Science of the Total Environment 955 (2024) 176765

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Global quantification and distribution of antibiotic resistance genes in oceans and seas: Anthropogenic impacts and regional variability

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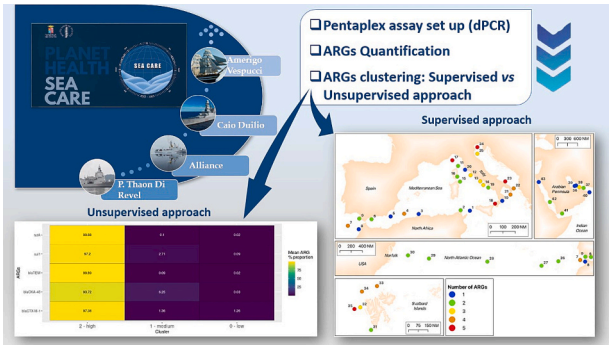
HIGHLIGHTS

- Novel pentaplex digital PCR assay enhances precise detection of multiple ARGs.
- Global analysis reveals widespread ARGs in marine environments, including remote regions.
- High ARGs contamination found in the Mediterranean Sea and Arctic Ocean near Svalbard
- Two clustering methods show 51 % concordance in classifying ARG contamination levels.
- Potential contamination hotspots linked to human activities found in marine ecosystems

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damia Barcelo

Keywords:
Antibiotic resistance genes (ARGs)
Marine environment
Distribution patterns
Anthropogenic impact
Clustering

ABSTRACT

The global spread of antibiotic resistance genes (ARGs) in the marine environment poses a significant threat to public health and natural ecosystems. This study quantified and analysed the distribution and co-occurrence patterns of ARGs in a wide range of oceans and high seas, including the Atlantic, Arctic and Indian Ocean, the Mediterranean Sea and the Persian Gulf. Focusing on beta-lactamases ($bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-1 group}}$, and bla_{TEM}), sulfonamides (*sul*1) and tetracycline (*tet*A), our results showed that *sul*1 was ubiquitous, indicating widespread dissemination. Notably, the Mediterranean Sea exhibited higher levels of multiple ARGs in single samples, suggesting significant anthropogenic impact. Interestingly, the Arctic Ocean, particularly around the Svalbard Islands, also showed the presence of multiple ARGs, highlighting the pervasive occurrence of antibiotic resistance in remote areas. We employed two clustering approaches to explore ARG patterns, primarily focusing on identifying geographic trends and differences in ARG abundance. Additionally, we investigated potential sources of contamination, including proximity to wastewater treatment plants, ports, marine traffic, and currents.

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https://doi.org/10.1016/j.scitotenv.2024.176765
Received 10 August 2024; Received in revised form 16 September 2024; Accepted 4 October 2024
Available online 10 October 2024

These findings clearly demonstrate that antibiotic resistance gene contamination is widespread across diverse marine environments, with significant regional variations. This underscores the urgent need for tailored intervention strategies and global collaboration to mitigate the spread of ARGs and manage their complex dynamics in marine ecosystems.

1. Introduction

Antimicrobial resistance (AMR) represents a complex global challenge that affects humans, animals and the environment, posing a significant threat to public health and the delicate balance of natural ecosystems. While clinical contexts typically receive the most attention, the environmental dimension has emerged as a critical area for understanding and addressing this multifaceted problem. Recognizing the role of the environment is essential for developing comprehensive strategies to face up to AMR. Research has demonstrated the widespread presence of antibiotic resistance genes (ARGs) in various aquatic environments, including raw and treated wastewater, as well as surface water such as rivers, lakes, seas and oceans. Understanding the impact of these genes requires a comprehensive approach to elucidate their pathways and effects in different ecosystems.

The World Health Organization's Global Action Plan on Antimicrobial Resistance (WHO, 2015) provides a framework for the development of national action plans. It outlines key actions that should be taken by different stakeholders to combat AMR. One of these actions is to understand the emergence and spread of resistance. This involves understanding how resistance moves within and between humans and animals, as well as through food, water, and the environment. In its Communication of 29 June 2017, entitled 'A European One Health Action Plan against AMR', the European Commission sets out in detail over 70 actions covering human health, animal health, and the environment (https://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX%3A52017DC0339). However, more actions are needed, particularly in the areas of human health and the environment. On 13 June 2023, the Council of the EU adopted a Recommendation (2023/C 220/01) to address antimicrobial resistance using a One Health approach. The Recommendation sets targets to be achieved in the EU by 2030 and recommends that Member States adopt a One Health approach, particularly in relation to measures concerning the environment, which are often underdeveloped or absent. There is growing evidence that the natural environment is a significant reservoir and a key driver of AMR. To better understand the role of antimicrobial residues in the emergence and spread of AMR, it is essential to monitor their presence in groundwaters, surface waters (including coastal waters), wastewaters, and even agricultural soils.

The oceans play a critical role in hosting and maintaining bacteria exhibiting both natural and acquired antibiotic resistance. These microorganisms, along with the genetic elements conferring resistance, persist and thrive in marine environments. Antibiotics introduced into marine ecosystems from various sources such as agricultural run-off, pharmaceutical waste, and human activities, create selective pressures that promote the survival and proliferation of ARBs and the increase of ARGs.

Horizontal Gene Transfer (HGT) plays a pivotal role in the rapid dissemination of antibiotic resistance genes among diverse bacterial populations. This process, which involves the exchange of genetic material between unrelated organisms, accelerates the evolution of multidrug-resistant bacteria. Marine environments provide ideal conditions for HGT from environmental bacteria to human pathogens and vice versa, facilitated by factors such as high bacterial densities, the presence of mobile genetic elements (e.g., plasmids, transposons), and environmental stressors.

Therefore, studying the dynamics of antibiotic resistance in marine ecosystems is crucial to understanding, with a comprehensive One Health approach, how these genes may impact human and animal health, and the environment. This study was carried out as part of the 'Sea Care' project, a collaboration between the Italian Military Navy and the National Institute of Health (Istituto Superiore di Sanità - ISS). The project aims to study the health risks associated with the environment and climate change from a 'planetary health' perspective (see the referenced Sea Care video). The main objective is to collect data on the health of the oceans, contributing to a comprehensive understanding of the potential health risks posed by environmental factors. The project, running from 2022 to 2025, involves the collection of samples along the regular routes of naval units of the Italian Military Navy in territorial and international waters. In this study, we investigated the distribution and abundance of ARGs in the marine environments across the oceans and seas. Understanding these dynamics is crucial for developing evidence-based policies and interventions across disciplines, in line with the One Health approach.

2. Materials and methods

2.1. Sampling and DNA isolation

Forty-three marine water samples were primarily collected in the open sea, away from direct sources of anthropogenic pollution. Specifically, in the Mediterranean basin, with few exceptions, sampling sites were selected outside the areas covered by the Marine Strategy Monitoring Network, which are beyond 12 nautical miles from the coast. Supplementary Table S1 shows the distances of all sampling points from the coast (average distance: 62.9 nautical miles). Expeditions were conducted aboard of four military naval vessels between May 2022 and January 2023, namely the Amerigo Vespucci, Caio Duilio, Alliance, and Paolo Thaon di Revel, covering a wide range of regions, including the Mediterranean Sea, the Persian Gulf, the Gulf of Oman, the Gulf of Aden, the Red Sea, the Atlantic Ocean, and the Arctic Ocean (Table 1).

Five liters of seawater were collected using a submersible pump (upper 20–30 cm of the water column) by the ISS researchers embarked on the ship. The filtration of the seawater samples was usually performed on the military ships immediately after sampling, except for a few cases of night sampling, which were filtered within 8 h from sampling. Samples were pre-filtered using 5 μm porosity mixed cellulose ester membranes to remove larger particles and prevent clogging of the finer filter. The prefilter was discarded after this step. Then the sample (3 to 5 L) was filtered through sterile 0.22 μm porosity mixed cellulose ester membranes (Whatman, UK). After filtration, the membranes were placed in Eppendorf tubes and frozen at −20 °C on board the ship. The samples remained frozen until the ship docked, at which point they were transported to ISS under refrigerated conditions. Upon arrival at ISS, nucleic acid extraction was performed immediately. Total DNA was extracted directly from the 0.22 μm membranes, using the DNeasy PowerWater Kit (Qiagen, Hilden, Germany), specifically designed to isolate genomic DNA from various types of filtered water samples. Each sample yielded 100 μL of final extract, which was then divided into two aliquots and stored at −80 °C for further analysis. A blank sample of sterile water was included to verify the absence of contamination during the DNA extraction process. The concentration of genomic DNA (gDNA) was determined using both the Qubit dsDNA HS Fluorometric Assay Kit (Invitrogen, USA) and the Agilent® High Sensitivity DNA kit.

2.2. Novel pentaplex digital PCR assay

Genes were selected for their ability to confer resistance to various

Table 1
Marine sampling site and naval vessel.

Naval vessel	Sample ID	Map ID	Sampling date	Ocean/sea	Site name	Latitude	Longitude	Distance from the nearest coast (nm)
Caio Duilio	ATL-1 V	26	13-May-22	Atlantic Ocean	East Atlantic Ocean	35°7′00" N	16° 7′00"W	167
	ATL-2 V	27	14-May-22		SW Azores Islands	33°47′60" N	22° 6′00" W	270
	ATL-3 V	28	17-May-22		Central Atlantic Ocean	35°1′3.96"N	41°23′3.96"W	735
	ATL-4 V	29	20-May-22		mid-way between ATL3V&5 V	35°54′16.80"N	62°9′47.40"W	280
	ATL-5 V	30	21-May-22		West Atlantic Ocean (Norfolk)	36°49′6.00"N	69°38′0.00"W	330
Amerigo Vespucci	MED all 7	7	2-Aug-22		Casablanca	35°28′60.00"N	8°20′60.00"W	105
	MED all 8	8	3-Aug-22		Morocco	35° 1′3.60"N	6°34′54.00"W	18
	MED all 9	9	10-Aug-22		Cádiz	36°18′37.80"N	6°34′60.00"W	21
Alliance	HN 22_002_SW_001	31	5-Jul-22	Arctic Ocean	Svalbard Island south	76°26.650′N	013°58.941′E	38
	HN 22_012_SW_003	32	7-Jul-22		Svalbard Island north west	79°00.810′N	008°11.322′E	30
	HN 22_046_SW_006	33	11-Jul-22		Svalbard Island north east	80°59.011′N	017°10.927′E	45
	HN 22_085_SW_007	34	16-Jul-22		Svalbard Island north	80°30.832′N	009°06.059′E	56
	HN 22_093_SW_008	35	17-Jul-22		Svalbard Island north west	78°50.145′N	005°03.232′E	70
Amerigo Vespucci	MED all 1	1	11-Jul-22	Mediterranean Sea	Strait of Sicily	37°27′7.86"N	11°31′50.10″E	37
	MED all 2	2	15-Jul-22		near Tunis	37°30′48.00"N	10° 1′24.00″E	15
	MED all 3	3	21-Jul-22		Algiers	37°1′18.00"N	3°11′6.00″E	15
	MED all 4	4	22-Jul-22		Algeria, (meridian 0.8°)	37°3′36.00"N	0°49′48.00″E	43
	MED all 5	5	23-Jul-22		Alboran Sea	36°42′12.00"N	1°23′28.20"W	38
	MED all 6	6	24-Jul-22		Strait of Gibraltar	36°19′30.00"N	4°31′24.00"W	14
	MED all 10	10	29-Aug-22		Catanzaro	38°43′0.00"N	16°49′0.00″E	105
	MED post 3	18	22-Oct-22		Strait of Messina	38°21′30.00"N	15°38′24.00″E	18
	MED post 4	19	7-Aug-22		Capri	40°22′60.00"N	14°31′60.00″E	21
	MED post 5	20	9-Sep-22		Elba	42°34′0.00"N	10°45′0.00″E	12
	MED post 6	21	29-Sep-22		Leuca	39°30′36.00"N	18° 8′60.00″E	6
	MED post 7	22	19-Oct-22		Otranto	40°16′60.00"N	18°40′60.00″E	17
	MED post 8	23	15-Oct-22		Monopoli	41°8′42.00"N	17°18′0.00″E	14
	MED post 12	24	10-Oct-22		Venice	45°13′0.60"N	12°44′49.20″E	22
	MED post 14	25	1-Oct-22		Ravenna	44°27′16.20"N	12°44′36.00″E	13
	MED pre 1	11	6-Jun-22		Corsica Channel	43°1′14.98"N	9°36′37.29″E	8
	MED pre 2	12	7-Jun-22		Civitavecchia	41°54′18.00"N	11°38′21.00″E	13
	MED pre 3	13	7-Jun-22		Gaeta	41°9′20.01"N	13°13′15.89″E	8
	MED pre 4	14	13-May-22		Ischia	40°41′53.00"N	13°43′25.00″E	7
	MED pre 5	15	17-Jun-22		Sardinia, La Maddalena	41°7′12.00"N	9°55′22.00″E	19
	MED pre 6	16	18-Jun-22		Corsica Island South East	41°23′43.00"N	9°28′27.00″E	4
	MED pre 7	17	19-Jun-22		Ligurian Sea	43°41′41.00"N	8°40′18.00″E	31
PaoloThaon di Revel	TDR 22–1	36	10-Nov-22	Persian Gulf	Dubai	25°20′42.18"N	54°49′53.52″E	27
	TDR 22–3	38	13-Nov-22		Lavan	26° 4′23.88"N	53°13′1.14″E	37
	TDR 22–4	39	24-Nov-22		Doha (Qatar)	25°51′21.18"N	52°30′39.18″E	54
	TDR 22–2	37	11-Nov-22	Oman Gulf	Hormuz	24°57′45.90"N	57° 0′54.96″E	57
	TDR 22–5	40	10-Jan-23	Arabic Sea	Oman	22°56′26.40"N	59°56′24.60″E	29
	TDR 22–6	41	12-Jan-23	Aden Gulf	Aden	12°49′0.00"N	46°10′60.00″E	43
	TDR 22–7	42	15-Jan-23	Red Sea	central Red Sea	18°23′30.00"N	40° 2′48.00″E	80
	TDR 22–8	43	19-Jan-23		Sharm El Sheikh	27°37′42.00"N	34°26′18.00″E	13

antibiotics, such as beta-lactamases (specifically $bla_{\text{OXA-48}}$, $bla_{\text{CTXM-1 group}}$, bla_{TEM}), sulfonamides (*sul1*), and tetracycline (*tetA*). Quantification of these genes was performed by digital polymerase chain reaction (dPCR) using the QIAcuity One five-plex device (Qiagen, Hilden, Germany), and Nanoplate with a 26 K configuration. Quantitative results were generated using the Quiacuity Software Suite (version 2.2.0.26) (Qiagen, Hilden, Germany). To ensure measurements remained within the reliable curve section of the instrument's dynamic range, 10-fold serial dilutions (from 10^{-1} to 10^{-8}) were prepared for both ARGs and 16S rRNA. The total bacterial load in the samples was estimated by quantification of 16S rRNA gene (Muyzer et al., 1993).

The dPCR Microbial DNA Detection Assays (Qiagen, Hilden, Germany) were used to quantify the target genes using five different fluorescent dyes (Table S2), and the QIAcuity UCP Probe PCR Kit (Qiagen) was used to prepare all pre-reactions, according to the manufacturer's instructions. Once the working dilutions were established, a pentaplex assay was set up and rigorously compared with each singleplex assay. One sample positive for the 5 ARGs panel was selected for these measurements, and the results were subjected to a non-paired t-Student test with 10 replicates for each group (pentaplex vs. singleplex); the significance level was set at alpha <0.05. The analysis of t-Student test was carried out using the *stats v.4.3.1* package for *R*.

The optimized pentaplex assay was performed in duplicate on the complete samples set, using 5 μL of template per sample. The target concentration (copies/μL) in each sample was calculated by utilising the instrument's output and applying the following formula:

$$\text{Sample concentration (copies}/\mu L) = \frac{\text{dPCR outcome (genome copies}/\mu L) \times (\text{Reaction vol.})}{\text{DNA vol.}}$$

To express the concentration in genome copies per liter of sea/ocean water, the following equation was applied:

$$\text{Concentration (copies}/L) = \frac{(\text{Sample concentration [copies}/\mu L] \times \text{Final extraction volume [}\mu L\text{]})}{\text{Original water volume [}L\text{]}}$$

The digital MIQE guidelines served as an established standard for the technical quality of dPCR (dMIQE Group and Huggett JF, 2020) (Table S3).

Salmonella enterica subsp. *enterica,* serovar Choleraesuis (ATCC™ 10708) strain was used as positive control for 16S rRNA, and a ceftazidime/avibactam-resistant KPC-producing *K. pneumoniae* CZA-R (Accession: PRJNA866305) strain CZ5 was used for bla_{OXA48}, $bla_{\text{CTX-M-1 group}}$, bla_{TEM}, *sul*1 and *tet*A (Bongiorno et al., 2023). A negative control (sterile water) was systematically included in each run.

Potential inhibition was evaluated by quantifying the linearity of target gene concentrations in 10-fold serial dilutions. ARG abundance was measured as both absolute (gene copies/L) and relative (gene copies/16S rRNA gene copies number). Quantitative data were plotted as heatmap representations using *R ggplot2 v3.4.3*.

At qualitative level, the presence of ARGs (from 1 to 5) was represented on a thematic map, created with *QGIS v3.34 "Prizren"*, visualizing the spatial distribution of ARGs across different sampling sites.

2.3. Sampling sites classification

An attempt was made to classify the 43 samples according to their level of ARGs contamination and to investigate whether geographic areas could play a role in the resulting categories. Two different approaches were used to achieve this objective: a supervised and an unsupervised approach. In the supervised approach, we predefined categories based on specific criteria before analyzing the data. Specifically, we established three levels of impact based on the number of ARGs detected in each sample: low impact (1 or 2 ARGs), medium impact (3 or 4 ARGs), and high impact (5 ARGs, representing a full panel). In the unsupervised analysis, the relative abundance of ARGs was assessed using a data-driven approach to classify the sampling sites. The process began by reducing the dataset dimensionality, which included five ARGs dimensions, to two dimensions using the t-SNE (t-distributed Stochastic Neighbor Embedding) algorithm. This facilitated the visualization of natural groupings and patterns in a scatterplot. Following this, the DBSCAN (Density-Based Spatial Clustering of Applications with Noise) algorithm was applied to identify clusters within the data. The results, including the mean abundance percentage proportion of ARGs within these clusters, were visualized using heatmaps and scatter plots created with R ggplot2 v3.4.3. The analysis was performed using Python v3.09 and the scikit-learn v1.2.2 library. This unsupervised method was then compared with the supervised approach to evaluate their effectiveness in categorizing the samples based on ARGs presence and abundance.

2.4. Mapping of potential determinant environmental factors

Thematic maps were created to highlight key environmental impact factors potentially influencing the sampling sites identified as high impact by both supervised and unsupervised approaches. These factors included proximity to wastewater treatment plant discharges, port, and aquaculture farming facilities, as well as the intensity and direction of marine currents, and the intensity of maritime traffic. The maps were plotted with QGIS using databases from the European Marine Observation and Data Network (EMODnet) (https://emodnet.ec.europa.eu/en/about_emodnet), Copernicus Marine Service (CMEMS) (https://marine.copernicus.eu/about) and the Datahub from the European Environment Agency (EEA) (https://www.eea.europa.eu/en/datahub). In particular, wastewater treatment plant discharges points were obtained from EEA: eea_v_4258_100_m_uwwtd-disc-pts_p_2019-2020_v08_r00; marine currents were obtained from CMEMS: doi:10.48670/moi-00016 and ports data were acquired from EMODnet (EMODnet: EMODnet_HA_Main_Ports_Traffic_20231106), aquaculture farming facilities of finfish production (EMODnet: EMODnet_HA_Aquaculture_Marine_Finfish_20210913), and marine traffic (EMODnet: EMODnet_HA_EMSA_Route_Density_Map_20191111). These maps provided a visual representation of the proximity and potential influence of each source of impact relative to the sampling sites at the selected scale.

3. Results

3.1. Pentaplex assay optimization and ARGs quantification

The optimal working dilutions for the ARGs dPCR assay were determined, with the undiluted samples emerging as the most suitable concentration. This concentration was subsequently employed to validate the pentaplex assay. No statistically significant difference was observed between the pentaplex and singleplex assays results (p-value >0.05, Table S4). Therefore, the pentaplex assay was utilized for its efficiency and reliability in simultaneously detecting multiple ARGs without compromising the accuracy of the analysis.

Analysis, performed in duplicate, detected the presence of ARGs in all 43 samples. The absolute ARGs abundance (g.c/L) and relative abundance (g.c/16S rRNA g.c) were determined. The 10-fold dilutions showed linearity, indicating no inhibition in the samples. Graphical outputs for positive and negative controls for all target ARGs are available in the Supplementary Material (Fig. S1). Coefficient of Variation (CV%) of data for each gene within assays are listed in Table S5.

The ARGs were identified in the samples as follows: *sul*1 (43/43), bla_{TEM} (24/43), bla_{OXA48} (19/43), *tet*A (16/43) and $bla_{\text{CTX-M-1 group}}$ (7/43). The mean concentrations and standard deviations of absolute and relative abundance were calculated (Tables S6 and S7, respectively). Fig. 1 shows the heatmaps of the absolute and relative abundance of the 5 target genes. For absolute abundance (panel a), concentrations, expressed in g.c/L of water, ranged as follows: 16SrRNA ($4{,}5 \times 10^7$ to

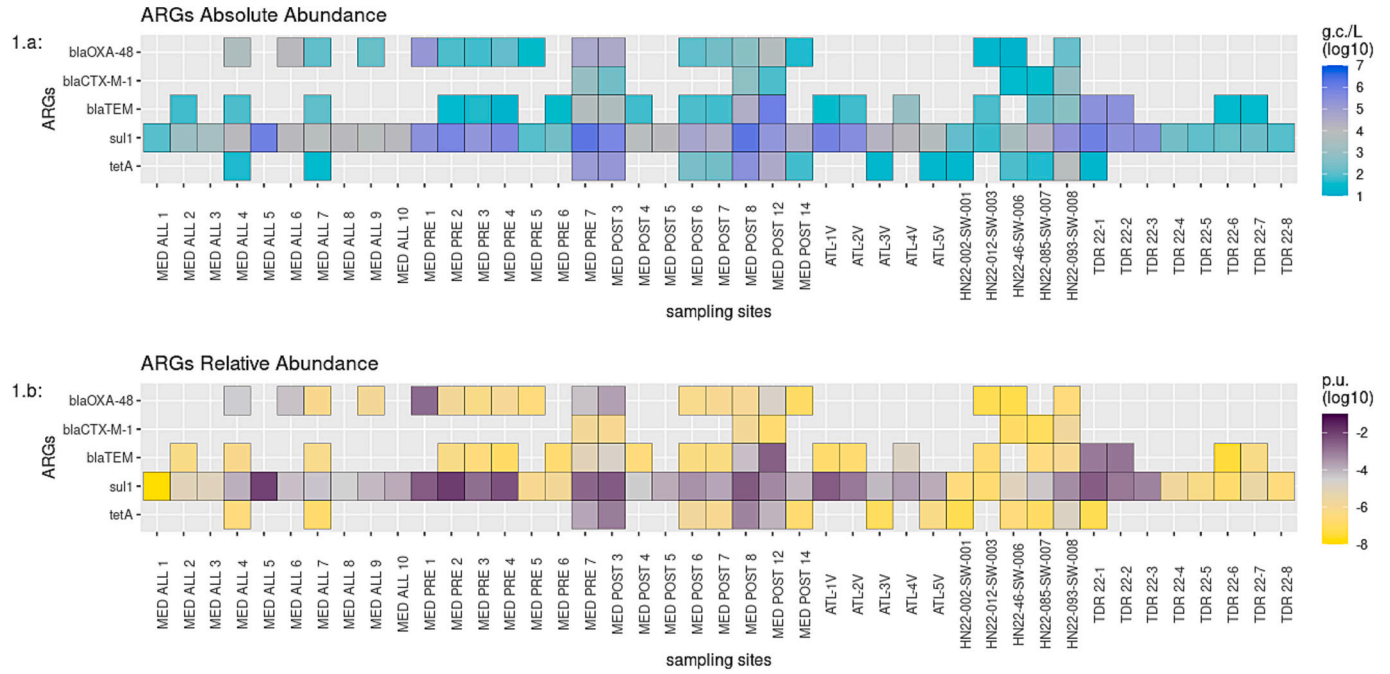

Fig. 1. ARGs abundance - a. ARGs Absolute abundance: values expressed in gene copies/L; b. ARGs relative abundance: gene copies normalized per unit (p.u.) of 16S rRNA gene copy.

$7{,}6 \times 10^{9}$); *sul*1 ($5{,}7 \times 10^{1}$ to $1{,}8 \times 10^{6}$); bla_{TEM} ($1{,}5 \times 10^{1}$ to 1×10^{6}); bla_{OXA48} ($1{,}5 \times 10^{1}$ to $1{,}7 \times 10^{5}$), *tet*A ($1{,}9 \times 10^{1}$ to $1{,}7 \times 10^{5}$) and $bla_{\text{CTX-M-1group}}$ ($3{,}1 \times 10^{1}$ to $1{,}1 \times 10^{3}$). For relative abundance (panel b), normalized gene copies ranged as follows: *sul*1 ($1{,}4 \times 10^{-8}$ to $1{,}2 \times 10^{-2}$); bla_{TEM} ($3{,}3 \times 10^{-8}$ to $1{,}1 \times 10^{-3}$); bla_{OXA48} ($5{,}8 \times 10^{-8}$ to $1{,}9 \times 10^{-3}$); *tet*A (9×10^{-8} to $1{,}5 \times 10^{-4}$); $bla_{\text{CTX-M-1 group}}$ ($9{,}3 \times 10^{-8}$ to $1{,}5 \times 10^{-6}$).

3.2. Sampling sites classification

The presence and distribution of ARGs at marine sampling sites, with the number of the detected ARGs ranging from 1 to 5, are shown in Fig. 2. Sampling sites are marked by coloured dots, with each colour representing the number of ARGs detected at that location. A total of 11 samples had only 1 ARG (represented by blue dots), while 5 samples had the highest number of 5 ARGs (represented by red dots). The *sul*1 gene was present in 100 % of the samples, demonstrating its widespread occurrence across all sites.

On a global scale, the impact categories, based on the supervised approach, were summarized as follows: low impact (26/43 samples, 60 %); medium impact (12/43, 28 %); high impact (5/43, 12 %). The high impact category was predominantly populated by samples from the Mediterranean Sea (4/5) and Svalbard Islands (1/5). In contrast, samples from the Atlantic Ocean, Persian Gulf, and Indian Ocean (Oman and Aden gulfs) mainly fell into the low impact category (Fig. S2). The results of the unsupervised approach, which considers the relative abundance of ARGs, are presented in Fig. 3. This figure identifies three clusters in panel 3a: cluster 0 (red circles), cluster 1 (green circles), and cluster 2 (blue circles). The five samples that showed contamination with all the genes all fall into cluster 2. Panel 3b shows that the geographical location of the samples has no influence on the clustering process, as samples from distinct geographical locations are grouped together within the main clusters. This indicates that the clustering is primarily driven by the ARG content and abundance, rather than by the geographical origin of the samples. Sampling sites associated with each cluster are shown in Table S8.

To further explore the significance of the clusters, we generated a heatmap showing the relative abundance of ARGs in the three clusters, as seen in Fig. 4. The heatmap reveals that cluster 0 had very low mean abundance percentage proportion across all ARGs, with the highest value being 1.2 % for $bla_{\text{CTX-M-1 group}}$. Cluster 1 showed an increase in relative abundance, with $bla_{\text{OXA-48}}$ reaching up to 6.2 %. Cluster 2, however, demonstrated a markedly higher relative abundance for the majority of ARGs, such as *tet*A at 99.8 % and *sul*1 at 97.2 %, accounting for over 93 % of the total abundance for each of the five ARGs. Consequently, similar to the supervised approach, we classified these three clusters as representing low (cluster 0), medium (cluster 1), and high (cluster 2) impact, respectively. Then, we compared the supervised and the unsupervised classification approaches to assess how concentration data influences the classification. A global concordance of 51 % was observed (Supplementary Fig. S2), indicating that the half of samples were recategorized, highlighting the impact of incorporating the relative abundance of ARGs in the unsupervised approach. All 5 samples classified as high impact by the supervised approach were also classified as high impact by the unsupervised approach. Among the 12 samples classified as medium impact in the supervised approach, 6 remained in the same category in the unsupervised approach, while 4 were reclassified to high and 2 to a low impact category. Similarly, of the 26 samples classified as low impact by the supervised approach, 11 retained their classification in the unsupervised approach, while the remainder 15 were reclassified into medium (9 samples) or high impact categories (6 samples).

3.3. Mapping of potential determinant environmental factors

Five samples were consistently classified in the high impact category by both the supervised and unsupervised classification. Potential environmental factors that could explain these results are shown in Figs. 5 (Mediterranean Sea) and 6 (Arctic Ocean). These factors included the proximity to sewage treatment plant discharges, ports, finfish aquaculture farms, and the intensity and direction of the major currents.

Four out of five high impact samples were collected in the Mediterranean Sea, namely MED PRE 7, MED POST 3, MED POST 8, MED POST 12, in areas along the Italian coast between 6 and 31 nautical miles, all characterized by a high density of WWTP discharges and proximity to commercial ports (Fig. 5). The map also shows the dominant local

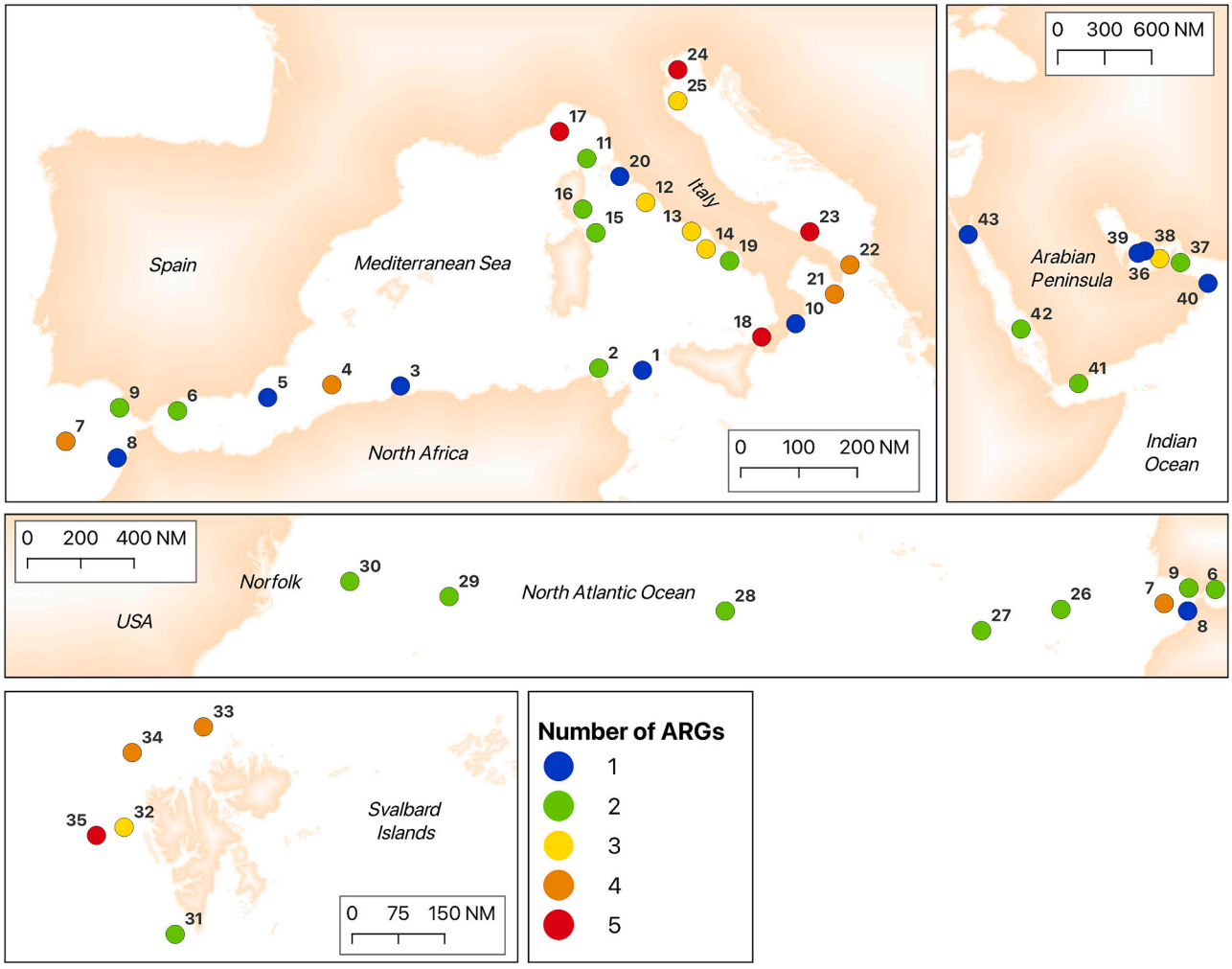

Fig. 2. Global Distribution of Antibiotic Resistance Genes (ARGs) in Marine Sampling Sites - Mediterranean Sea: 1 = MED all 1; 2 = MED all 2; 3 = MED all 3; 4 = MED all 4; 5 = MED all 5; 6 = MED all 6; 10 = MED all 10; 11 = MED pre 1 12 = MED pre 2; 13 = MED pre 3; 14 = MED pre 4; 15 = MED pre 5; 16 = MED pre 6; 17 = MED pre 7; 18 = MED post 3; 19 = MED post 4; 20 = MED post 5; 21 = MED post 6; 22 = MED post 7; 23 = MED post 8; 24 = MED post 12; 25 = MED post 14. Atlantic Ocean: 26 = ATL-1 V; 27 = ATL-2 V; 28 = ATL-3 V; 29 = ATL-4 V; 30 = ATL-5 V; 7 = MED all 7; 8 = MED all 8; 9 = MED all 9. Arctic Ocean: 31 = HN 22_002_SW_001; 32 = HN 22_012_SW_003; 33 = HN 22_046_SW_006, 34 = HN 22_085_SW_007; 35 = HN 22_093_SW_008. Persian Gulf and Indian Ocean: 36 = TDR 22–1; 37 = TDR 22–2; 38 = TDR-22-3; 39 = TDR 22–4; 40 = TDR 22–5; 41 = TDR 22–6; 42 = TDR 22–7; 43 = TDR 22–8.

currents, which could play a role in dispersing contaminants from WWTPs, ports, and other coastal sources towards the sampling sites. For example, in the Adriatic Sea, the local currents near Venice could be transporting pollutants from discharge points into sampling station 24.

Fig. 6 illustrates the potential environmental drivers in the Svalbard Islands, which include nearby ports and WWTP discharges. Additionally, aquaculture operations in the nearby Norwegian coastal areas may indirectly contribute to the ARGs detected in the region. Although the aquaculture facilities are not located close to the sampling site, the currents are such that they can transport pollutants from these facilities towards the sampling area. Finally, the maritime traffic cannot be overlooked, as shown in Supplementary Figs. S3 (Mediterranean) and S4 (Arctic). All sampling stations in the Mediterranean are located in regions with high marine traffic density, averaging over 200 routes/km^2 per year, with peaks exceeding 500 routes/km^2 near Venice and the Strait of Messina. In contrast, the sampling station in the Svalbard region experiences much lower traffic densities, with most areas showing <20 routes/km^2 per year.

4. Discussion

The growing issue of antibiotic resistance in the marine environment is a major concern, as it poses a threat not only to marine ecosystems but also to human health through the potential spread of resistant pathogens. Information on the status of antimicrobial resistance and environmental transmission risks in the marine environment is not monitored by the national surveillance systems, mainly based on the Marine Strategy Framework Directive [*Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008 establishing a framework for community action in the field of marine environmental policy*].

The spread of ARB and ARGs from the marine environment to humans poses a significant threat to public health (Leonard et al., 2022), as demonstrated by a study showing that surfers are more likely to be exposed to ARGs than non-surfers in swimming areas in England (Leonard et al., 2018).

While recognizing the presence of intrinsic antibiotic resistance - the natural ability of certain bacterial strains to survive antibiotic exposure using pre-existing genes (Darby et al., 2023) -, it is crucial to understand the factors that drive acquired resistance, particularly the role of ARGs that transfer between different bacteria carried by mobile genetic elements. In the marine ecosystem, the occurrence of ARGs has been documented in various environments, including the open oceans (Moore et al., 2020; Toth et al., 2010), estuaries and coastal waters (Carney

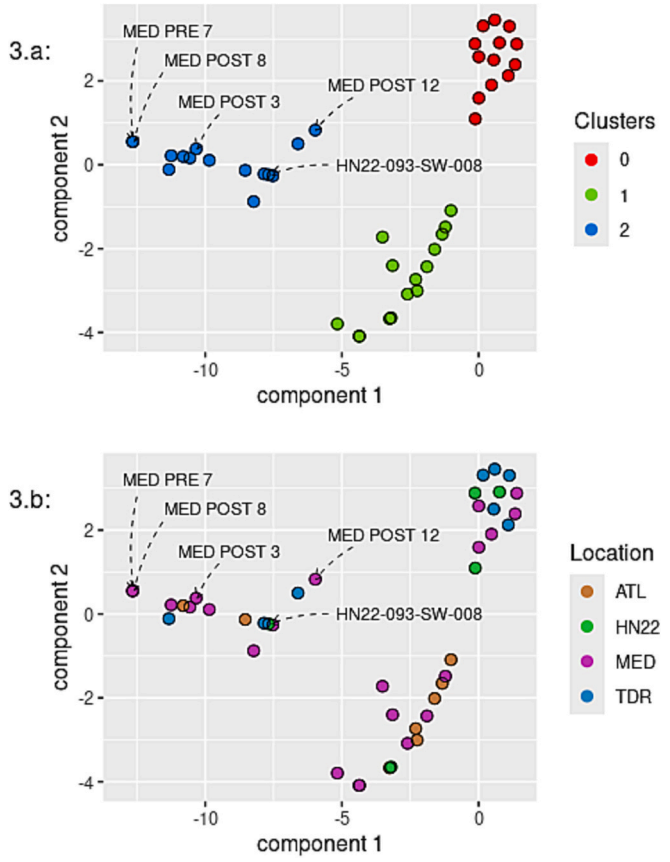

Fig. 3. Unsupervised approach clustering – a. DBSCAN main clusters: sampling sites coloured by 3 different impact class; b. sampling sites labelled by location (ATL = Atlantic Ocean, HN22 = Artic Ocean, MED = Mediterranean see, TDR = Persian Gulf and Arabic see). Points with extra annotation with label and dashed arrow represent the samples classified as high impact also by supervised approach.

et al., 2019; Fresia et al., 2019; Jang et al., 2021; Zhang et al., 2020), and even in marine sediments (Rahman et al., 2008; Yang et al., 2013). Indeed, the marine environment has been shown to account for a significant proportion, approximately 28 %, of the total ARGs (Hatosy and Martiny, 2015). Although dilution may reduce the concentrations of ARGs as they enter the ocean (Gao et al., 2018), the ability of these genes to spread over long distances highlights their potential for global dispersal.

Most existing studies on ARGs in the marine environment focus on specific local areas, particularly coastal areas which are heavily impacted by human activities and are easily accessible for sampling. For instance, Jang et al. (2022) conducted a comprehensive analysis of ARGs in the western Pacific and Southern Oceans, highlighting the influence of hydrometeorological factors on ARG prevalence. Similarly, Zhang et al. (2020) focused on ARGs in coastal bays in China, identifying wastewater pollution as a primary source of ARGs. Fresia et al. (2019), uncovered ARG reservoirs in coastal beach and sewage waters in Uruguay, through urban metagenomics. Peng et al. (2024) investigated the resistome, mobile genetic elements (MGEs), and microbiomes in a subtropical coastal ecosystem of the Beibu Gulf, China. However, further research is needed to gain a deeper understanding of the broader trends of ARGs in the marine environment, including open ocean areas far from the coast. Our work covers a broader range of geographical locations, providing a more extensive understanding of ARGs distribution on a global scale.

Thanks to the Sea Care project, marine sampling sites have been selected over a wide area of the globe, including the Mediterranean Sea, the Persian Gulf, the Gulf of Oman, the Gulf of Aden, the Red Sea, the Atlantic Ocean, and remote regions such as the Arctic Ocean. This comprehensive sampling in distant and peripheral areas, has provided a thorough overview of marine environments worldwide. Among the selected ARGs, $bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-1 group}}$ and bla_{TEM} all encode β-lactams enzymes. These ARGs, of clinical interest, confer bacterial resistance to β-lactams antibiotics, such as penicillins and cephalosporins routinely used for treatments of human bacterial infections. The *sul*1 gene encodes resistance to sulfonamide antibiotics, which inhibit folate synthesis. Sulfonamides have historically been used to treat a wide range of bacterial infections, making *sul*1 a valuable proxy for assessing anthropogenic impacts due to its long selective pressure in the environment. The *tet*A gene provides bacteria with resistance to tetracycline by encoding a protein that actively pumps tetracycline out of the bacterial cell, thereby reducing the antibiotic's intracellular concentration and mitigating its inhibitory effects.

Our analysis highlighted the global distribution and relative abundance of ARGs, revealing a distinct pattern in their prevalence and concentration levels. Specifically, results showed the following descending order of ARGs positivity: *sul*1 (100 %) > bla_{TEM} (56 %) > $bla_{\text{OXA-48}}$ (44 %) > *tet*A (37 %) > $bla_{\text{CTX-M-1 group}}$ (16 %). In terms of absolute abundance (g.c./L of seawater samples), this ranking remains consistent.

The *sul*1 gene was both ubiquitous and more abundant than other ARGs. Along with its closely related gene, *sul*2, *sul1* is recognized as a

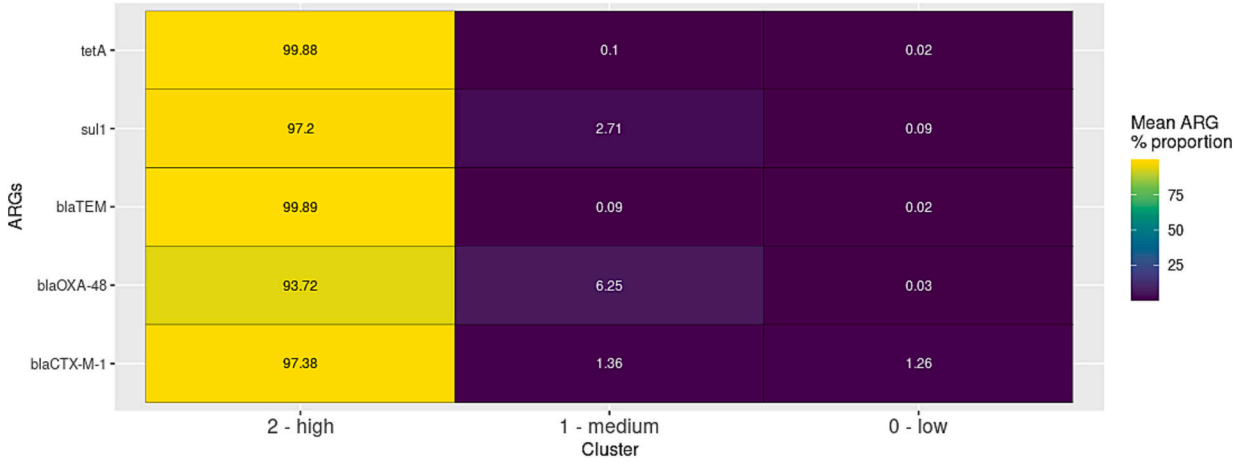

Fig. 4. Mean concentration of different ARGs in the three categories identified through unsupervised clustering and their assignment to low, medium, and high impact.

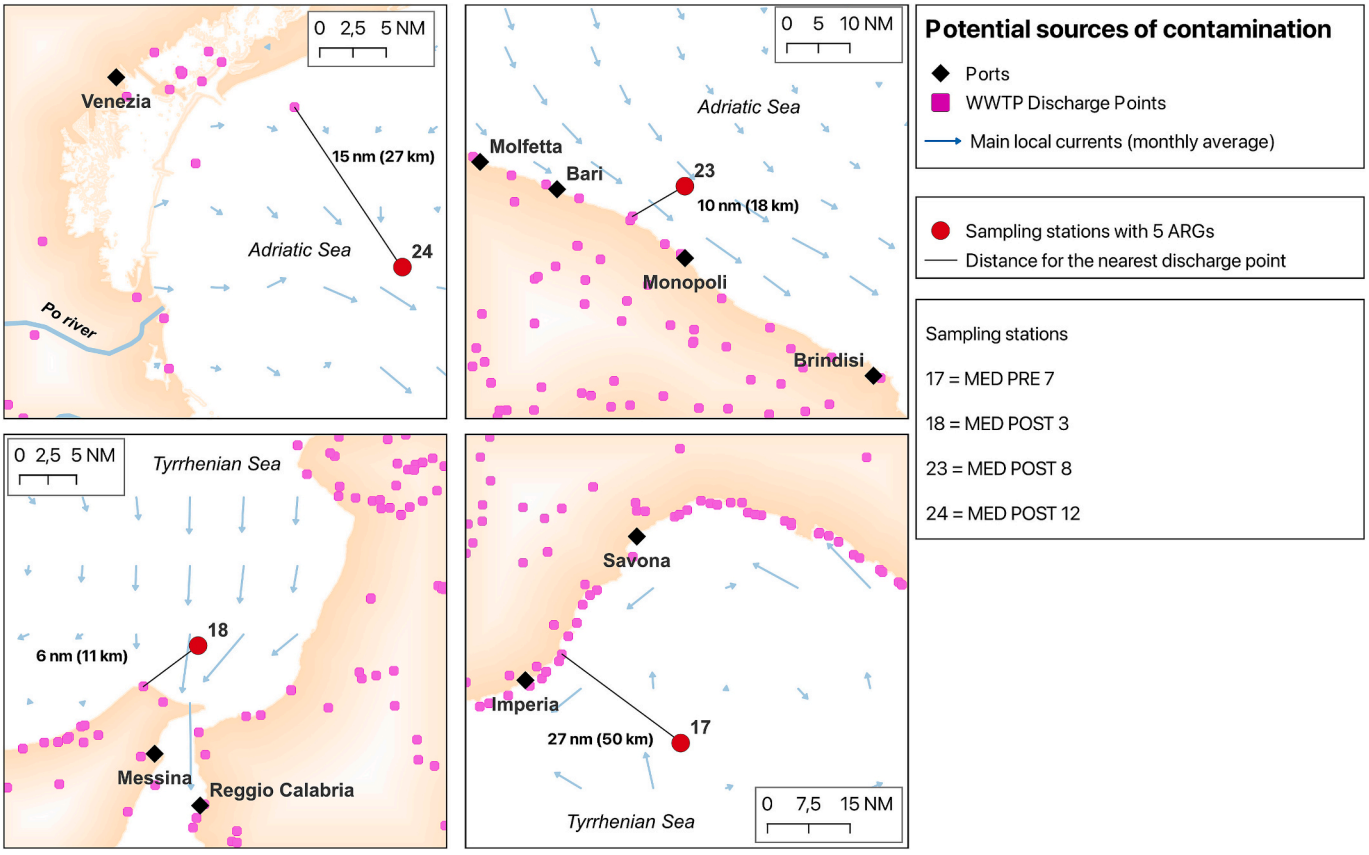

Fig. 5. Potential sources of impact on ARGs in the Mediterranean Sea. Sampling stations with 5 ARGs (in red) are shown alongside wastewater treatment plant (WWTP) discharge points (in purple) and major ports (in black). Arrows indicate the main local currents (monthly average), while black lines connect the sampling stations to the nearest discharge points, with distances expressed in nautical miles (nm).

key indicator of environmental pollution by antimicrobial agents (Haenelt et al., 2023a, 2023b). In addition, both the *sul*1 gene and the mobile genetic element *Int*1 have been identified as robust indicators of AMR contamination in the marine environment (Bourdonnais et al., 2024). Our results confirm that the *sul*1 gene emerges as an effective proxy for assessing anthropogenic impact on oceans, given its ubiquity and abundance, making it a robust indicator of environmental pollution by antimicrobial agents. The *sul*1 gene can enter water environment from a variety of sources, including WWTP discharges (Bonanno Ferraro et al., 2024), aquaculture facilities where sulphonamides are used as prophylactics to prevent bacterial infection in farmed species (Gao et al., 2018), and maritime traffic discharges, such as ballast water and blackwater (Lv et al., 2020; Westhof et al., 2016). Additionally, *sul*1 can be transported attached to plastic biofilm carried by currents reaching areas far from their original anthropogenic sources of impact (Lebreton and Andrady, 2019; Onink et al., 2021).

The bla_{TEM} gene was identified as one of the most frequently occurring antibiotic resistance gene in marine samples, second only to *sul*1. Our findings are consistent with previous studies conducted in the oceans (Jang et al., 2022) and the Mediterranean Sea (Alves et al., 2014; Gambino et al., 2022; Sucato et al., 2021), where bla_{TEM} has also been reported in fish and other marine organisms (Alduina et al., 2020).

The bla_{OXA-48} gene was found in 44 % of the tested samples, and was exclusively detected in coastal marine areas, which are often more heavily impacted by human activities. This finding aligns with a study conducted in the central Adriatic Sea (Mediterranean), where underwater effluent from WWTPs was identified as a source of bla_{OXA-48} carrying *Enterobacteriaceae* in coastal waters (Kvesić et al., 2022). Additionally, another study reported the detection of both bla_{OXA-48}-like-producing ST131 *E. coli* and bla_{OXA-48}-like producing ST101 *K. pneumoniae* in Irish recreational waters, highlighting the widespread presence of these resistant bacteria in environments heavily influenced by human activities (Mahon et al., 2019).

The $bla_{CTX-M-1\ group}$ gene was detected in only 7 samples (16 %) and at the lowest concentrations compared to other genes, appearing exclusively in the Mediterranean Sea and the Arctic Ocean. This gene has previously been found in *Enterobacteriaceae* isolates from seawater samples collected in the Adriatic Sea (Maravić et al., 2015) and the Caribbean Sea (Atlantic Ocean), particularly in surface waters impacted by wastewater treatment plant (WWTP) effluents (Guyomard-Rabenirina et al., 2017). These findings align with earlier research that identified the $bla_{CTX-M-1\ group}$ gene in WWTP effluents (Amos et al., 2014; Bonanno Ferraro et al., 2024).

The *tet*A gene was detected in 37 % of the samples, with the highest concentrations observed in the Mediterranean Sea. It was also detected in the Atlantic Ocean, the Arctic Ocean, the Persian Gulf, and the Arabian Sea. Previous studies have similarly reported the presence of this gene along the Mediterranean Coast and in the Oceans (Sucato et al., 2021; Jang et al., 2022).

To further investigate, we mapped potential sources of anthropogenic impact in the areas with the highest contamination, such as the presence of ports, WWTP discharges, areas designated for finfish farming, maritime traffic, as well as major currents. One of the sampling sites (MED POST 3) is located near the Strait of Messina, a narrow passage between the eastern tip of Sicily and the western tip of Calabria in southern Italy. The high levels of contamination in this area are likely due to a combination of discharges and port activities, all facilitated by the dynamic local currents. Sampling sites MED PRE7 and MED POST 8 were both located in areas susceptible to the impact of numerous WWTP discharges. Additionally, their proximity to the coast and the circulation

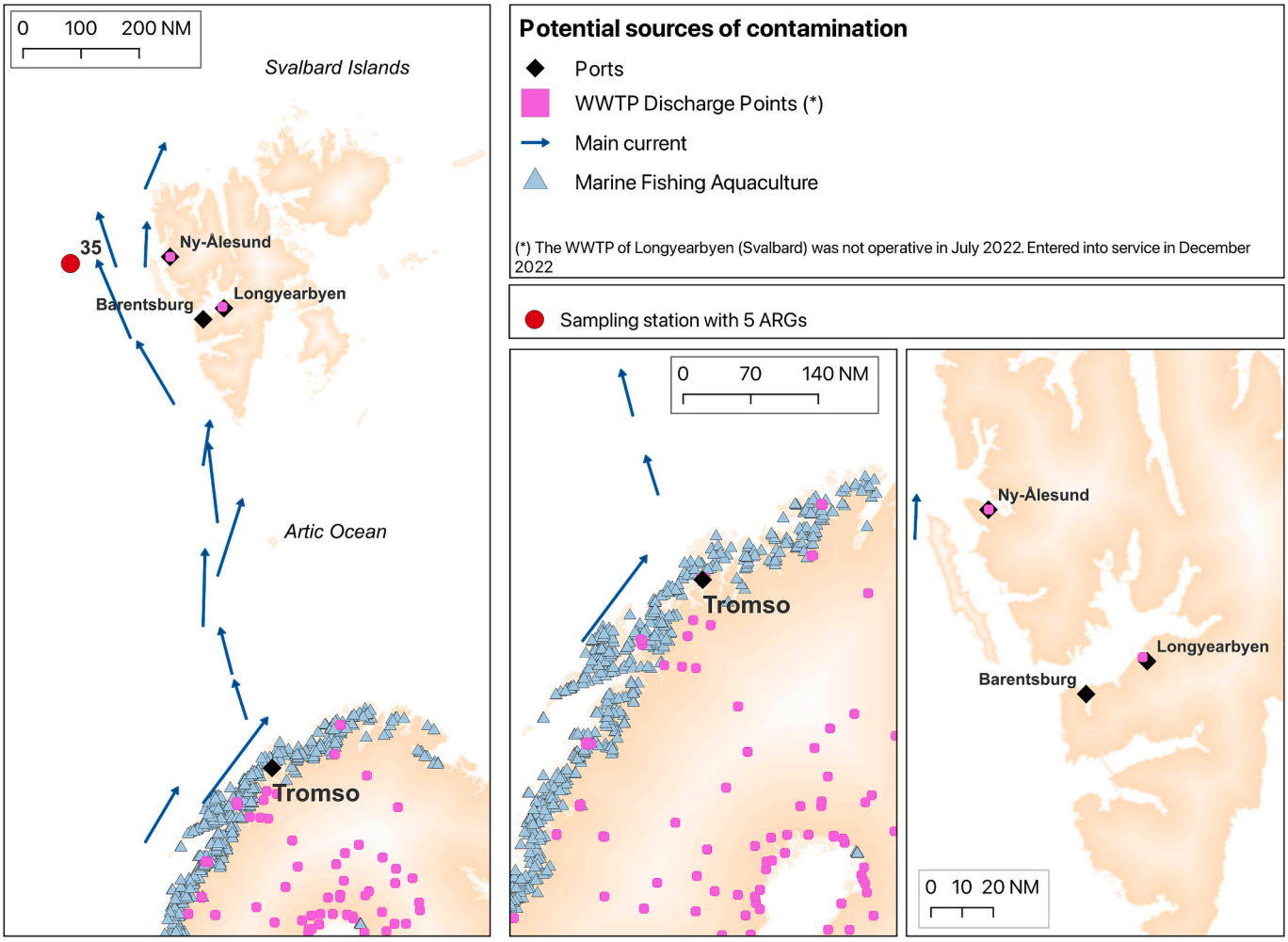

Fig. 6. Potential sources of impact on ARGs in Arctic Ocean. The sampling stations with 5 ARGs (in red) is shown alongside wastewater treatment plant (WWTP) discharge points (in purple) and major ports (in black). Arrows indicate the main local currents (monthly average), while black lines connect the sampling stations to the nearest discharge points, with distances expressed in nautical miles (nm).

of marine currents may have further contributed to the observed contamination levels. MED POST 12, situated in the Gulf of Venice, is characterized by shallow waters and is subjected to a typical vortex circulation of the currents that confine most of the waters of the upper Adriatic Sea. This circulation is further influenced by the substantial inflow from the River Po and other rivers, which leads to variations in water density and a decrease in salinity. Finally, sample ID HN22–093-SW-008 from the Arctic Ocean near the Svalbard Islands, showed unexpectedly high contamination levels for such a remote area. Hot spot maps reveal nearby sources of impact, including ports and WWTP discharges. Aquaculture operations along the Norwegian coast may also contribute indirectly, as currents can transport pollutants from these facilities to the sampling area (Zambrano, 2023). Although the marine traffic density in the area is not particularly intense, it could potentially play a role in introducing pollutants, including ARB and ARGs, through ballast water, septic discharges, and other ship-related activities. The contribution of ballast water should not be overlooked, as it may serve as a vector for the long-distance transport of antibiotic resistance genes (Lv et al., 2024; Lv et al., 2018; Ng et al., 2015).

The comparison between supervised and unsupervised classification approaches for assessing the impact of ARGs in marine samples provides valuable insights into the strengths and limitations of each method. The supervised classification method primarily categorizes samples into low, medium, and high impact groups, based on the presence of ARGs. Notably, the geographical regions with the highest ARGs contamination were the Mediterranean Sea and the Arctic Ocean around the Svalbard Islands, where all five high-impact samples were located. In contrast, the unsupervised classification method considers both the number and the relative abundance of ARGs. This approach revealed deeper relationships and clustering patterns based on ARGs abundance that were not evident with the supervised method, allowing for a more comprehensive understanding of the distribution and impact of ARGs in marine environments. Indeed, some samples classified as medium impact by the supervised approach were reclassified as high impact due to their high ARG abundance. Similarly, a significant number of samples that were previously categorized as low impact were reclassified into medium or high impact categories, revealing that even genes occurring at low frequencies can pose a significant environmental risk when present in high concentrations. In summary, the supervised approach has strengths in its simplicity and clarity of classification, making it effective for quickly identifying broad patterns of contamination based on the presence of ARGs. However, it may overlook significant contamination where ARGs are present in low variety (i.e. just one or two ARGs) but high abundance.

As for the geographical patterns of ARG contamination, the results indicate that geography alone does not drive the clustering of antibiotic resistance gene levels. Low and medium impact samples are geographically mixed across regions, suggesting that factors other than location are more influential in determining ARG prevalence. The only clear geographical pattern is observed in the Mediterranean samples with 5

ARGs, which cluster together in the high impact group, likely due to specific local factors increasing contamination levels.

Our findings confirm and expand upon previous studies that have documented the presence of ARGs in the Mediterranean Sea. For instance, Alves et al. (2014) and Maravić et al. (2015) reported the spread of extended-spectrum beta-lactamase-producing Enterobacteriaceae in coastal marine waters, underscoring the impact of human activities. More recent work by Gambino et al. (2022) demonstrated the prevalence of ARGs conferring resistance to beta-lactams, tetracyclines, and sulfonamides, aligning with our observations of widespread *sul*1 and bla_{TEM} distribution in Mediterranean waters. Additionally, our results are consistent with those of Dželalija et al., who quantified clinically relevant ARGs associated with resistance to five major classes of antibiotics, including sulfonamide and tetracycline resistance genes, as well as the *int*1 integrase gene. Their study also highlighted that ARGs, particularly intl1 and β-lactam resistance genes, were strongly linked to human activities, with higher concentrations found in eutrophic, nutrient-rich coastal waters, particularly those impacted by wastewater. Furthermore, our detection of ARGs in the Arctic Ocean extends the understanding of their global distribution, consistent with previous findings in this remote environment. Studies by Poulain et al. (2015) and Su et al. (2017) have documented the presence of ARGs in Arctic marine waters and sediments, suggesting that both human activities and long-range transport mechanisms contribute to the spread of resistance even in the Arctic. Similarly, Murray et al. (2020) highlighted the widespread occurrence of integrons in Arctic marine sediments, indicating a high potential for ARG dissemination over long distances through ocean currents. However, it is important to consider that ARGs in such remote and pristine environments may not be solely the result of anthropogenic influences. For example, Van Goethem et al. (2018) reported the presence of naturally occurring ARGs in undisturbed soils in Antarctica, which are likely to represent ancestral genetic diversity rather than recent human impacts. This highlights the possibility that some of the ARGs detected in the Arctic may be of ancient origin, inherited over generations, and not exclusively the result of contemporary human activity.

This study has some limitations. The limited number of samples may not fully represent the broader marine environment, and the unequal distribution of samples across different geographical areas, with a larger number collected from the Mediterranean Sea, may affect the generalisability of the findings. The results should be interpreted with these limitations in mind, especially when making comparisons across different geographic regions. Furthermore, the focus on a few selected resistance genes, may not capture the full spectrum of ARGs present in marine environments. However, the study also has notable strengths. The project was made possible by the Sea Care initiative, which enabled the collection of samples from a vast array of distant and isolated locations, offering invaluable insights into the global distribution of ARGs. The study conducted a thorough analysis of the environmental factors contributing to the distribution of ARGs and provided an advanced clustering of impact categories, enhancing our understanding on how different factors influence ARGs prevalence. A significant methodological advancement was the development of a pentaplex assay, which represents a major step forward in the simultaneous detection and analysis of multiple ARGs, improving the efficiency and comprehensiveness of ARGs monitoring.

Future studies should aim to include a larger number of samples to provide a more robust and representative analysis of the distribution of ARGs in the marine environment. Efforts should also be made to ensure a more balanced distribution of samples across different geographical areas to improve the generalisability of the results. In addition, future research should consider including a wider range of resistance genes, such as those conferring resistance to aminoglycosides, vancomycin, and methicillin, to provide a more comprehensive picture of ARGs prevalence and impact. By addressing these limitations and building on the strengths of this study, future research can provide a more detailed and accurate assessment of ARGs in marine environments, ultimately contributing to the development of more informed strategies for managing antimicrobial resistance.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.176765.

CRediT authorship contribution statement

G. Bonanno Ferraro: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **D. Brandtner:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation. **A. Franco:** Writing – review & editing, Methodology, Investigation. **M. Iaconelli:** Writing – review & editing, Methodology, Investigation. **P. Mancini:** Writing – review & editing, Methodology, Investigation. **C. Veneri:** Writing – review & editing, Methodology, Investigation. **R. Briancesco:** Methodology, Writing – review & editing. **A.M. Coccia:** Methodology, Writing – review & editing. **E. Suffredini:** Writing – review & editing, Validation, Formal analysis, Conceptualization. **A. Muratore:** Writing – review & editing, Visualization, Software. **F. Ferrara:** Writing – review & editing, Project administration, Formal analysis, Data curation. **L. Lucentini:** Writing – review & editing, Supervision, Formal analysis. **A. Piccioli:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **G. La Rosa:** Writing – review & editing, Writing – original draft, Validation, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Funding

This project has been made possible through the financial support of the “Sea Care project: Health, Environment and Climate Research in the Vision of Planetary Health”, a collaborative initiative between the Italian Navy and the National Institute of Health - Istituto Superiore di Sanità (ISS), with the main contribution from the Italian Navy. We also thank the European Union – NextGenerationEU under the National Recovery and Resilience Plan (NRRP) PE13 INF-ACT.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Special thanks to the Italian Navy and its Chief of Staff, Vice Admiral Enrico Credendino, for their role in the Memorandum of Understanding between the Italian Navy and the ISS.

Special recognition is due to Rear Admiral Cristiano Nervi (Head of the Naval Support and Experimental Centre of the Italian Navy and of the Operational Structure of the National Pole of the Underwater Dimension) for promoting and supporting the project's approval. We also thank Vice Admiral Roberto Dattola (EP&OHS General Office of Navy General Staff) for ensuring maximum support for the project's activities and success, and Rear Admiral (LH) Massimiliano Lauretti (Head of the Third Division "Plans, Operations and Maritime Strategy) for providing the decisive stimulus for implementing the project during the Amerigo Vespucci's World Tour Campaign.

Our gratitude goes to the commanders of the naval vessels that hosted the Sea Care project teams: Capt. Jacopo Rollo (Commanding Officer of the Destroyer Caio Duilio), Capt. Massimiliano Siragusa, Capt. Luigi Romagnoli, Capt. Giuseppe Lai (Commanding Officers of the Training Ship Amerigo Vespucci), Cdr. Maurizio Demarte (Commanding Officer of the Oceanographic Vessel Alliance, Italian Navy Hydrographic Institute), Cdr. Emanuele Morea, Cdr Alessandro Serrani (Commanding Officers of the Offshore Patrol Vessel Paolo Thaon di Revel). The team's expertise and professionalism were crucial to the successful completion of all planned activities, especially considering that military vessels are not typically equipped for research purposes.

We would also like to express our gratitude to Cdr. Giuseppe Aceto (Head of the Environmental Protection Office, Manager of Operation of the Sea Care Project) for his invaluable support in coordinating and facilitating relations within the Naval General Staff and High Commands, as well as with all the naval vessels in which the scientific teams were embedded. His problem-solving skills were greatly appreciated. Finally, we would like to thank all the military personnel, both on board and on shore, for their support, passion, and professionalism in ensuring the success of this project.

Data availability

All data are shown in the main text and in Supplementary Material

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