar WWTP and the Francavilla al Mare WWTP included the sewage bio-oxidation in activated sludge tanks and/or other plant units for the abatement $BOD_5$ (i.e., secondary treatments). Treated sewage in the Francavilla al Mare WWTP underwent disinfection with peracetic acid prior to unloading into the discharging pipeline.

A total of 29 treated sewage samples and 29 seawater samples were collected in the sampling points located in the central Adriatic area and, subsequently, processed for DNA isolation. However, due to the purchase of defective DNA extraction kits, a group of samples were extracted with low efficiency, causing the loss of 15 samples (1 of treated sewage and 14 of seawater). For this reason, data was screened for potential batch effects.

## 3.1 Characterization of microbial communities by High-Throughput Sequencing

Bacterial community structure in treated sewage was mainly dominated by Proteobacteria (29.0–75.9%), Firmicutes (1.4–49.2%), Bacteroidota (2.9–40.4%), and Actinobacteriota (1.2–20.8%), as shown in Figure 1. A PCoA based on Bray-Curtis distances (Fig. 2) showed that treated sewage samples obtained from Katalinića and Stobreč WWTPs (i.e., primary treatment only) were very similar in bacterial community structure and different from those obtained by Francavilla and Zadar WWTPs, which formed two separated clusters. In the presence of secondary treatment (i.e., Francavilla and Zadar WWTPs) Firmicutes decreased significantly (SIMPER, fdr corrected p-value < 0.001) by 24.7% on average, in favor of Actinobacteriota, Chloroflexi, Patescibacteria and Planctomycetota (SIMPER, fdr corrected p-value < 0.01 for Actinobacteriota, p-value < 0.001 for the others).

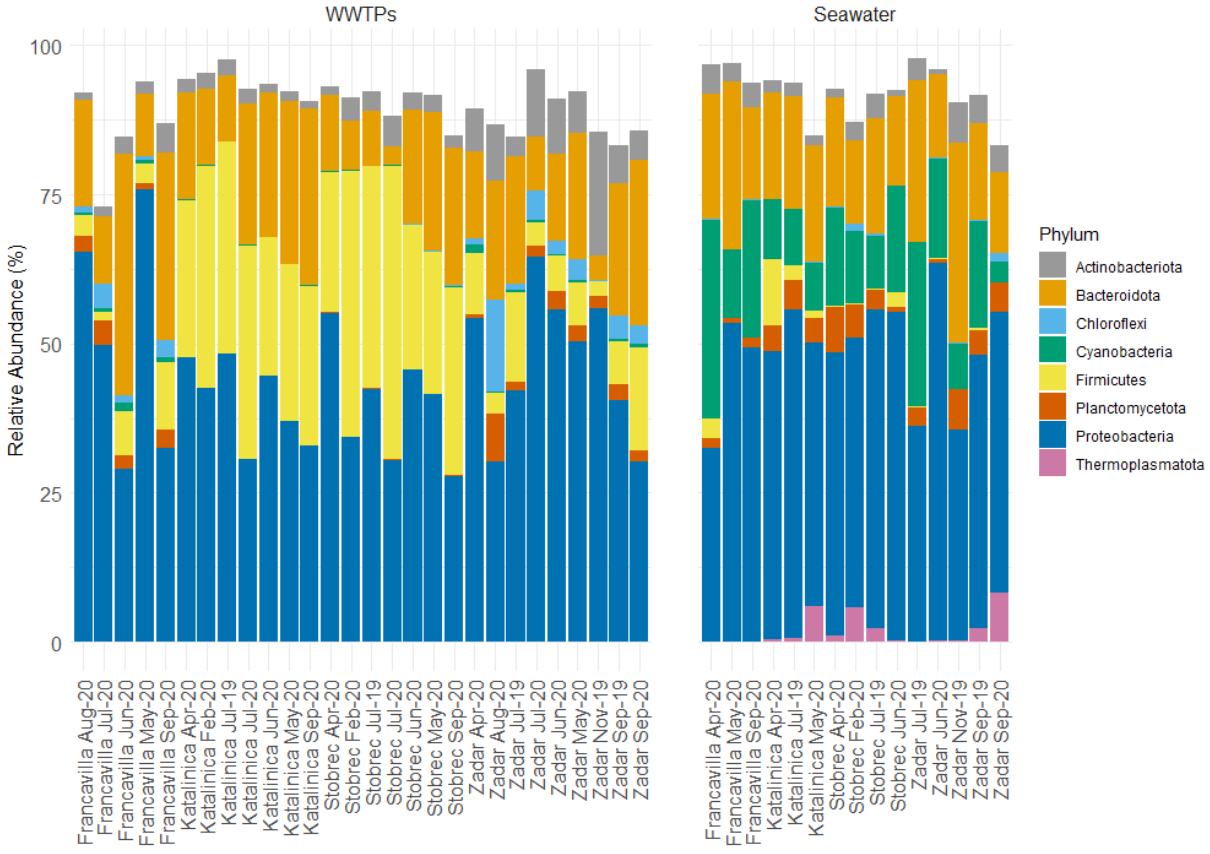


**Figure 1.** Prokaryotic community composition at phylum level of treated sewage (WWTPs) and seawater samples based on 16S rDNA amplicon sequencing. Only top phyla are depicted.

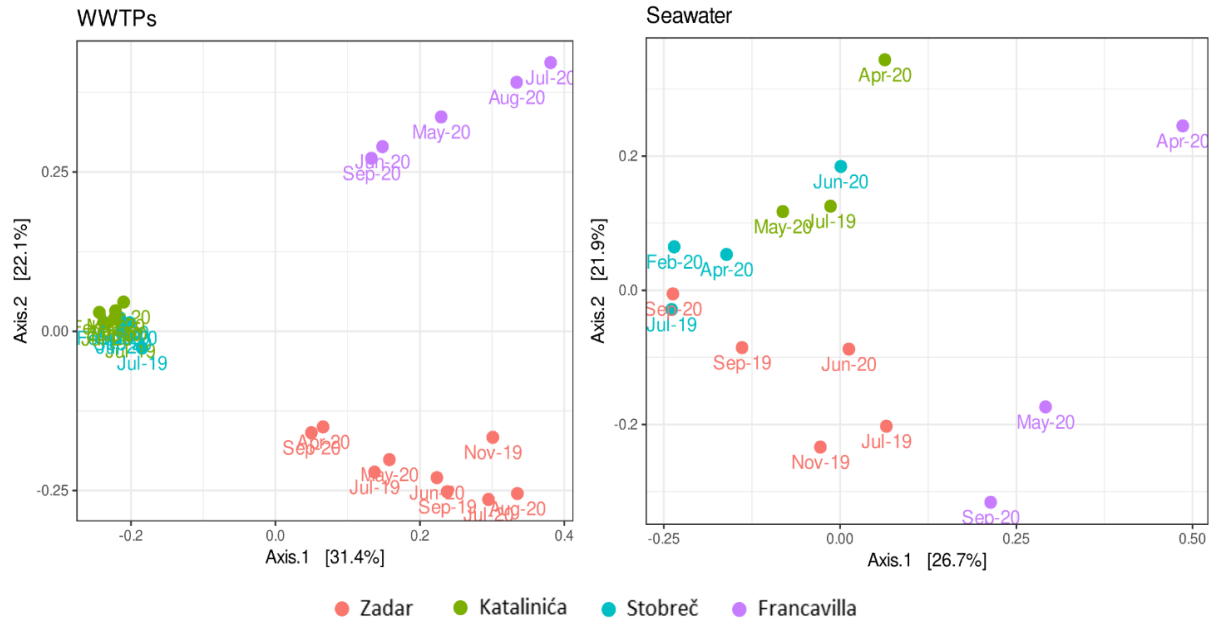


**Figure 2.** Sample ordination by PCoA on Bray-Curtis distances. In Katalinića and Stobreč WWTPs, sewage undergoes only basic mechanical treatments (i.e., "primary treatments"). Zadar and Francavilla WWTPs performed activated sludge treatment subsequently to primary treatments. Only in the case of Francavilla WWTP treated sewage undergoes a final disinfection step.

Francavilla WWTP effluent had an average percent contribution of Actinobacteriota lower than the Zadar WWTP by 10.1% (SIMPER, fdr corrected p-value < 0.01). Deinococcata, Acidobacteriota, and Myxococcota contribute to minor (< 5%), yet significant, differences between the two WWTPs.

The bacterial community structure in seawater samples collected next to WWTP outfalls was mainly dominated by Proteobacteria (45.1–50.2%), Bacteroidota (16.6–21.6%), Cyanobacteria (9.2–22.6%), and in lower contribution by Actinobacteriota (2.0–4.1%) and Planctomycetota (1.3–4.5%). Firmicutes accounted for relative abundances less than 1%, except for samples collected next to the outfall pipe of Katalinića WWTP (i.e., average relative abundance 4.9%, Fig. 1). None of the phyla that contributed the most to the within-group similarities (i.e.,> 70% as assessed by SIMPER) varied in a statistically significant fashion. Fig.2 suggests showed a mild differentiation based on sampling site or type of treatment performed in the corresponding WWTP.

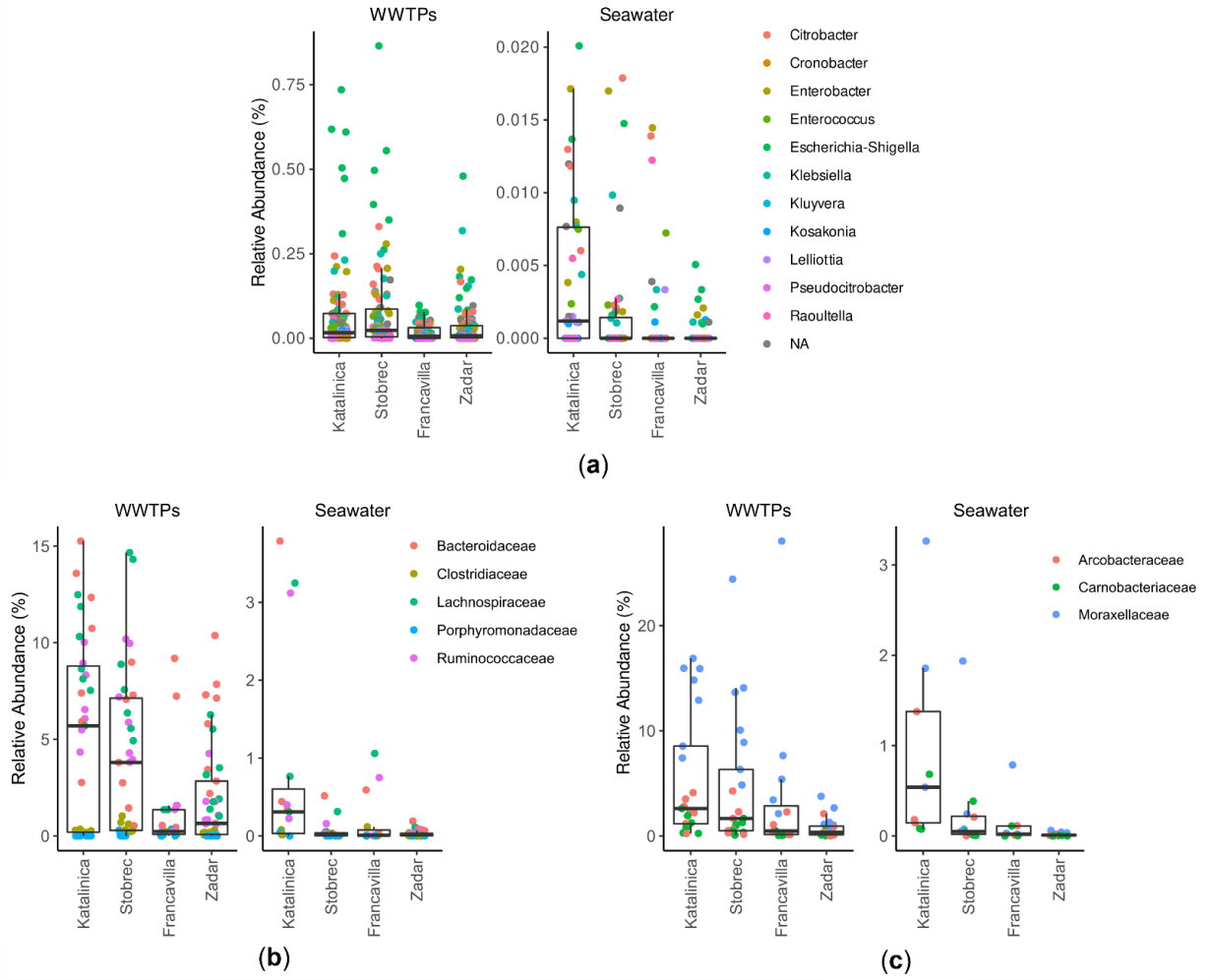


**Figure 3**. Fecal indicator bacteria in treated sewage (WWTPs) and seawater samples. (a) Traditional fecal indicator bacteria (i.e., families Enterobacteriaceae and Enterococcaceae). (b) Alternative fecal indicator taxa: feces-associated families. (c) Alternative fecal indicator taxa: sewage-associated genera. Data are the average relative abundance of each taxon over time.

Compared to the traditional fecal indicator taxa, the alternative fecal indicator taxa (both the five feces-associated families and the three sewage-associated genera) covered much higher proportions of the bacterial diversity in all treated sewages here investigated, despite differences between types of

treatments (Fig. 3). However, both the alternative and the traditional fecal indicator taxa showed statistically lower relative abundances in treated sewage undergoing secondary treatment (ANOSIM, R = 0.5852, 0.4685, and 0.4839 with p-value < 0.001, for traditional indicator taxa, feces-associated and sewage-associated taxa, respectively).

Seawater samples collected next to WWTP discharging points revealed a marked fecal signature, despite evident mitigation due to the dilution of the treated sewage plumes (Fig.3). Alternative fecal indicator taxa provided a better description of the fecal bacterial load in marine samples compared to traditional bacterial indicators.

Traditional fecal indicator bacteria accounted for low relative abundances (sum of taxa per sample up to 0.1%), with differences between the type of sewage treatment performed by the WWTP discharging in the area and with Katalinića seawater samples with the highest relative abundances over time (ANOSIM, R = 0.278, p-value < 0.05). However, we did not find any read affiliated with traditional fecal indicator bacteria in a few seawater samples. Enterococcaceae were significantly lower than Enterobacteriaceae (as observed for WWTP samples) and absent in the majority of the seawater samples. On the contrary, alternative fecal indicator taxa (both feces- and sewage-associated) were found in all seawater samples collected in this study.

As observed for the treated sewage, Bacteroidaceae, Lachnospiraceae, and Ruminococcaceae were the main feces-associated families contributing to fecal pollution (Figure 3B). The distribution of feces- and sewage-associated taxa was not affected by the type of sewage treatment, but it was site-specific (ANOSIM, R = 0.265, p-value < 0.05), with the Katalinića seawater samples with the highest contribution of both feces and sewage-associated families (i.e., average overtime was 4.3% and 2.7%, for feces- and sewage-associated taxa, respectively). Statistically significant differences between Katalinića and all the other seawater samples suggested scarce dilution of the sewage plume probably due to local hydrographic characteristics.

We screened the treated sewage samples for a list of 60 genera of PPB (679 ASVs), and then we looked at their presence and distribution in seawater samples. Out of the 42 PPB genera found in the dataset (Figure 4), *Alcaligenes*, *Propionibacterium*, *Ralstonia*, *Leptospira*, and *Serratia* were not found in the marine samples. *Acinetobacter*, *Coxiella*, *Prevotella*, *Streptococcus*, *Pseudomonas*, *Vibrio*, *Empedobacter*, *Paracoccus,* and *Leptotrichia* were among the most abundant. Conversely, FIB *Escherichia-Shigella* and *Enterococcus* ranged between 0.002–0.03% and 0–0.003%, respectively. The overall distribution of investigated PPB genera differentiated seawater samples based on the type of treatment performed in WWTPs discharging next to seawater sampling points (ANOSIM 0.2216, p-value < 0.05), with Katalinića seawater samples characterized by the highest relative abundances for several genera of PPB. However, despite differences in PPB distribution between samples, one-way ANOVA (on ranks, Kruskal-Wallis test, fdr correction applied) did not highlight any statistical difference associated with a specific site or type of treatment (performed in the WWTP discharging next to seawater sampling points). On the contrary, a differential abundance analysis based on the negative binomial distribution suggested that *Acinetobacter* was strongly associated with the seawater affected by the discharge from WWTP performing only primary treatment of sewage (Benjamini-Hochberg corrected p-values < 0.001), while *Sphingomonas*, *Legionella*, *Vibrio*, *Brevundimonas*, and *Pseudomonas* were associated with the presence of a secondary treatment (Benjamini-Hochberg
corrected p-values < 0.001).

The large majority of PPB phylotypes were shared between treated sewage and seawater samples, except for Vibrio and Coxiella, suggesting that WWTPs may represent the primary source for PPB in

marine areas investigated in this study. Indeed, the presence of Vibrio, Pseudomonas, and Coxiella phylotypes in marine waters is not uncommon due to the ubiquitous nature of representatives of these genera (Bacosa et al., 2015; Bonadonna et al., 2002; Fabbro et al., 2012). However, deeper investigations and the use of long-read sequencing approaches may reveal differences that could not be appreciated with the methodologies chosen for the purposes of this study.

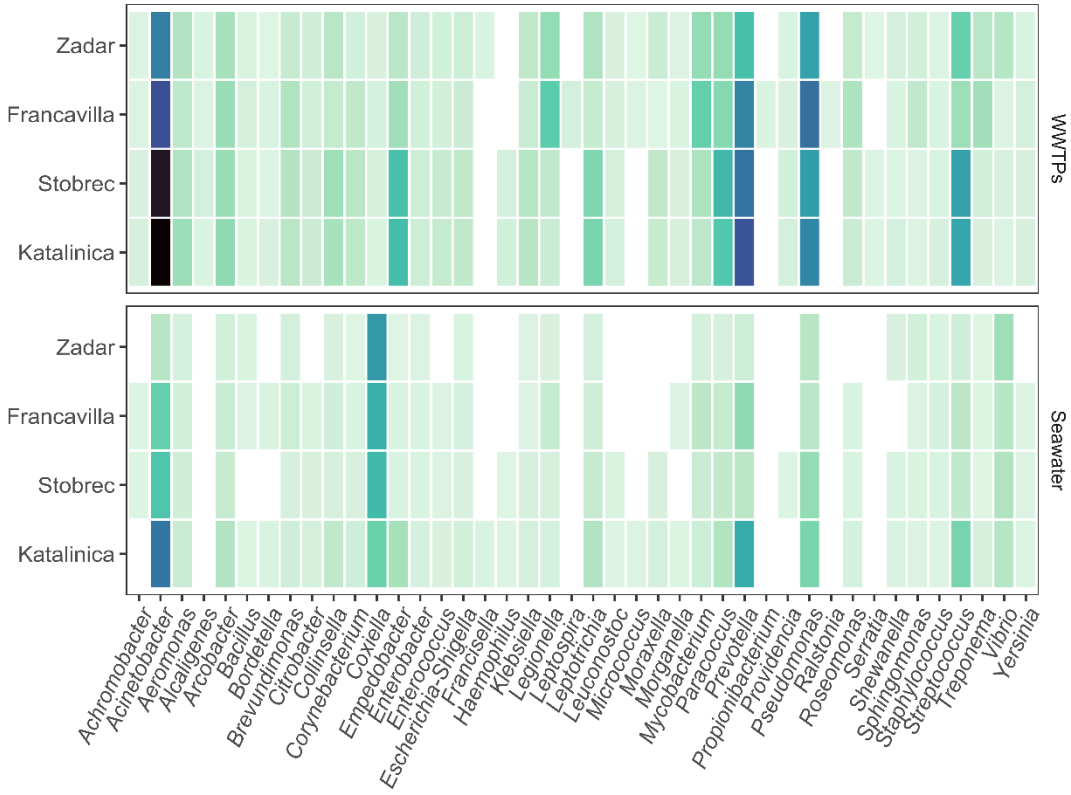


**Figure 4.** Potentially pathogenic bacteria in treated sewage (WWTPs) and seawater samples. Out of the 60 genera investigated *Bacteriodes*, *Borrelia*, *Brucella*, *Burkolderia*, *Chlamydia*, *Enterobacter*, *Laclercia*, *Listeria*, *Orientia*, *Rosemonas*, and *Salmonella* were not found in the dataset. ASV raw counts are log(1 + x) transformed to improve visualization and comparisons between samples. Tiles represent the average count over time per site. Higher values correspond to darker colors.

## 3.2 ARGs IN TREATED SEWAGE AND RECEIVING MARINE WATER

WWTPs represented continuous sources of *erm*B, *qnr*S, *sul*2, and *tet*A genes for coastal marine water (Figure 5). The three Croatian WWTPs (both with and without secondary biological treatment) were also important sources of the *bla*TEM gene. The *bla*CTX-M gene was found in low concentrations in the Katalinića WWTP effluent and in <LOQ or null concentrations in the other WWTPs.

The two WWTPs with only primary treatments (i.e., Katalinića and Stobreč) were characterized by a more constant presence of ARGs over time, but differences between genes were often not associated with the type of treatment (i.e., primary vs. secondary), except for *sul*2 (significantly higher in the presence of secondary treatment; $H = 7.509246$, p-value $< 0.05$) and *erm*B (significantly lower in the presence of secondary treatment; $H = 32.50548$, p-value $< 0.001$). On the contrary, patterns were gene-specific and were likely related to the geographical origin of the corresponding wastewater streams. In particular, *qnr*S did not vary in a statistically significant way among all tested treated sewage ($H = 5.160097$, p-value $= 0.16$). We did not observe statistically significant differences in *tet*A content among

treated sewage from Zadar, Francavilla, and Katalinića treated sewage (H = 7.509246, p-value = 0.0573), but *tet*A was significantly lower in Stobreč effluent (H = 6.706772, p-value < 0.01). Francavilla WWTP was characterized by the absence or by low concentrations of *erm*B, *bla*TEM, *bla*OXA, and *bla*CTX-M. The colistin-resistance gene, *mcr*-1, was only found in 4 samples of treated sewage, all <LOQ, but never found in samples collected at Francavilla WWTP.

Figure 6 shows ARGs in marine sites. As observed for the treated sewage, *erm*B, *qnr*S, *sul*2, and *tet*A were found at sea in higher concentrations and more constantly than the other ARGs, despite the evident mitigation due to dilution. On the contrary, the most abundant gene in marine samples was *sul*2, with concentrations up to 1.6 × $10^{-3}$ copies/16S rDNA copy (Stobreč, February 2020).

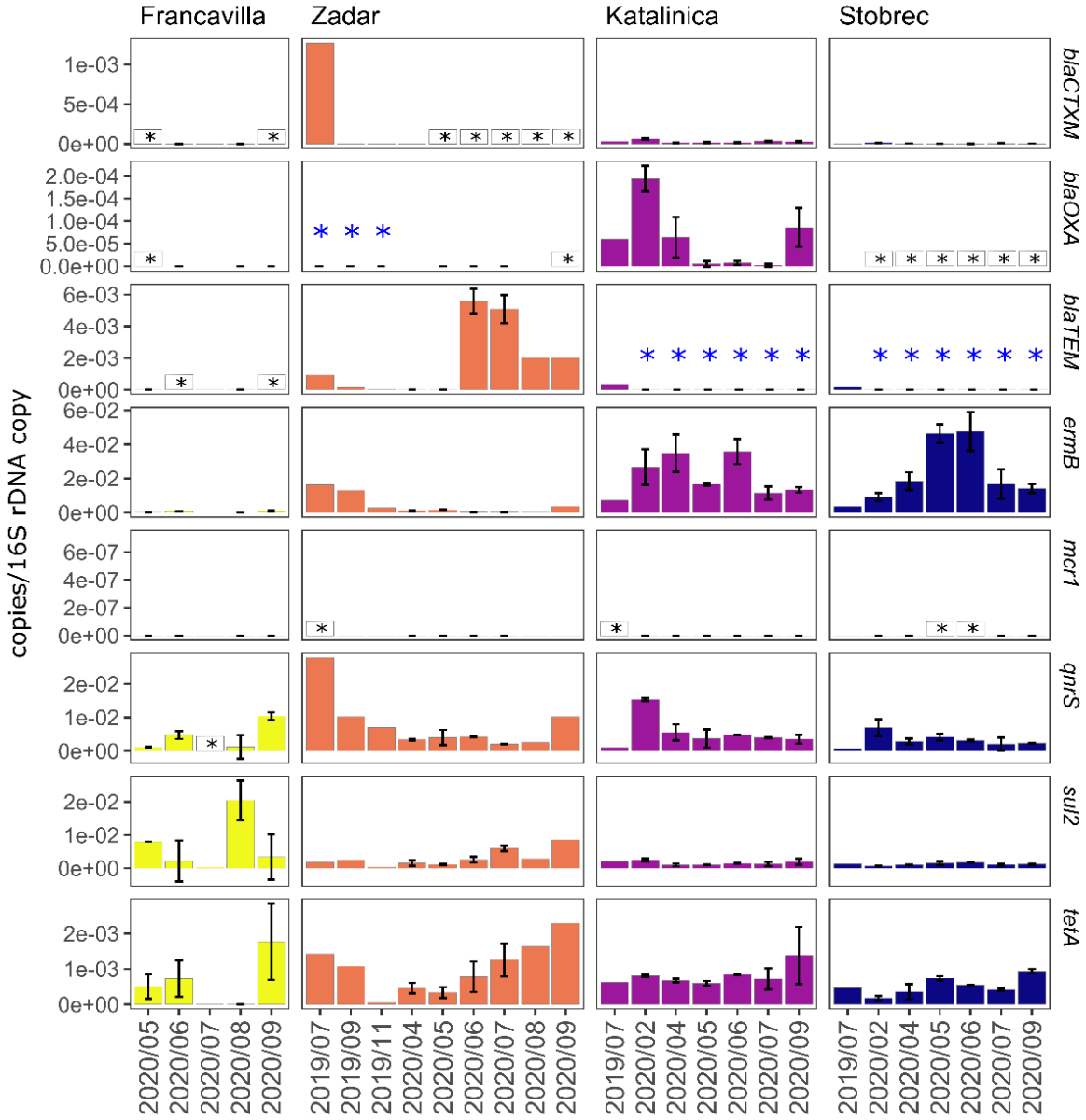


**Figure 5.** Content of ARGs in treated sewage samples over time. Black stars in white boxes are <LOQ values. Blue stars indicate not quantifiable samples due to primer-specificity issues.

Only Katalinića seawater samples were always positive to *erm*B, *qnr*S, *sul*2, *tet*A and *bla*TEM, probably because of the hydrographic characteristics of the area. Interestingly, *mcr*-1 was rarely found and in concentration usually <LOQ, although few seawater samples collected next to Francavilla and Zadar WWTP discharging points were higher than LOQ, with average values of $1.9 \times 10^{-4}$ and $1.4 \times 10^{-4}$ copies/16S rDNA copy, respectively, thus higher concentrations than their corresponding effluents. That could suggest that other environmental sources are relevant for *mcr*-1 (and, thus, other ARGs) in marine environments.

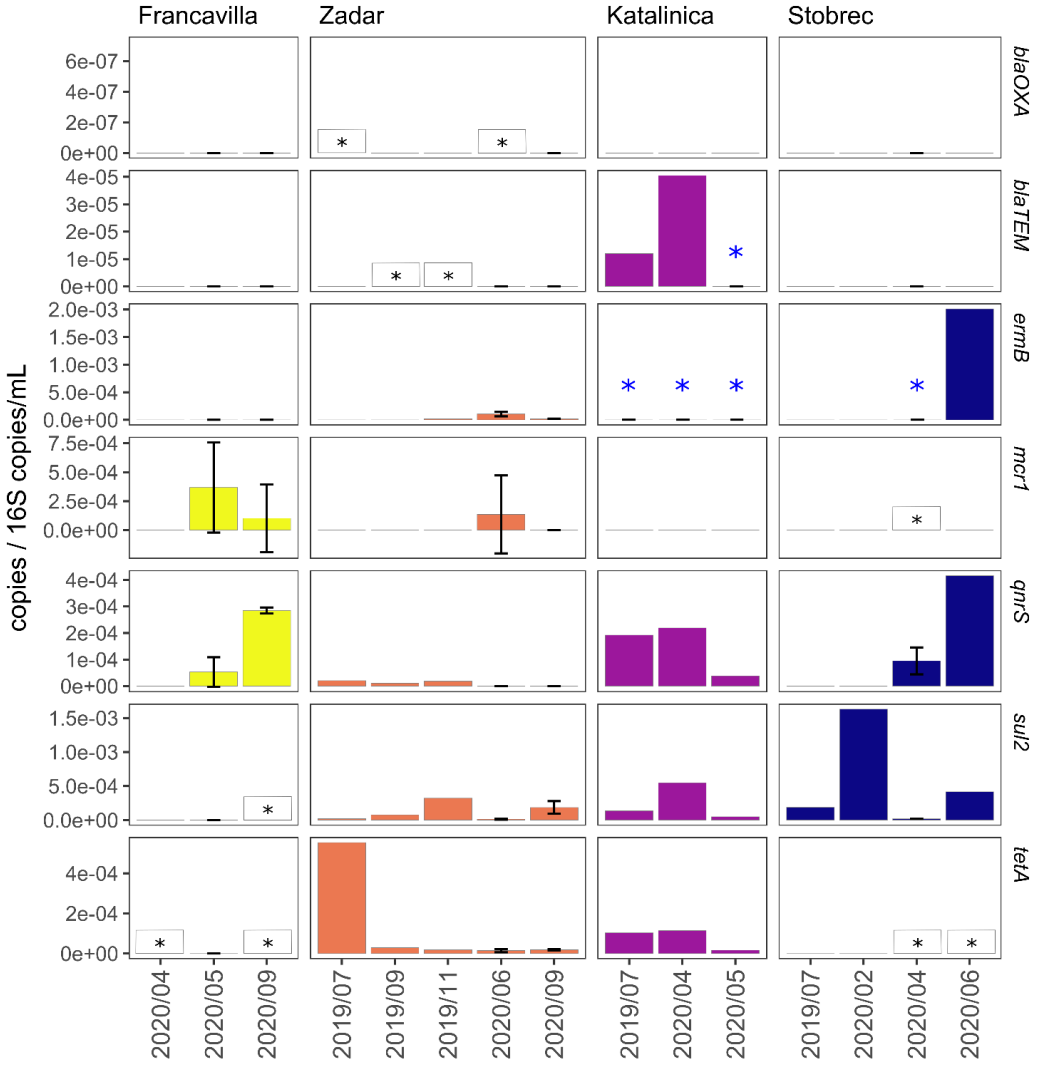


**Figure 6.** Content of ARGs in seawater samples over time. Black stars in white boxes are <LOQ values. Blue stars indicate not quantifiable samples due to primer-specificity issues.

# 4 RESULTS: NORTH ADRIATIC AREA

Two WWTPs were investigated in the N-Adriatic area: Lignano Sabbiadoro and San Giorgio di Nogaro WWTPs (Italy), both included the sewage bio-oxidation in activated sludge tanks and disinfection of treated sewage prior to unloading into the discharging pipeline. A total of 24 treated sewage samples and 24 seawater samples were collected in the sampling points located in the N-Adriatic and, subsequently, processed for DNA isolation.

## 4.1 CHARACTERIZATION OF MICROBIAL COMMUNITIES BY HIGH-THROUGHPUT SEQUENCING

Bacterial community structure in treated sewage was mainly dominated by Proteobacteria (25.7–77.4%), Firmicutes (1.3–22.6%), and Bacteroidota (8.9–41.2%), as shown in Figure 7. Chloroflexi and Plantomycetota reached high relative abundances in Lignano Sabbiadoro treated sewage (0.4–9.6% and 0.4–11.1%, respectively), while they were very low in San Giorgio di Nogaro (0.02–0.4% and 0.3–1.5, respectively).

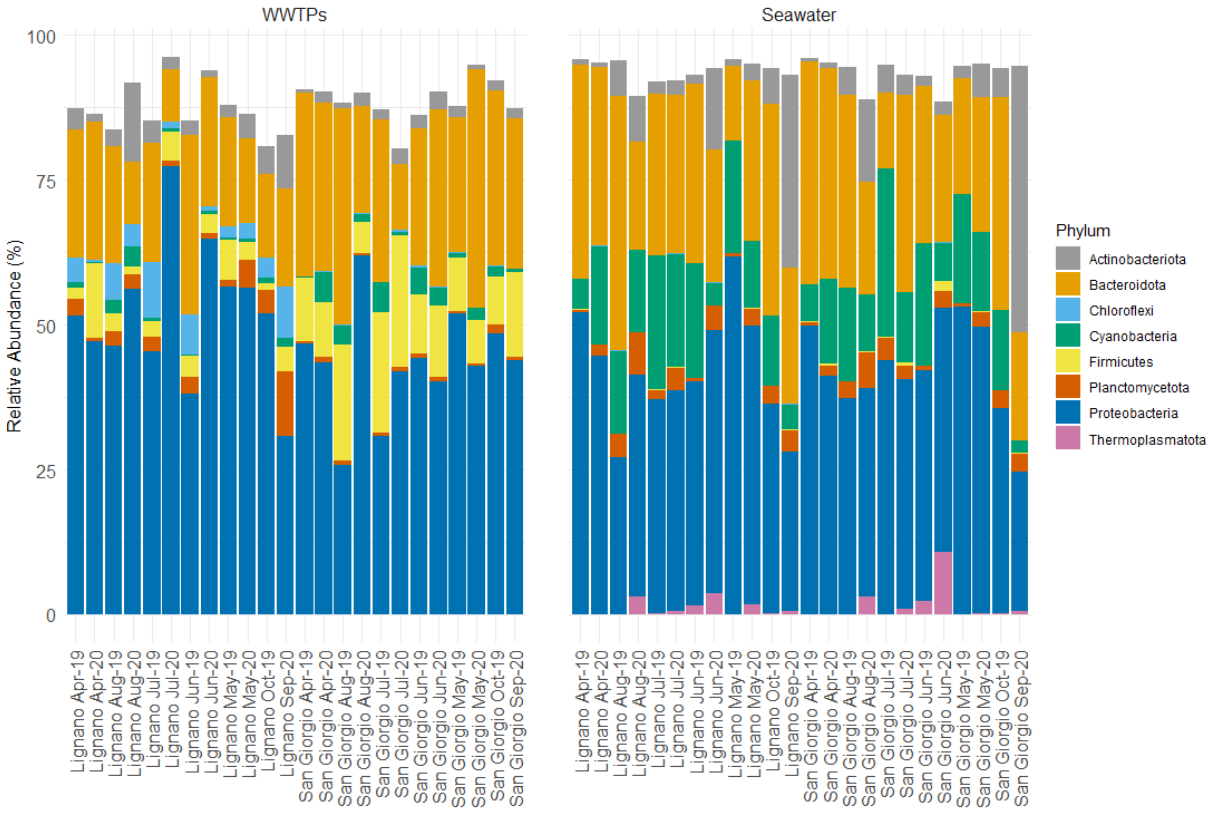


**Figure 7.** Prokaryotic community composition at phylum level of treated sewage (WWTPs) and seawater samples based on 16S rDNA amplicon sequencing. Only top phyla are depicted.

A PCoA based on Bray-Curtis distances (Fig. 8) showed that bacterial community structure in treated sewages tended to separate based on the WWTP (first PC explained about 40% variance), with statistically significant differences between Lignano Sabbiadoro and San Giorgio di Nogaro treated sewages in their content of Bacteroidota, Firmitus, Chloroflexi, and Actinobacteriota among the main phyla, as assessed by SIMPER followed by false discovery rate correction. Planctomycetota, Campylobacterota, Desulfobacterota, Nitrospirota, Acidobacteriota, Patescibacteria, and

Bdellovibrionota phyla contribute to minor (< 5%), yet significant, differences between the two WWTPs (SIMPER).

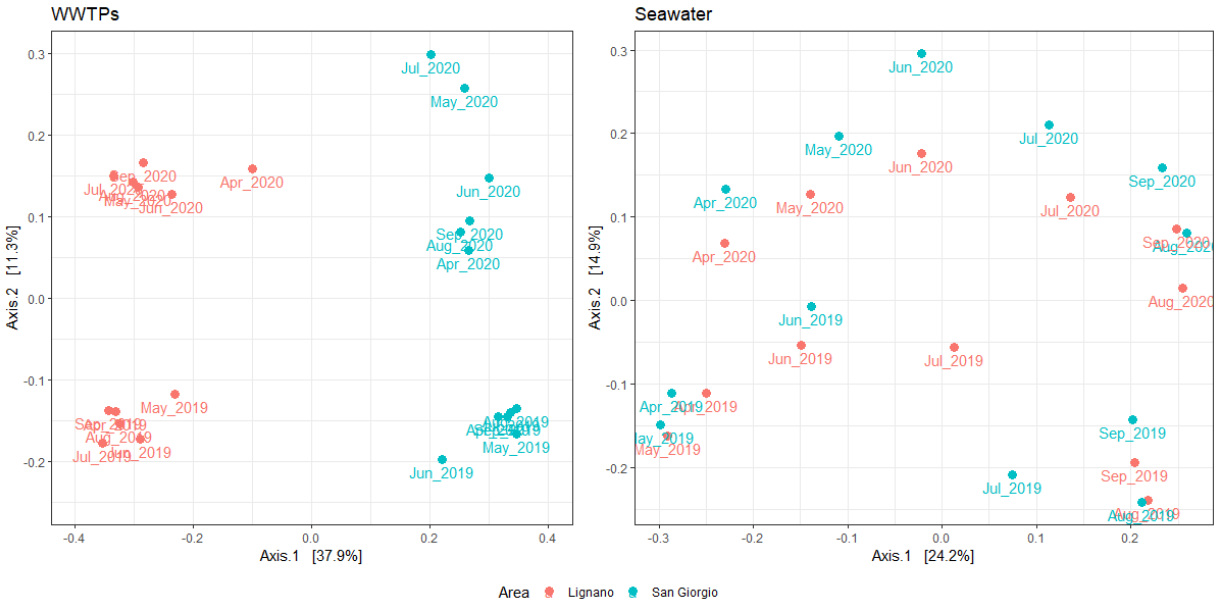


**Figure 8.** Sample ordination by PCoA on Bray-Curtis distances. Both Lignano Sabbiadoro and San Giorgio di Nogaro WWTPs, sewage undergoes activated sludge treatment subsequently to primary treatments, and a final disinfection step.

The bacterial community structure in seawater samples collected next to WWTP outfalls was mainly dominated by Proteobacteria (24.0–61.8%), Bacteroidota (12.8–43.9%), Cyanobacteria (2.2–29.0%), Actinobacteriota (0.6–46%), and in lower contribution by Planctomycetota (0.4–7.3%). Firmicutes accounted for relative abundances less than 2%. A PCoA (Fig.8) suggested that variation in community structure was driven by seasonal drivers, while the sampling site showed no effect on community differences. None of the phyla that contributed the most to the within-group similarities (i.e., >70% as assessed by SIMPER) varied in a statistically significant fashion.

Compared to the traditional fecal indicator taxa, the alternative fecal indicator taxa (both the five feces-associated families and the three sewage-associated genera) covered much higher proportions of the bacterial diversity both in seawater and sewage samples (Fig. 9). Moreover, traditional fecal indicator taxa weren't useful in highlighting community differences between Lignano Sabbiadoro and San Giorgio di Nogaro treated sewage (ANOSIM, R = 0.0161, p-value = 0.33167). On the contrary, both sewage- and feces-associated taxa showed statistically significant differences between the two treated sewages (ANOSIM, R = 0.4821 and 0.3572 with p-value < 0.001, for alternative fecal- and alternative sewage-associated indicator taxa, respectively). We did not find any read affiliated with traditional fecal indicator bacteria in a few seawater samples. Enterococcaceae were significantly lower than Enterobacteriaceae (as observed for WWTP samples). On the contrary, alternative fecal indicator taxa (both feces- and sewage-associated) were found in all seawater samples collected in this study. However, the distribution of traditional and alternative fecal indicator taxa did not show any significant differences between the two marine sampling sites (ANOSIM, R = -0.04427, 0.003051, and 0.04388, p-value = 0.68831, 0.39461, and 0.14486, for traditional, alternative fecal-associated, and alternative sewage-associated fecal indicator taxa, respectively).

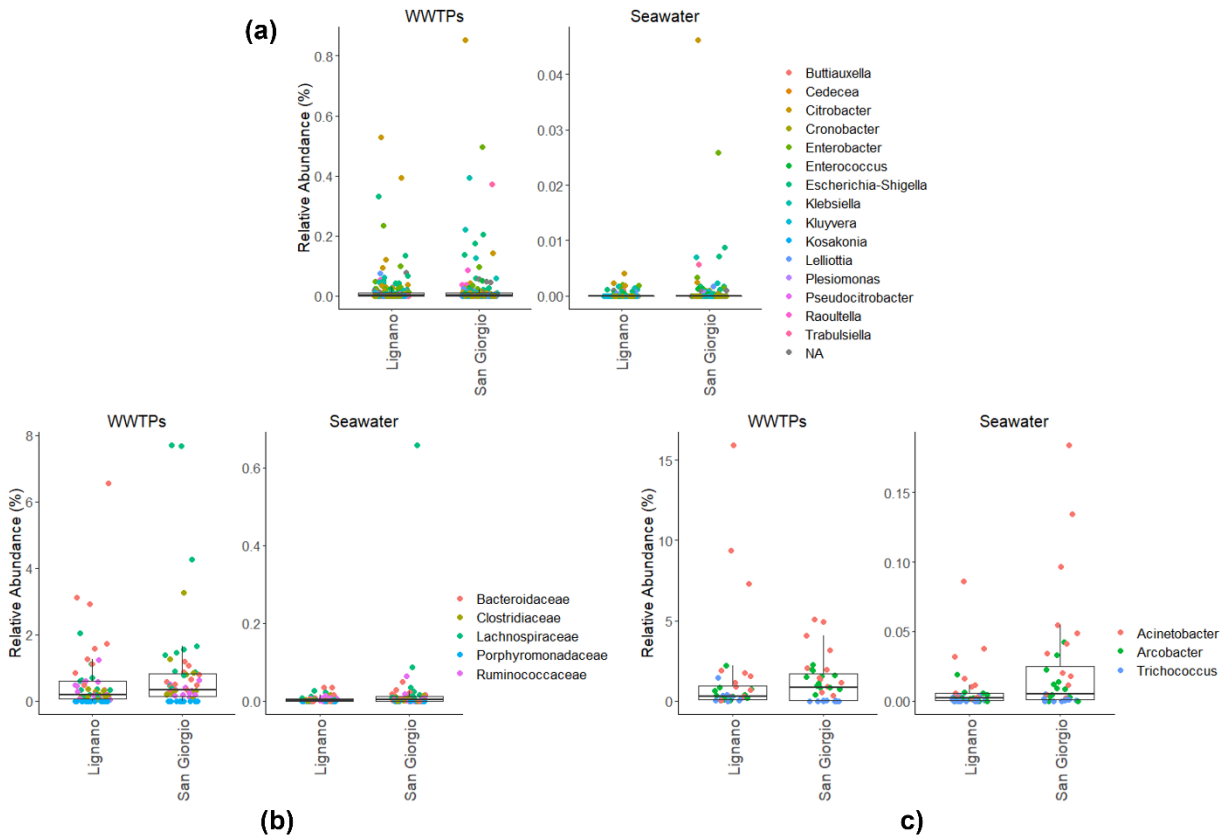


**Figure 9**. Fecal indicator bacteria in treated sewage (WWTPs) and seawater samples. (a) Traditional fecal indicator bacteria (i.e., families Enterobacteriaceae and Enterococcaceae). (b) Alternative fecal indicator taxa: feces-associated families. (c) Alternative fecal indicator taxa: sewage-associated genera. Data are the average relative abundance of each taxon over time.

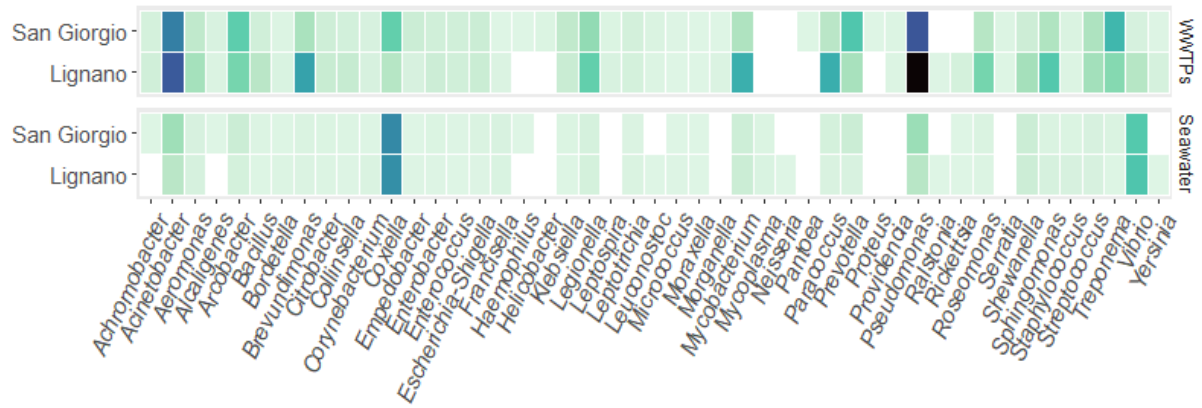


**Figure 10.** Potentially pathogenic bacteria in treated sewage (WWTPs) and seawater samples. Out of the 60 genera investigated *Bacteriodes*, *Borrelia*, *Brucella*, *Burkolderia*, *Chlamydia*, *Laclercia*, and *Salmonella* were not found in the dataset. ASV raw counts are log(1 + x) transformed to improve visualization and comparisons between samples. Tiles represent the average count over time per site. Higher values correspond to darker colors. White tiles indicate 0 reads.

We screened the treated sewage samples for a list of 60 genera of PPB (1270 ASVs), and then we looked at their presence and distribution in seawater samples. Out of the 47 PPB genera found in the dataset

*Acinetobacter*, Arcobacter, *Coxiella*, *Morganella*, *Pseudomonas*, *Shewanella*, and *Vibrio* were among the most abundant genera found in seawater samples collected next to WWTP outfalls. Although Lignano Sabbiadoro and San Giorgio di Nogaro showed statistically significant differences in the overall distribution of PPB genera (ANOSIM, R = 0.6687, p-value < 0.001), the pool of PPB genera found in sweater samples was substantially similar in the two marine sampling sites (ANOSIM -0.0362, p-value = 0.6983).

## 4.2 ARGs IN TREATED SEWAGE AND RECEIVING MARINE WATER

As for the four WWTPs in the central Adriatic area, Lignano and San Giorgio WWTPs represented continuous sources of *erm*B, *qnr*S, *sul*2, and *tet*A genes for coastal marine water (Figure 11). However, in WWTPs in the North Adriatic were lower than those of the central Adriatic area of at least one order of magnitude. The *bla*CTX-M and *bla*OXA genes were found in low concentrations, often <LOQ or null. Only the genes *sul*2, *erm*B and *bla*OXA had statistically higher concentrations in Lignano treated sewage than in San Giorgio's (H = 6.76176, 4.815425, and 3.965517; p-value < 0.01, 0.05 and 0.05, respectively). The colistin-resistance gene, *mcr*-1, was found only in 1 treated sewage sample (i.e., San Giorgio di Nogaro WWTP, September 2020).

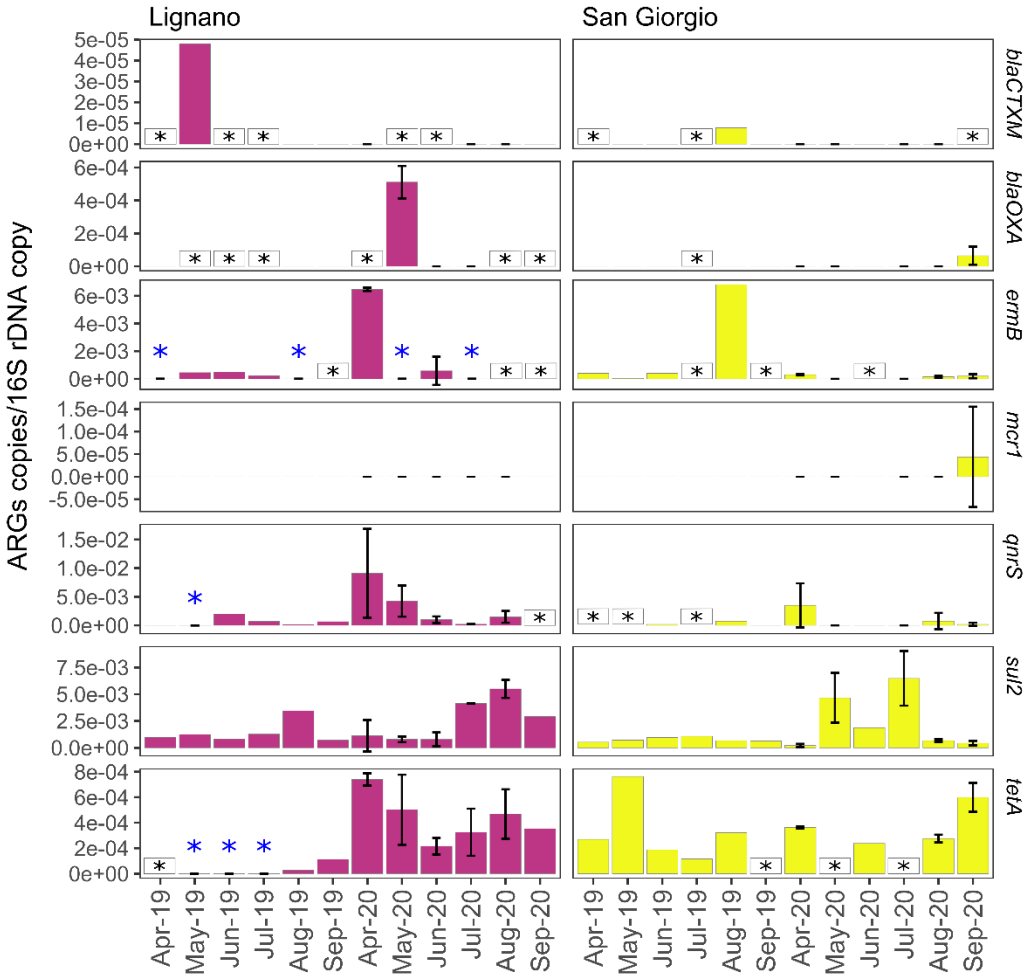


**Figure 11.** Content of ARGs in treated sewage samples over time. Black stars in white boxes are <LOQ values. Blue stars indicate not quantifiable samples due to primer-specificity issues.

As regards the seawater samples collected next to Lignano and San Giorgio WWTP outfalls, only *sul*2, *tet*A, and *qnr*S genes were found in quantifiable concentrations, with no statistically significant differences between the two sampling sites (H = 1.474359, 0.0356405, and 0.00144002; p-values = 0.2246592, 0.8502598, and 0.9697295, respectively). Concentrations ranged between 0 - 2.7 × $10^{-3}$, 0 - 6.3 × $10^{-4}$, and 0 - 9.1 × $10^{-5}$, for *sul*2, *tet*A, and *qnrs*, respectively. The *bla*OXA gene could not be quantified due to concentrations <LOQ or due to primer specificity issue. Genes *bla*CTX-M, *bla*TEM and *erm*B were not found in the sweater samples, while the colistin-resistance gene *mcr*-1 was found only in 1 sample (i.e., San Giorgio di Nogaro, June 2019).

# 5 SUPPLEMENTARY ACTIVITIES

During the implementation of AdSWiM two circumstantial events led to the development of experimental activities, not originally present in the AF. These are described in detail in the following sections.

## 5.1 SEARCH FOR THE VIRUS SARS-COV-2 IN THE NORTHERN ADRIATIC TREATED WASTEWATER AND SEAWATER

As it is sadly well-known, in the first weeks of year 2020 the virus SARS-CoV-2 originally discovered in China started its spread throughout Europe, showing its first diagnosed infections in NW Italy. Here, the first 'wave' of the COVID-19 reached its peak in early Spring 2020, right before the start of the scheduled project activities for that year. At that time, it was already known that genetic elements of the virus could be found in wastewater due to the fact that COVID-19-positive people could release large amounts of the virus through the sewage systems. However, no data on the potential transfer of the virus to the sea through DPs pipelines was available. Thanks to a collaboration in place with the University of Trieste, we took the opportunity to investigate the presence of SARS-CoV-2 in the samples collected for the other activities of WP4.5 (i.e., Lignano Sabbiadoro and San Giorgio di Nogaro treated wastewater and seawater at the discharge point). We extended our field of investigation including also surface seawater at two popular beaches in the Trieste city proximity and treated wastewater from the Trieste Depuration plant together with seawater at the discharge point. A summary of the collected samples for this activity is reported in Fig. 12 and Tab. 3.

For each sample, 1.8 L were collected and processed within few hours. All the plasticware was cleaned in 10% bleach and then rinsed in DI water. Samples were filtered through a GF/F filter to remove debris and microorganisms, nominally larger than 0.7 µm. Then 10 % of chloroform stabilized with amylenes was added and incubated at 4°C for at least 30 min, inverting every 15 min. The water phase was then transferred into new bleach-clean bottles for the PEG-NaCl precipitation step, following Thurber et al (2009). 10 % w/v PEG-8000 and enough NaCl to reach a final concentration of 1 M was added to the sample and incubated in the dark at 4°C overnight. Then the sample was centrifuged to precipitate the phage lysate at 11,000 x g at 4 °C for 10 min. The pellet was resuspended in DNA/RNA ShieldTM and then the RNA was extracted using a commercial kit (ZymoBIOMICSTM RNA Miniprep Kit). RNA was then stored at -80°C until RT-PCR analysis that was performed using the IP2 primer set (Corman et al., 2019), designed to target RdRp gene. According to the protocol and manufacturer instructions of the 1-Step RT-qPCR q Script ™ XLT (Quanta Bio), the reaction mix volume was set to 10 µL as follow: $H_20$ 2.5 µL,

Reaction Mix 6.25 µL, IP2Fw 0.5 µL, IP2Rw 0.5 µL, IP2Probe 0.25 µL and 2.5 µL of samples. RT-PCR conditions were: reverse transcription at 50 °C for 20 min, then denaturation step at 95 °C for 1 min, followed by 50 amplification cycles at 95 °C for 10 sec and 60 °C for 30 sec and a cooling step at 40°C for 30 sec. Melting curves were performed regularly at the end of the run. Quintuplicate samples were run and standard curves using a synthetic RNA from the Pasteur Institute were set up to quantify the products. In each sample, total prokaryotes and total viruses were enumerated by flow cytometry as detailed in Manna et al (2019).


**Figure 12**: Sampling points in the Gulf of Trieste (N Adriatic Sea). Green and blue waves indicate the location of Depuration Plants and their discharge points at sea, respectively. Blue swimmers indicate the location of the two sampled beaches.

| Site | Dates |
|---|---|
| Lignano Sabbiadoro, Lignano Sabbiadoro DP, San Giorgio di Nogaro, San Giorgio di Nogaro DP | 27/05/2020<br>24/06/2020<br>29/07/2020<br>02/09/2020<br>01/10/2020 |
| Trieste, Trieste DP | 19/06/2020<br>05/08/2020 |
| Barcola, S. Croce | 09/06/2020<br>22/06/2020<br>19/08/2020<br>15/09/2020 |

**Table 3.** Sampling dates and location.

In seawater the abundances of total prokaryotes ranged between 0.5 and 2.0 × $10^9$ cell $L^{-1}$ whereas viruses were in the range 1.5 – 3.8 × $10^{10}$ virus $L^{-1}$ (Fig. 13), in line with previously reported data for the Gulf of Trieste (Turk et al., 2021). The abundance of prokaryotes in treated wastewater was strongly dependent on the Plant. In Lignano Sabbiadoro and Trieste, cell numbers were similar and displayed the same order of magnitude of seawater (range 1.2 – 3.7 × $10^9$ cell $L^{-1}$) whereas in San Giorgio di Nogaro significantly higher values were detected (mean ± standard deviation = 2.6 ± 1.2 × $10^{10}$ cell $L^{-1}$). Viral

populations in treated wastewater were less variable and ten times more concentrated than at sea, displaying an average abundance of 2.6 (± 1.8) × $10^{11}$ virus $L^{-1}$. The gene targeted for the quantification of the virus SARS-CoV-2 was detectable only in one depuration plant sample (San Giorgio di Nogaro, in May), with an estimated number of 3.2 × $10^5$ copies $L^{-1}$. All seawater samples were SARS-CoV-2-free.

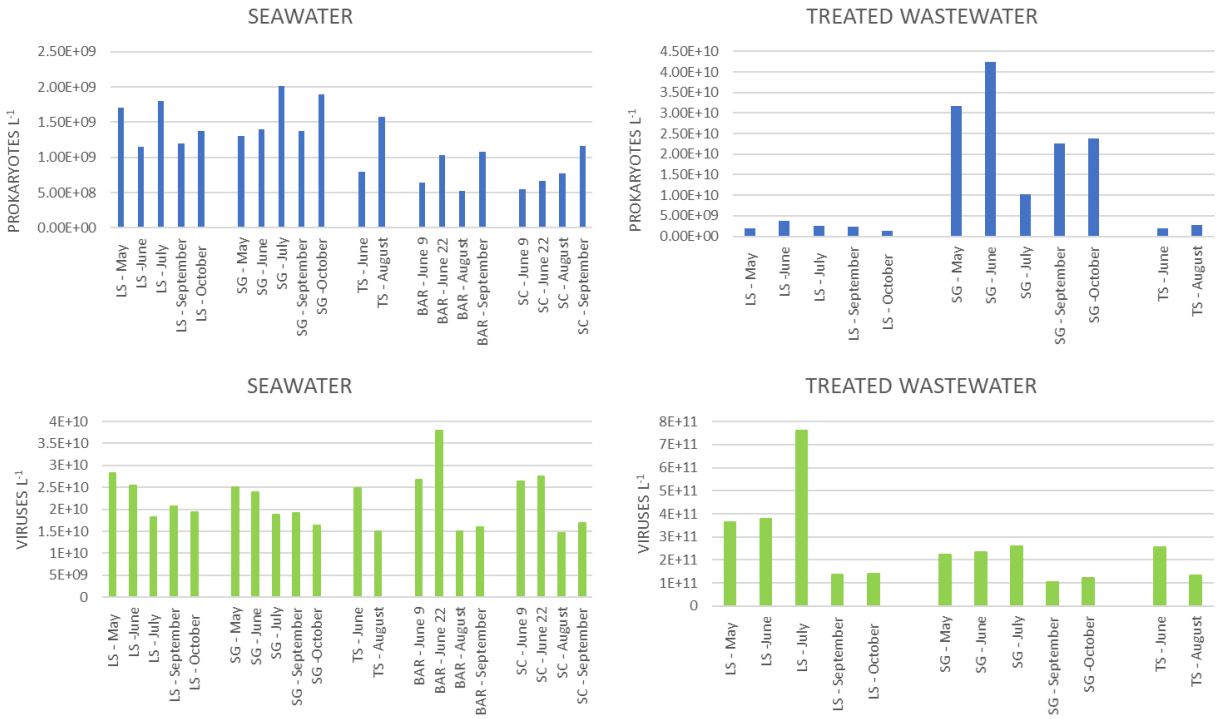


**Figure 13**. Abundances of prokaryotes (in blue) and viruses (in green) in treated wastewater (right panels) and seawater (left panels). LS = Lignano Sabbiadoro, SG = San Giorgio di Nogaro, TS = Trieste, BAR = Barcola, SC = S. Croce.

In conclusion, we observed that Depuration Plants are in general not so efficient in removing viral particles from wastewater (although we cannot assess their integrity). However, even though viruses are abundant in treated wastewater and we could detect traces of the virus SARS-CoV-2 in one out of twelve samples, their transfer to seawater and potential infection in recreational areas are highly unlikely.

## 5.2 Effect of wastewater discharge on the coastal microbiome of the Gulf of Trieste

The second case study dealt with the potential effect of treated wastewater on the ecology of the coastal microbiome. This activity took advantage of ongoing collaborations between OGS and the Slovenian National Institute of Biology based in Piran. For the purposes of this study, an incubation experiment was set up utilizing coastal seawater collected nearby the oceanographic buoy Vida (https://www.nib.si/mbp/en/oceanographic-data-and-measurements/buoy-2/general-2) and treated wastewater (WW) from the Piran Depuration Plant. WW was subjected to two kinds of filtrations:

- onto 5 µm in order to remove large particles and maintain organic matter, inorganic nutrients, viruses and prokaryotes (WW< 5 um)
- onto 0.2 µm in order to remove large particles and prokaryotes while keeping in the filtrate dissolved organic matter, inorganic nutrients and viruses (WW< 0.2 um)

The two size-fractionated WW were mixed with seawater at 1:10 v:v. Therefore, three experimental conditions, run in triplicated microcosms, were created: seawater+prokaryotes+nutrients (SW+Prok+Nut), seawater+nutrients (SW+Nut) and sweater itself (SW - control). Several microbiological and chemical parameters were monitored for 32 h with 6 discrete samplings within microcosms. The OGS team were in charge for the quantification of prokaryotes and viruses and for the assessment of the presence of antibiotic-resistance genes, with the methods described in Manna et al (2019) and Fonti et al (2021), respectively.

Results obtained for the counts of prokaryotes and viruses are reported in Fig. 14. Prokaryotes in WW were not highly abundant, as a consequence of an efficient depuration treatment and the filtration on 0.2 µm was efficient in removing all cells. An increase in cell abundance characterized all experimental microcosm and it was slightly faster in the SW+Nut treatment, indicating that the (inorganic and organic) nutrients from WW are *per se* able to stimulate prokaryotic growth. As observed in the previous case study the abundance of viral particles in WW were in the order of hundreds of billions per liter and this affected significantly the measurements from the treatments SW+Prok+Nut and SW+Nut. However, the decreasing trend in time of viruses in the treatments suggests that they were not able to infect prokaryotes and trigger lytic phenomena in the tested time span.

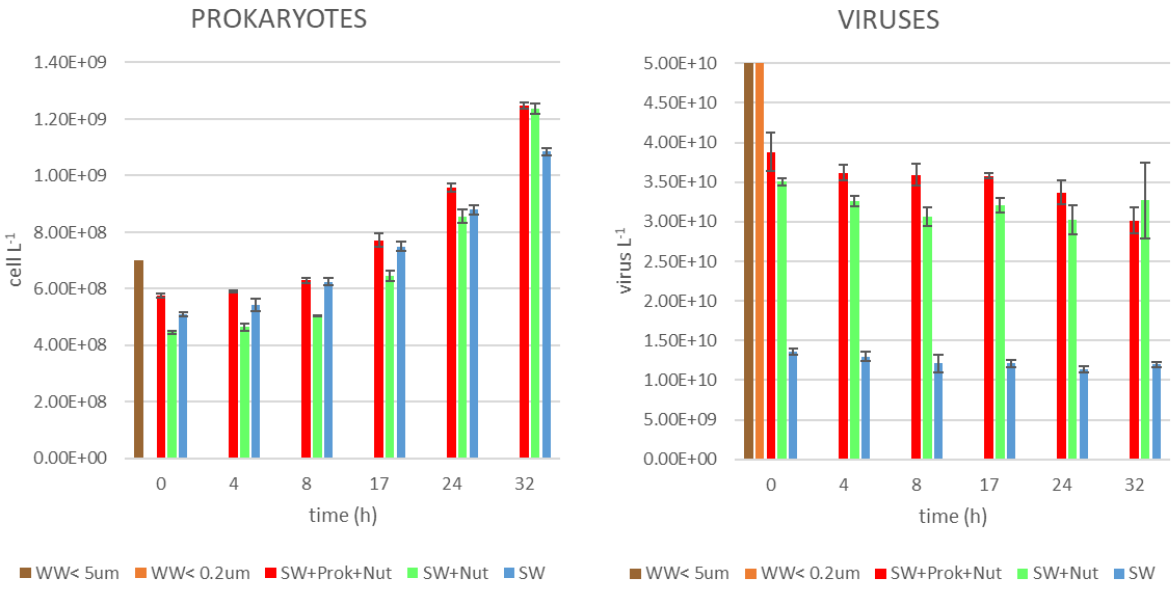


**Figure 14**. Abundances of prokaryotes (left) and viruses (right) in treated wastewater and in the experimental microcosms at T0 and after 4, 8, 17, 24, and 32 hours. For visualization purposes, the abundance of viruses in WW<5 µm and in WW< 0.2 µm exceeds the y-axis maximum values: the data from these two samples are 2.4 and 2.1 × $10^{11}$ virus $L^{-1}$, respectively.

The quantification of specific genes was carried out on the WW<5 µm sample collected at T0 and in the different experimental enclosures at the end of the experiment. The 16S rRNA gene was tested as a highly conserved signature, present in all prokaryotes. Five genes conferring resistance to commonly used antibiotic families were also quantified: *qnrS* (fluoroquinolones), *sul2* (sulfonamides), $bla_{TEM}$ (beta-lactams) *ermB* (macrolides) and *mcr*-1 (colistin). The outcomes of the analyses on the 16S rRNA gene roughly paralleled the total prokaryotic counts, with slightly higher abundances in the WW-treated microcosm than in the control (SW) (Tab. 4). Genes conferring resistance to beta-lactams and colistin were not quantifiable in any of the samples. *qnrS* and *sul2* were present in wastewater and in the

SW+Prok+Nut samples, displaying a strong decreasing trend over time. *ermB* was detected in wastewater and in the two treatments at the end of the experiment, even in SW+Nut. Collectively, these results indicate that the persistence of antibiotic-resistance genes are carried by wastewater-associated prokaryotes and horizontal gene transfer operated by viruses or other small-sized vectors is not detectable in the timescales examined in this experiment.

| | 16S | qnrS | sul2 | blaTEM | ermB | mcr-1 |
|---|---|---|---|---|---|---|
| WW<5um | 1.4E+09 | 3.8E+05 | 1.2E+06 | NQ | 1.1E+06 | 0 |
| SW | 7.9E+07 | 0 | 0 | <LOQ | 0 | <LOQ |
| SW+Prok+Nut | 1.9E+08 | <LOQ | 2.1E+04 | <LOQ | 1.4E+04 | 0 |
| SW+Nut | 2.0E+08 | 0 | 0 | <LOQ | 2.2E+03 | 0 |

**Table 4**. Results of the gene quantification analyses carried out during the experiment. Results are provided in copies $L^{-1}$. Data about SW, SW+Prok+Nut and SW+Nut are reported as means calculated among the experimental replicates. < LOQ: below the limit of quantification of the method. NQ: the gene is present but it is not quantifiable due to specificity issues in the reaction

# 6 CONCLUSIONS

In this study we investigated WWTPs over time as continuous point sources of microbial pollution for coastal waters of the Adriatic Sea. In the absence of secondary treatments, marine coastal waters received high inputs of fecal indicator bacteria (Fonti et al., 2021) and potentially pathogenic bacteria (including the emerging opportunistic pathogen *Pseudomonas aeruginosa*). Moreover, WWTPs under study represented continuous sources of genetic elements conferring resistance to major classes of antibiotics, with potential consequences on the emerging and the establishment of AMR pathways in the environmental microbial communities. NGS-based approaches provided broader detection of specific taxa than classic microbiological techniques, especially in the presence of a final disinfection step, which could lead to an underestimation of fecal pollution. In particular, the exploration in the microbiomes of alternative fecal indicator taxa and potentially pathogenic bacteria allowed a more thorough understanding of emerging and potential pathogens spread into the environment from either low-efficiency and conventional WWTPs. Overall, our results highlight the need for an improvement of wastewater treatment technologies, especially for those plants which perform primary treatment only, and stress the importance of dedicated monitoring programs at the WWTP discharge points at sea.

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