Antibiotic Resistance in Black Sea Microbial Communities

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Background: Antibiotic resistance genes (ARGs) are considered as pollutants and are found in natural and anthropogenically impacted environments. Distribution of ARGs in marine environment poses a threat to human health turning the water body into a pool for the ARGs' transmission.

Objectives: A large-scale study of antibiotic resistance in microbial communities has been performed in the Black Sea, both in the coastal and offshore regions.

Methods: The quantitative distribution of the genes responsible for the inactivation of the beta-lactam (bla_{CMY}, bla_{SHV}), vancomycin ($vanA$, $vanB$), macrolides ($ermB$) and colistin ($mcr-1$) was assessed with real-time quantitative PCR. Concentrations of the antibiotics belonging to the classes of beta-lactam/cephalosporin/carbapenem, macrolides and glycopeptides were determined by LC-ESI-QTOF-MS.

Results: The present study revealed the distribution of antibiotic resistance genes targeting the response to all antibiotics included in our analysis at various locations across the Black Sea. According to the ARGs copy number normalized to the 16S rRNA, $vanB$ ($2 \times 10^{-1} \pm 1 \times 10^{-1}$) and bla_{SHV} ($4 \times 10^{-2} \pm 1 \times 10^{-2}$) were the most numerous genes, followed by bla_{CMY} ($1 \times 10^{-2} \pm 3 \times 10^{-3}$) and $mcr-1$ ($3 \times 10^{-2} \pm 2 \times 10^{-2}$). The less abundant gene was $ermB$ ($1 \times 10^{-3} \pm 5 \times 10^{-4}$) and $vanA$ ($1 \times 10^{-5} \pm 5 \times 10^{-4}$). The $mcr-1$, bla_{CMY} and bla_{SHV} had moderate positive correlation with markers of ruminant faecal pollution. The concentration of antibiotics in seawater was below the detection limit. The abundance of all ARGs included in the study was significantly higher (p-value<0.05) within the northwest coastal area when compared to the offshore stations. The results clearly indicate an alarming antibiotic resistance problem in the region and call for a regular monitoring of ARGs abundance in the Black Sea and its major freshwater tributaries.

Keywords: ARGs, beta-lactam, macrolide, colistin, vancomycin, qPCR, Black Sea

INTRODUCTION

The Black Sea is a large semi-closed European sea with an area of 436,400 km^2. It is virtually isolated from other seas and ultimately drains into the Mediterranean Sea via the narrow Bosphorus channel. The drainage basin of the Black Sea is ca. 2,000,000 km^2 and covers regions with intensive agriculture and industrial activities. Riverine input, especially of the major rivers Danube, Dnieper and Dniester,

is a considerable source of the Black Sea pollution (Shimkus and Trimonis, 1974; Alygizakis et al., 2019; Diamanti et al., 2020). Ports and coastal cities contribute to the pollution as well; persistent organic pollutants, metals and pharmaceuticals were frequently found in the Black Sea water, sediment and biota compartments (Bakan and Ariman, 2004; Ozkoc et al., 2007; Stoichev et al., 2007). Considering the overall pollution pattern, contamination of the Black Sea with antibiotic resistance genes (ARG) can be expected (Kümmerer, 2009; Kraemer et al., 2019; Zheng et al., 2021).

Antibiotic resistance is one of the biggest threats to global health, emerging as one of the major causes of death in the coming decades (https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance). ARGs have burst outside the clinic environment long ago (Kraemer et al., 2019), and numerous studies report distribution of ARGs in different water environments (Tang et al., 2015; Szekeres et al., 2018; Griffin et al., 2019) etc. Dissemination of ARGs in the natural environment implies a direct hazard to human health due to the possibility of ARGs and antibiotic resistance bacteria (ARBs) intake with food or by direct contact (Amarasiri et al., 2020; Zheng et al., 2021).

The Black Sea region has already been in focus in terms of the antibiotic resistance., however, most of the studies imply antibiotic resistant testing or ARG profiling of bacteria isolated from fish farms, marine biota, seawater and freshwater from the Danube river (Kayis et al., 2009; Kittinger et al., 2016a; Kittinger et al., 2016b; Türe and Alp, 2016; Terzi and Isler, 2019; Kayis et al., 2021). ARGs distribution in the Black Sea water was studied by Sabatino et al. (2020) in the eastern and western part of the Black Sea. bla_{CTXM}, $sul2$, and $tetA$ were the most abundant ARGs, being present in at least 43% of the samples, while $ermB$ and $qnrS$ were not detected. A wide-scope chemical screening together with analysis of ARGs was carried out in the effluents of wastewater treatment plants (WWTPs) in the Danube river basin (Alygizakis et al., 2019). According to the study, $aph\ (III)a$, bla_{OXA}, $ermB$, $ermF$, $sul1$ and $tetM$ had widespread occurrence, while bla_{SHV}, $mecA$, $qnrS$, $tetB$ and $vanA$ were detected sporadically. In general, there is a lack of data on the ARGs distribution in the Black Sea environment.

Antibiotics are considered as important driving factors for ARG proliferation, as they pose selective pressure on microbes and enrich ARGs in estuarine and coastal environments (Zheng et al., 2021). Moreover, antibiotics may encourage the horizontal gene transfer (HGT), which facilitates the dissemination of ARGs (Szczepanowski et al., 2009). Faecal pollution is another factor of ARGs distribution (Khan et al., 2019). According to the publicly available metagenomic data, the presence of ARGs can simply result from faecal contamination with resistant bacteria (Karkman et al., 2019). Poorly treated or untreated sewage discharge containing faecal bacteria may fuel the pool of ARGs in anthropogenically impacted environments, especially in low-income countries.

To facilitate an overview of ARGs distribution in the Black Sea environment, 12 water samples were collected in the eastern, central and northwestern parts of the Black Sea in the course of the EU/UNDP EMBLAS-Plus project (https://emblasproject.org/).

TABLE 1 | Coordinates and regions of the sampling sites.

Station	Longitude	Latitude	Region
1A	30,2518	45,3386	Northwest shelf
2A	31,0021	46,3333	Northwest shelf
2	31,2350	45,2159	Northwest shelf
3	31,3041	44,2162	Northwest shelf
4	31,5632	44,1216	Open waters
5	31,8195	43,3998	Open waters
7	34,7733	43,3697	Open waters
8	36,0586	43,5384	Open waters
9	39,8810	42,2357	Eastern shelf
10	40,3369	42,1137	Eastern shelf
11	40,8367	41,9331	Eastern shelf
12	41,2196	41,7836	Eastern shelf

The research aimed at studying the spatial distribution of ARGs and associated factors contributing to their persistence in the Black Sea. The objectives of this study were to estimate whether: 1) ARGs targeting the antibiotics of wide usage and last-resort antibiotics are present in the Black Sea; 2) there is a difference in ARGs number in the open water and anthropogenically impacted shelf zone of the Black Sea; 3) the number of ARGs is associated with concentration of antibiotics in the water or with the markers of the faecal contamination.

MATERIALS AND METHODS

Sampling
The samples were collected during the Joint Black Sea Survey on the RV "Mare Nigrum" carried out within the EMBLAS-Plus project in July and August 2019. Altogether 12 sampling stations were investigated on a transect from Constanta (Romania) to Odessa (Ukraine) and Batumi (Georgia) (**Table 1**; **Figure 1**). 2 L of seawater were taken from the surface and passed through the Isopore PC 0.22 µm filters (Millipore, United States) using Microsart e. jet vacuum pump (Sartorius Stedim, Germany). Filters with bacterial biomass on them were immediately frozen at −180°C after filtration.

DNA Extraction
DNA was extracted from seawater using DNAeasy PowerSoil® Kit (Qiagen, Germany) according to the manufacturer's instructions with the adaptations to the material used. Filters were cut with sterile scissors prior to the lysis step. The DNA quantity and quality were estimated using NanoDrop Spectrophotometer 2000 (Thermo Fisher Scientific, United States). All samples had sufficient DNA concentration (with most above 30 ng/µL) and A260/280 ratio ~1.8.

Quantitative Real-Time PCR
ARGs that encode resistance to beta-lactams/cephalosporins (bla_{CMY}, bla_{SHV}), vancomycin ($vanA$ and $vanB$), macrolide-lincosamide ($ermB$) and colistin ($mcr-1$) were enumerated by RT qPCR. Primers CMY_fwd/CMY_rev, SHV_fwd/SHV_rev (Roschanski et al., 2014), vanAF/vanAR, vanBF/vanBR (Mirzaei et al., 2015), erm (B)-91f/erm (B)-454r and mcr1FP/

FIGURE 1 | Locations of the sampling sites.

mcr1RP (Hembach et al., 2017) were used correspondingly (**Supplementary Table S1**).

The list of targeted genes was based on the antibiotic resistance prevalence data from the European Resistance Surveillance Network (EARS-NET, 2019) and Joint Danube Survey 3 reports (Zarfel et al., 2015).

Gut host-specific bacteria of the *Bacteroidetes* phylum were used as markers of faecal pollution. To quantify human *Bacteroidetes* primers HF183 and BacR287 were used (Epa and of Science, 2019). BacR/BacF (Reischer et al., 2006) and Pig-2-Bac41F/Pig-2-Bac163Rm (Mieszkin et al., 2009) primers were used to quantify ruminant and pig *Bacteroidetes* respectively. Primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 were used to estimate the quantity of the 16S rRNA gene (Klindworth et al., 2013). Primer sequences and annealing temperatures are presented in **Supplementary Table S1**.

RT qPCR was set using the QuantiFast SYBR Green PCR Kit (Qiagen, Germany) according to the standard manufacturer's procedures. The standards were created for each of the targeted genes using the DNA from the samples. Each 25 µL PCR reaction contained the following components: 2x QuantiFast SYBR Green PCR Master Mix—12.5 µL, Primer Reverse −2.5 µL, Primer Forward −2.5 µL, template DNA −1 µL, RNase-free water −6.5 µL. The initial concentration of DNA in each reaction was 5 ng/µL. The thermal conditions were different for all primers and are presented in **Supplementary Table S1**. Negative controls contained no DNA template.

The standards for each gene analyzed were purified with QIAquick PCR Purification Kit (Qiagen, Germany) and 10-fold serially diluted ranging from 1.0×10^3 to 1.0×10^7 to be used for standard curve generation in quantitative PCR. The threshold value (Ct) was used to determine the copy numbers of targeted genes in the environmental subsamples based on the standard curves.

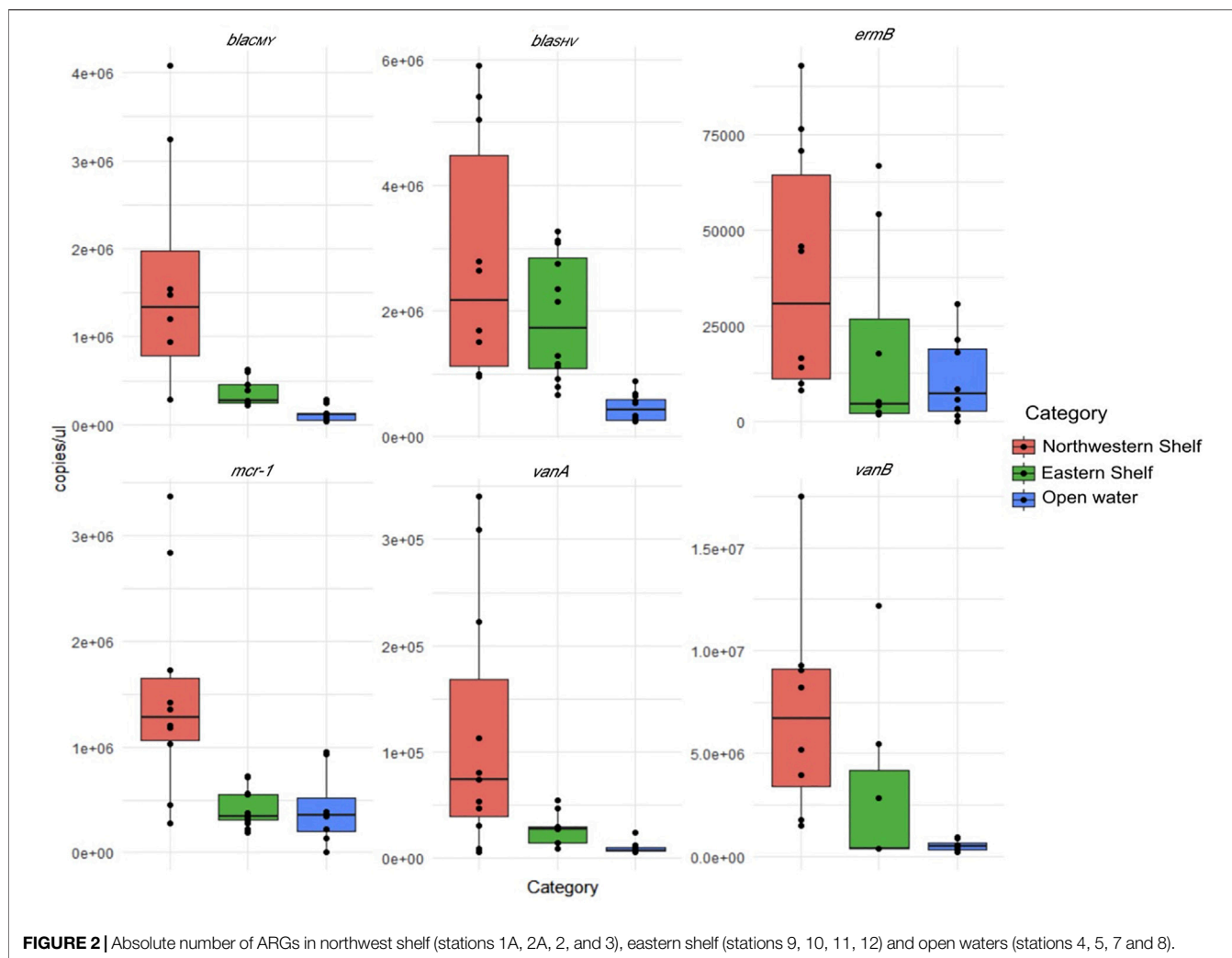Melting curve analysis was performed at the end of the amplification cycles in order to assess primer specificity and to ensure proper amplification of all target fragments. The PCR reactions were performed on the Qiagen Rotor-Gene Q (Qiagen, Germany). All reactions were performed in triplicate.

Concentrations of Antibiotics

Concentrations of the antibiotics belonging to the classes of β-lactam/cephalosporin/carbapenem (Amoxicillin, Ampicillin, Cefaclor, Cefadroxil, Cefalonium, Cefazolin, Cefoperazone, Cefquinome, Ceftazidime, Ceftiofur, Dicloxacillin, Meropenem, Oxacillin, Penicillin V), macrolides (Azithromycin, Clarithromycin, Erythromycin, Rifaximin, Roxithromycin, Tilmicosin, Tylosin), glycopeptides (Vancomycin), were determined by liquid chromatography-electrospray ionization-quadrupole time of flight-mass spectrometry (LC-ESI-QTOF-MS). All seawater samples were extracted on board using HORIZON SPE-DEX 4790 device (United States). The samples were spiked with internal standards for quality assurance and quality control purposes and were concentrated on Atlantic HLB-M Disk with 47 mm disk holder according to an automated extraction programme (Alygizakis et al., 2019). The extracts were evaporated using nitrogen and reconstituted in methanol:water (50:50 v/v) to the final volume of 500 µL achieving concentration factor of 4,000. The samples were filtered through 0.2 µm RC syringe filter before the LC-ESI-QTOF-MS analysis. The instrumental setup and gradient programme can be found elsewhere (Gago-Ferrero et al., 2020). The method was previously validated for linearity, accuracy using recovery experiments, repeatability and sensitivity (Gago-Ferrero et al., 2020).

Statistical Analysis

The obtained data was analyzed in the RStudio (version 3.6.0, packages: vegan, tidyverse, ade4, geosphere, corrplot, ggplot2). The data on the ARG abundance was tested for the normality by the Shapiro-Wilk normality test, which revealed non-normal distribution (*p*-value <0.05). Wilcoxon rank sum test was

FIGURE 2 | Absolute number of ARGs in northwest shelf (stations 1A, 2A, 2, and 3), eastern shelf (stations 9, 10, 11, 12) and open waters (stations 4, 5, 7 and 8).

applied (q-value = 0.05), p-value adjustment method: fdr (q-value <0.05), to test for the difference in the genes' abundance in the water from different Black Sea locations. Spearman correlation was used to test the correlation between ARGs' abundance and number of the faecal pollution markers. Mantel test was performed with the purpose to infer whether the variance in genes' number is explained by geographical distance, using the abundance matrix (based on Bray–Curtis measure) and the geographical distance matrix (based on Haversine distance).

RESULTS

Number of Antibiotic Resistance Genes in the Black Sea Water Samples

All target antibiotic resistance genes were found in all Black Sea water samples (**Figure 2**). Absolute values of the ARGs in each sample are presented in **Supplementary Table S2**.

The average values normalized to the 16S rRNA gene number indicate that the *vanB* ($2 \times 10^{-1} \pm 1 \times 10^{-1}$) was the most
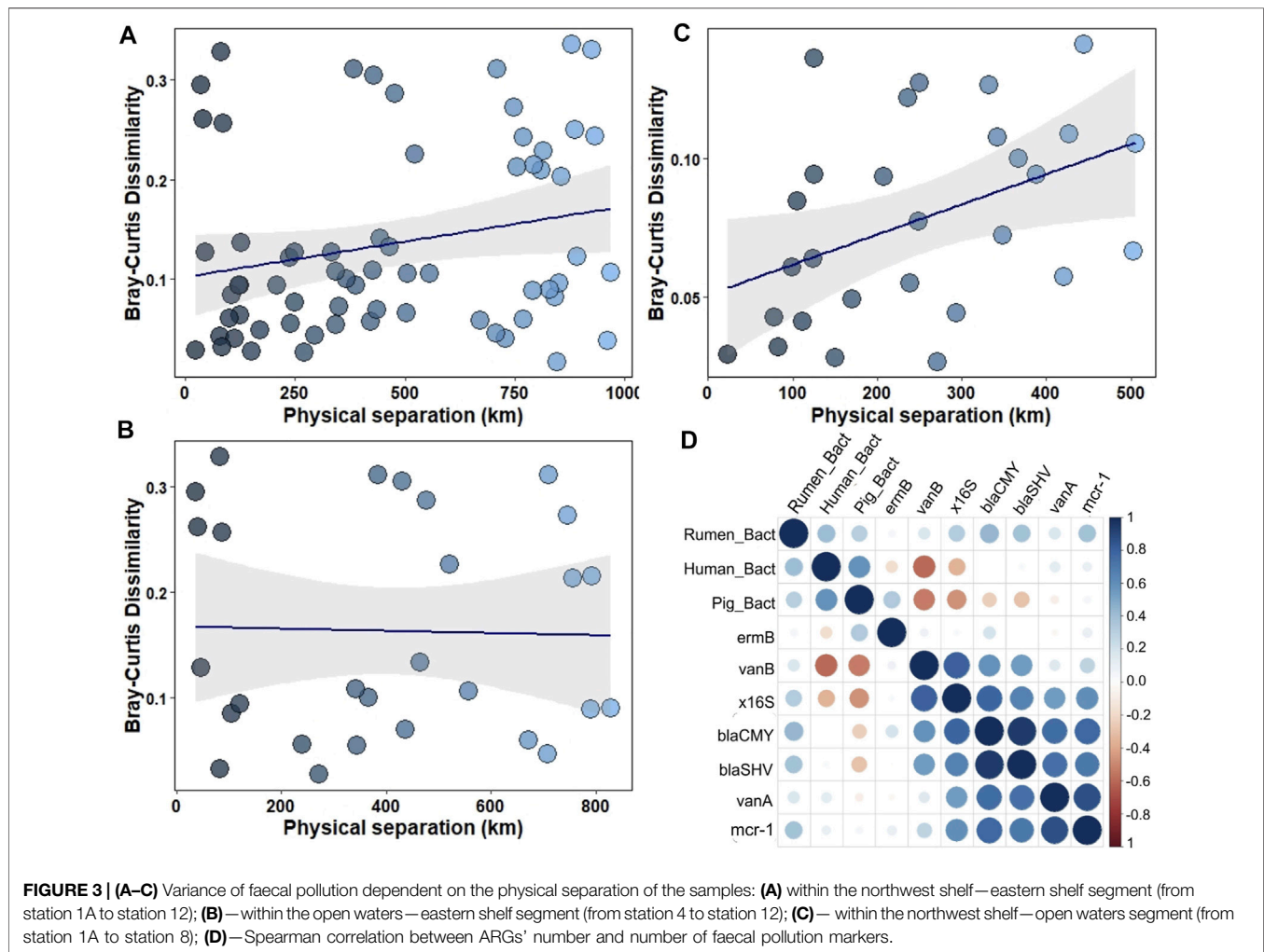
numerous gene followed by bla_{SHV} ($4 \times 10^{-2} \pm 1 \times 10^{-2}$), *mcr-1* ($5 \times 10^{-2} \pm 3 \times 10^{-2}$) and bla_{CMY} ($1 \times 10^{-2} \pm 3 \times 10^{-3}$). *ermB* ($1 \times 10^{-3} \pm 5 \times 10^{-4}$) and *vanA* ($1 \times 10^{-5} \pm 5 \times 10^{-4}$) were the least numerous genes.

Considering the absolute values, differential abundance of ARGs in the waters of eastern, northwest shelf and open waters was pronounced. The higher number of ARGs in the waters of the northwest shelf was especially notable (**Figure 2**). Open waters contained the least number of all ARGs. Wilcoxon pairwise comparison proved that blaCMY, vanA and mcr-1 had significantly higher abundance in the northwest shelf coastal area compared to the open waters and the eastern shelf (Wilcoxon, $p < 0.05$—**Table 2**). Bla_{SHV} and bla_{CMY} had significantly higher estimates in the eastern shelf compared to the open waters, while bla_{CMY} and *mcr-1* significantly varied in their abundance in the eastern and northwestern shelf.

The data normalized to the 16S rRNA did not exhibit a pronounced difference in the three Black Sea areas due to the uneven distribution of 16S rRNA gene. It had the highest estimates in the northwest shelf ($3 \times 10^8 \pm 6 \times 10^7$ copies/μL) and the lowest in the open waters ($3 \times 10^7 \pm 8 \times 10^6$ copies/μL).

TABLE 2 | Wilcoxon rank sum test comparison of the AGS' absolute number in eastern shelf, open waters, northwest shelf. *p*-value adjustment method: fdr (q-value<0.05).

Gene/Target	Groups of comparison		
	Northwest shelf vs. Open waters	Eastern shelf vs. Northwest shelf	Eastern shelf vs. Open waters
bla$_{SHV}$	3.1×10^{-6}	0.38	5.2×10^{-6}
bla$_{CMY}$	8.2×10^{-5}	0.005	0.002
vanA	0.05	0.04	0.05
vanB	0.01	0.28	0.63
mcr-1	0.005	0.002	0.77
ermB	0.04	0.07	0.95
Human *Bacteroidetes*	0.03	0.11	0.003
Pig *Bacteroidetes*	0.63	0.51	0.18
Rumen *Bacteroidetes*	0.03	0.16	0.26
16S rRNA	6.0×10^{-4}	0.02	0.05

FIGURE 3 | (A–C) Variance of faecal pollution dependent on the physical separation of the samples: **(A)** within the northwest shelf—eastern shelf segment (from station 1A to station 12); **(B)**—within the open waters—eastern shelf segment (from station 4 to station 12); **(C)**— within the northwest shelf—open waters segment (from station 1A to station 8); **(D)**—Spearman correlation between ARGs' number and number of faecal pollution markers.

The 16S rRNA copy number was $1 \times 10^8 \pm 5 \times 10^7$ copies/µL in the eastern shelf.

Pollution by Faecal Markers

Genetic markers for human-, rumen- and pig-specific faecal members of the *Bacteroidetes* phylum were quantified to identify the sources of faecal contamination. Anaerobic bacteria belonging to *Bacteroidetes* phylum inhabit the faeces and exhibit host adaptation on the genetic level (Dick et al., 2005). For this reason *Bacteroidetes*' markers (specific regions of the 16S rRNA gene) are used as indicators that can discriminate between human and nonhuman sources of faecal

contamination (Reischer et al., 2006; Mieszkin et al., 2009; Epa and of Science, 2019).

Gene sequences of human-associated *Bacteroidetes* are usually found in the humans' faeces and environments polluted with human faeces. Human-associated *Bacteroidetes* can be present in other animals' sources (Layton et al., 2013). Similarly, pig-associated or cow-associated *Bacteroidetes* can have cross-reaction in other types of faeces due to the common omnivorous diet and similar digestive tract (Dick et al., 2005; Mieszkin et al., 2009). Nevertheless, presence of human-specific *Bacteroidetes* in environmental samples is usually associated with human faecal pollution (Layton et al., 2013).

Number of human associated *Bacteroidetes* (per μL) was $7 \times 10^3 \pm 9 \times 10^2$ in the northwest shelf, $7 \times 10^3 \pm 1 \times 10^2$ in the open water and $6 \times 10^3 \pm 2 \times 10^3$ in the eastern shelf. Number of pig associated *Bacteroidetes* did not vary in samples taken from different locations, presenting $1 \times 10^2 \pm 5 \times 10^1$, $2 \times 10^2 \pm 1 \times 10^2$, $1 \times 10^2 \pm 5 \times 10^1$ marker copy/μL in the northwest shelf, open waters and eastern shelf respectively. Number of rumen-specific *Bacteroidetes* was $6 \times 10^2 \pm 2 \times 10^2$, $4 \times 10^2 \pm 4 \times 10^2$, $4 \times 10^2 \pm 2 \times 10^2$ copies/μL in the three respective studied areas.

Human-specific and rumen-specific *Bacteroidetes* differed in quantity in the northwest coastal waters and open waters according to the Wilcoxon rank sum test (**Table 2**). Mantel test revealed moderate positive correlation (r = 0.52, *p*-value = 0.0033) between the faecal markers and physical separation within the northwest shelf—open waters segment (from station 1A to station 8). There was no correlation if the whole distance (from station 1A to station 12) or open water—eastern shelf segment (from station 4 to station 12) was considered (**Figure 3**). This finding suggests that markers of the faecal pollution mainly enter the Black Sea environment from the northwest coast.

Rumen-specific *Bacteroidetes* had moderate positive correlation (0.4, *p* < 0.05) with bla_{CMY}, bla_{SHV} and *mcr-1* genes (**Figure 3D**). The 16S rRNA gene had strong correlation with bla_{CMY}, bla_{SHV}, *van*A and *mcr-1* genes (**Figure 3D**).

Concentrations of Antibiotics

None of the tested antibiotics exceeded the screening detection limit (1.25 ng/L) possibly due to the high dilution of the analytes in seawater.

DISCUSSION

The distribution of ARGs in the Black Sea was evaluated on the sea region-wide scale involving sampling locations in the eastern shelf, open waters and northwest shelf. The list of genes included bla_{CMY} and bla_{SHV} genes that are responsible for the inactivation of beta-lactam antibiotics, *ermB* targeting macrolides, *van*A and *van*B genes that inactivate glycopeptides and *mcr-1* that compromises effectiveness of colistin.

All studied ARGs were found in the seawater samples. *van*B, bla_{SHV} and bla_{CMY} were among the most numerous ARGs. *mcr-1* had also high estimates, which is of particular interest since, to

our knowledge, this is the first report of the *mcr-1* distribution in the Black Sea. Results of the study reveal that *van*A and *ermB* were the least numerous genes among the tested ones, but they were also found in all analysed samples.

The obtained data was compared to ARGs distribution in other aquatic environments summarized in the holistic analysis of the 122 metagenomic sets from lakes and seas (Yang et al., 2019). According to Yang et al. (2019) the relative abundance of beta-lactams' and aminoglycosides' resistance genes was in the range from 1×10^{-3} to 2×10^{-2} copies normalized to the 16S rRNA. The copy number of polymyxin and vancomycin resistance genes was on average by one order of magnitude lower (up to 3×10^{-3}). Our data on genes coding for aminoglycosides' resistance is in consistence with that of Yang et al. (2019). The sum of bla_{SHV} and bla_{CMY} varied from 7×10^{-3} to 2×10^{-1} copies that is higher compared to the sum of beta-lactam resistance genes presented by Yang et al. (2019). The copy number of beta-lactam resistance genes (bla_{OXA}) in the Danube River Basin had wider variation compared to our data: 2.4×10^{-8}–3.1×10^{-2} (Alygizakis et al., 2019). bla_{CTXM} was detected in the Black Sea water by Sabatino et al. (2020), but its quantity was not estimated.

The present study revealed higher *van*B and *mcr-1* copy number compared to the estimates of vancomycin and polymixyn resistance genes presented by Yang et al. (2019). According to our data *van*B reached $2 \times 10^{-1} \pm 1 \times 10^{-1}$ copies normalized to 16S rRNA in the Black Sea water. This gene was not detected in the effluents of WWTPs in the Danube River Basin, while *van*A occurred sporadically (Alygizakis et al., 2019). That indicates that the Danube river is likely not a source of the *van*B in the Black Sea, and the other rivers (Dnieper, Dniester or Western Bug) on the northwest site should be considered in this regard. The number of *mcr-1* genes varied from 2×10^{-3} to 3×10^{-2} copies normalized to 16S rRNA.

The copy number of *ermB* (1.8×10^{-5}–4.9×10^{-3}) in the effluents of WWTPs in the Danube River Basin (Alygizakis et al., 2019) may indicate the origin of this gene distribution. Contrasting with our data, no *ermB* was detected in the Black Sea water samples according to (Sabatino et al., 2020). This indicates that *ermB* dissemination in the Black Sea might be possibly on its rise.

High number of ARGs in the Black Sea is likely associated with prescription rates and poorly controlled antibiotic consumption in the riparian countries. Amoxicillin, ceftriaxone and cefuroxime were the most consumed antibiotics during 2013–2018 in Ukraine (Yakovlieva and Bahlai, 2019), which is in parallel with the increase in macrolides consumption in this region (Matyashova and Iakovlieva, 2015). According to (Tarcea Bizo et al., 2015), vancomycin is the most prescribed antibiotic in a university tertiary hospital in Cluj-Napoca, Romania. Meanwhile, European Resistance Surveillance Network (EARS-NET, 2019) reports distribution of hospital isolates resistant to beta-lactams and vancomycin in Black Sea countries. About 63% of *E. coli* isolates were resistant to amoxicillin/ampicillin in hospitals of Romania and Bulgaria. From 64.1 to 75.7% of *K. pneumoniae* isolates were resistant to cefotaxime/ceftriaxone/

ceftazidime in these two countries respectively. About 35.7% isolates of *E. faecium* from hospitals of Romania were resistant to vancomycin. Joint Danube Survey 3 results indicate the distribution of the beta-lactam and carbapenem resistant *Pseudomonas* spp., *Klebsiella* spp. and *E. coli* in the Danube water (Kittinger et al., 2016a; Kittinger et al., 2016b). Isolates with resistance patterns normally associated with intensive care units were also found in the Danube river water (Kittinger et al., 2016a). Unfortunately, data reporting the colistin resistance is not presented in the EARS-NET Report. Though, besides the human treatment, colistin is widely applied in veterinary medicine, which fuels the development of the resistance to this antibiotic (Kempf et al., 2016).

The differential abundance of all tested ARGs according to the location of the sampling stations is notable. ARGs were significantly higher (Wilcoxon rank sum test, $p < 0.05$) in number in the northwest shelf compared to the open waters. bla_{CMY} and bla_{SHV} were considerably more abundant in the eastern shelf than in the open waters. It goes in line with the data on *sul1* distribution in the coastal waters of the Black Sea (Sabatino et al., 2020). Similar effect was observed in coastal and open waters of the Bohai Sea and Yellow Sea areas (Lu et al., 2019). The difference in ARG number in the open and coastal waters clearly evidences the anthropogenic impact on the ARGs' distribution in the Black Sea environment.

The main sources of the coastal and estuarine pollution with ARGs are riverine runoffs, WWTPs, aquaculture and untreated sewage (Zheng et al., 2021). Big cities such as Odessa (Ukraine) and Constanta (Romania) along with a number of smaller towns (total population more than 1.5 million) are located on the northwest bank of the Black Sea. Moreover, Dnieper, Western Bug, Dniester and Danube rivers (total basin area is 1,437,790 km^2) fall into the Black Sea on this side providing input of ARGs and other pollutants. Analysis of effluent wastewater from WWTPs in the Danube River Basin has shown the abundance of ARGs (*aph(III)a*, *blaOXA*, *ermB*, *tetM* etc.,) in the wastewater released into the Danube river and its tributaries (Alygizakis et al., 2019). Similar accumulation of the ARGs is likely to occur in the other rivers. The eastern bank of the Black Sea is less populated with Batumi (Georgia) as the biggest city. Rivers Chorokhi and Rioni fall into the Black Sea on the eastern side and may provide input of the ARGs collected along their basins (total area of basins is 35,500 km^2). The higher number of the *vanA* and *mcr-1* in the northwest shelf compared to the eastern shelf is worth noting. Wider area of river basins falling on the north in the Black Sea, extensive agriculture and peculiarities of antibiotic consumption in the region can cause the differences in ARGs' abundance.

ARGs can persist in the environment due to the presence of antibiotics or disinfectants (Zheng et al., 2021), which provide the selective pressure and facilitate the HGT. On the other hand, faecal pollution is a straightforward source of ARGs dissemination in the environment since gut microbiota is a reservoir of antibiotic-resistant bacteria (Karkman et al., 2018; Khan et al., 2019). The concentrations of 22 targeted antibiotics were below the detection limit (1.25 ng/L) in all tested samples. This excludes the influence of antibiotics on the ARGs proliferation, while the ARGs' number decrease in the open waters evidences the elimination of the ARGs from the environment.

Previous studies indicated that faecal pollution (from urban areas, animal farms and pasture) was a crucial problem throughout the Danube River Basin (Kirschner et al., 2009). Obviously, the Black Sea is impacted by faecal polluted water from the Danube river and other tributaries, which is reflected in elevated concentration of the markers of faecal pollution in the northwest shelf area. bla_{CMY}, bla_{SHV} and *mcr-1* had moderate positive correlation with the *Bacteroidetes* common to the ruminant microbiome. The positive moderate correlation may evidence the impact of the beta-lactams and colistin use in the veterinary pharmaceuticals on the dissemination of these ARGs in the Black Sea. However, the faecal pollution is likely not the major reason for increased concentration of the ARGs in the coastal waters. Among the other reasons can be pollution with ARBs harbouring the corresponding ARGs since all ARGs had a strong positive correlation with 16S rRNA gene. It can be noted that ARGs could be selected for and transmitted to the environmental bacteria before entering the Black Sea (*via* WWTPs, sewage, etc.,), while the Black Sea receives the ARBs together with riverine and sewage input.

CONCLUSION

The distribution of ARGs targeting a wide spectrum of antibiotics (beta-lactams, macrolides, glycopeptides, colistin) including both first-line and last-resort antibiotics was investigated in the Black Sea water samples at a region-wide scale, including shelf and offshore areas. All studied ARGs were detected in all of the studied samples. The number of ARGs inactivating beta-lactams, vancomycin and polymyxin was higher compared to the previous studies performed in the region. The elevated number of colistin resistance *mcr-1* gene is of particular interest, as this gene was reported in Black Sea microbial communities for the first time.

ARGs were significantly higher in number in the shelf areas compared to the open waters, which signals involvement of the anthropogenic impact on the natural resistome of the water ecosystem.

The ARGs targeting beta-lactams, vancomycin and last resort antibiotic colistin were of the highest abundance in the northwest shelf, which might be attributed to poorly controlled antibiotic consumption in the riparian countries.

The observed correlation between bla_{CMY}, bla_{SHV} and *mcr-1* with the *Bacteroidetes* common to the ruminant microbiome indicates that the potential sources of ARGs in the Black Sea are the riverine and sewage inflow and, to the lesser extent, application of veterinary pharmaceuticals.

The results are of high environmental concern, highlighting an alarming presence of antibiotic resistance in the Black Sea. An inclusion of ARGs into regular legacy monitoring of the Black Sea and major inflowing rivers is recommended.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

IP: Formal analysis (lead); Investigation (equal); Methodology (equal); Visualization (equal); Writing—original draft (lead). MP: Investigation (equal); Methodology (equal); Validation (equal); Visualization (supporting); Writing—original draft (supporting). AD: Investigation (supporting); Validation (supporting); Visualization (supporting). ED: Investigation (supporting); Project administration (equal); Supervision (equal). NA: Formal analysis (supporting), Methodology (supporting), Writing—original draft (supporting). JS: Formal analysis (supporting); Project administration (equal); Resources (lead); Writing—original draft (supporting).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.823172/full#supplementary-material

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