Seasonal and spatial distribution of antibiotic resistance in marine microbial communities along a trophic gradient in central Adriatic Sea

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DOCTORAL DISSERTATION

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Supervisor:

Ana Maravić, PhD, Assoc. Prof.

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“This doctoral dissertation was made at the University of Split, Faculty of Science, Department of Biology, under the supervision of Ana Maravić, PhD, Assoc. Prof. as a part of the Doctoral programme of Biology at the University of Zagreb, Faculty of Science, Department of Biology. “

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SUPERVISIOR INFORMATION

Ana Maravić, Ph.D., is an Associate Professor in Microbiology at Faculty of Science, University of Split. She earned her doctoral degree at Faculty of Science, University of Zagreb in 2013, with a dissertation titled "Mechanisms of resistance to antibiotics of Gram-negative bacteria in the coastal area of Kaštela Bay." She teaches courses in microbiology at undergraduate, graduate, and doctoral studies at Faculty of Science University of Split.

Ana Maravić has made significant contributions to the field of environmental microbiology, focusing on the study of antibiotic resistance in the environment. She has authored and co-authored 52 scientific articles published in journals indexed in Web of Science database and over 40 scientific conference proceedings. Furthermore, she has supervised 18 final and master's theses. She supervised PhD thesis entitled "The impact of submarine outfalls of treated wastewater on the microbial community structure and the resistome of the coastal marine environment" which was defended in 2023 at Faculty of Science University of Split.

She was a researcher on several research projects, including those of Ministry of Science, Education and Sports, Croatia, and Croatian Science Foundation (IP-8481, IP-2019-04-5539). As a project leader, Dr. Maravić directed the Croatian Academy of Sciences and Arts project "Carbapenemase-producing and colistin-resistant opportunistic pathogenic bacteria in the coastal sea in Croatia" (2018-2019). Currently, she leads an installation research project "Seasonal and spatial distribution of antibiotic resistance genes in marine microbial communities along a trophic gradient in the central Adriatic Sea" (ARGAS) (UIP-2019-04-9778) funded by Croatian Science Foundation.

In addition to her academic and research endeavors, she serves as a peer reviewer for various international journals, including the Water Research, Journal of Hazardous Materials, Science of the Total Environment, Environmental Pollution, Nature Scientific Reports, Chemosphere, and Microbial Ecology. Ana Maravić received several awards and accolades, including the Annual Award for Best Young Scientist from the Croatian Microbiological Society in 2014, and the 2019 and 2020 Award for Scientific Research from the Faculty of Science, University of Split.

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Bez vas i vaše ljubavi bila bih … poput mjedi što ječi ili cimbala što zveči.

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University of Zagreb
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Doctoral dissertation

Seasonal and spatial distribution of antibiotic resistance in marine microbial communities along a trophic gradient in central Adriatic Sea

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Abstract

Marine and ocean environments are the most widespread habitats in the world but are still among the least studied from the aspect of antibiotic resistance (AR). The aim was to investigate the seasonal and spatial features of AR in microbial communities along trophic gradient of central Adriatic encompassing a 68-km transect from wastewater-impacted estuary to coastal and pristine open sea. Eighty microbiomes were profiled by using Illumina-based 16S rRNA amplicon sequencing revealing that biogeographical shifts in microbial community are largely driven by the seasonality, featured by thermohaline stratification and nutrients in particular. Proteobacteria were the predominant along the gradient and major contributors to the marine resistomes. Targeted resistance genes were quantified using RT-qPCR, among which distribution of *tetA*, $bla_{\text{CTX-M}}$ and bla_{TEM} correlated with higher temperature, nutrient load and lower salinity. WHO 'high' and 'critical priority' pathogens including vancomycin-resistant *Enterococcus faecium* and carbapenemase-producing Enterobacteriaceae were isolated from wastewater-impacted coastal waters, pointing to their sewage-driven dissemination in marine environment. Enterobacteriaceae carried carbapenemase genes ($bla_{\text{KPC-2}}$, $bla_{\text{OXA-48}}$ and $bla_{\text{VIM-1}}$) on diverse conjugative plasmids favouring their horizontal spread as revealed by conjugation experiments and *de novo* plasmid sequencing. These results show a complex pattern of AR persisting along the trophic gradient and an importance of anthropogenic influence on its dissemination in marine ecosystems, accentuating the imperative for comprehensive strategies congruent with the One Health paradigm to combat AR in the environment.

(181 pages, 43 figures, 29 tables, 340 references, original in English)

Keywords: amplicon sequencing, carbapenemase-producing Enterobacteriacae, environmental factors, marine environment, microbiome, resistome, VREfm, qPCR

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Doktorska disertacija

Sezonska i prostorna raspodjela rezistencije na antibiotike u morskim mikrobnim zajednicama duž trofičkog gradijenta u srednjem Jadranu

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Mora i oceani su najrasprostranjeniji prirodni ekosustavi na Zemlji, a ipak su među najmanje istraženima s aspekta antibiotske rezistencije (AR). Cilj je bio istražiti sezonske i prostorne značajke AR u mikrobnim zajednicama duž trofičkog gradijenta srednjeg Jadrana duž 68 km od estuarija pod utjecajem otpadnih voda do obalnog i otvorenog mora. Osamdeset mikrobioma profilirano je korištenjem 16S rRNA sekvenciranja, otkrivajući da su biogeografski pomaci u mikrobnoj zajednici uvelike potaknuti sezonskim promjenama, osobito termohalinom stratifikacijom i hranjivim tvarima. Proteobakterije su prevladavale duž gradijenta i bile glavni čimbenici morskog rezistoma. Ciljani geni AR kvantificirani su pomoću RT-qPCR, pri čemu je distribucija *tetA,* $bla_{\text{CTX-M}}$ i bla_{TEM} korelirala sa višom temperaturom, povećanom koncentracijom hranjivih tvari i nižim salinitetom. Patogeni 'visokog' i 'kritičnog prioriteta', uključujući vankomicin-rezistentni *Enterococcus faecium* i karbapenemaze-producirajuće Enterobacteriaceae izolirani su iz obalnog područja pod utjecajem otpadnih voda, što ukazuje na njihovo širenje u morskom okolišu putem kanalizacije. Izolati enterobakterija nosili su karbapenemaza-kodirajuće gene ($bla_{\text{KPC-2}}$, $bla_{\text{OXA-48}}$ and $bla_{\text{VIM-1}}$) na različitim konjugacijskim plazmidima što pogoduje njihovom horizontalnom širenju, a što je potvrđeno eksperimentima konjugacije i *de novo* sekvenciranjem plazmida. Ovi rezultati pokazuju složeni obrazac AR duž trofičkog gradijenta i važnost antropogenog utjecaja za njezino širenje u morskim ekosustavima te nužnost sveobuhvatnih strategija usklađenih s paradigmom „Jednog zdravlja“ (*eng.* One health) radi rješavanja problema AR u okolišu.

(181 stranica, 43 slika, 29 tablice, 340 referenci, jezik izvornika: engleski)

Ključne riječi: karbapenem-producirajuće Enterobacteriaceae, morski okoliš, mikrobiom, okolišni čimbenici, rezistom, sekvenciranje amplikona, VREfm

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CONTENTS

1. INTRODUCTION

Antibiotic resistance (AR) has become one of the greatest global threats in the 21st century. The overuse of antimicrobial drugs in human and veterinary medicine, agriculture, and aquaculture over the past seventy years has led to the emergence of antibiotic resistance in a significant number of human pathogens, severely limiting treatment options and outcomes. According to the most recent data, a total of 4.95 million deaths worldwide in 2019 were associated with infections caused by antibiotic-resistant pathogens[1], most of which were due to critical- and high-priority pathogens such as carbapenem-resistant Enterobacterales, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant enterococci[2]. As a result, the World Health Organization (WHO) identified the spread of antibiotic resistance as an urgent problem that requires a global and coordinated plan of action[2] launching a One Health concept as a valuable framework to track down and limit the further spread of antibiotic resistance by reinforcing the global need for simultaneous surveillance and control of antibiotic resistance in humans, animals, and the natural environment.

Antibiotic resistance genes (ARGs) have been identified in a variety of environments, among which are clinical[3], natural[4] and wastewater-related ones[5]. Importantly, anthropogenic activities were found to be one of the main drivers of antibiotic resistance[6], accelerating the proliferation of antibiotic-resistant bacteria (ARB) and spread of plasmid-based ARGs[7] from environmental bacteria to pathogens through horizontal gene transfer in particular[6]. Namely, mobile genetic elements, including insertion sequences, integrons, and transposons, enable successful horizontal transfer of ARGs in association with plasmids and phages[8].

Marine and ocean environments are the most widespread habitats in the world but are still the least studied from the aspect of AR, pointing to the importance of future studies to expand knowledge of the repertoire of ARGs and their hosts available in this ecosystem[9]. It has been shown that the natural process of resistance selection due to antibiotic production by environmental bacteria, mixing with antibiotic-resistant terrestrial bacteria, and environmental contamination with commercially used antibiotics play key roles in the occurrence of ARGs in aquatic environments[10,11]. Moreover, a significant correlation was established between environmental pollution and the presence of ARGs[12,13], especially in polluted aquatic

ecosystems where successful exchange of ARGs occurs under selection pressure between autochthonous environmental bacteria and those of human and animal origin[14,15]. Indeed, recent metagenomic studies evidenced high diversity of ARGs in microbial communities from human-influenced surface waters, including the coastal marine ones[5,14] which could be involved in the transfer towards human pathogens[16]. Among others, ARGs encoding resistance to macrolides, glycopeptides, and peptide drug classes have been detected in the microbiome of marine sediments, with the richest and most diverse ARGs found in coastal areas with anthropogenic activity[17]. In addition, marine bacteria have been found to have similar mobile genetic elements including transposons and plasmids as human pathogens, suggesting that they may have introduced and/or acquired resistance genes from pathogens and thus are involved in the global exchange of AR[16].

However, we still lack fundamental data on the dynamics of the microbial communities that harbour ARGs in marine environment, as well as the data on the temporal and spatial distribution of ARGs and ARB in this natural ecosystem. It is known that bacterial abundance in marine environment is controlled by the physical and chemical factors, nutrients, and their main predators heterotrophic nanoflagellates[18,19]. Moreover, biogeographical studies provided evidence that microorganisms display spatial biogeographical patterns in nature and would decline in similarity with the increasing geographical distance[20]. Consequently, different locations would harbour microbial communities that differ in taxonomic and functional composition which could further relate to the distribution of ARGs. The researchers speculate that anthropogenic factors have the most significant effects on the spatial distribution of ARGs, which are followed by the spatial, bacterial and physicochemical factors, indicating that multiple ecological mechanisms, including environmental selection as a consequence of human activities and local physicochemical parameters, as well as dispersal limitation, influence the distribution of ARGs in aquatic habitats[21].

Nevertheless, scientific literature search revealed a lack of studies that comprehensively address this problem in the marine environment, as most authors have focused on particular types of marine ecosystems such as estuarine[22], coastal[23] or deep-sea areas[24]. To the best of our knowledge, the structure of the bottom layer and surface marine microbiome and resistome have not yet been studied simultaneously in terms of seasonality and relationship to trophic status.

Previously, the Jadro River estuary[25] and the sewage-receiving coastal waters of the central Adriatic[26–28] were found to harbour clinically relevant pathogens and ARGs. However, distribution of ARB and ARGs in open waters of Adriatic has never been investigated. Moreover, metagenomic studies focusing on the structure of microbial community were carried out in deep waters of central and southern Adriatic[19] and the shallow coastal area of the northern Adriatic[29], whereas the resistome structure was not the subject of these studies.

Giving the importance of the study of AR in marine environment following the One Health concept, the main objective of this study is to identify seasonal and spatial variations in the taxonomic structure and ARG repertoire of marine bacterial communities inhabiting the bottom layer and surface waters along the trophic gradient of central Adriatic, hypothesizing that specific physicochemical and biological factors influence the dynamics of marine bacteria as carriers of resistance determinants, thus impacting the marine resistome at seasonal and spatial scales.

1.1 Objectives and hypotheses

Given the importance of the One Health approach in studying the AR phenomenon and the fact that marine microbial communities remain among the least explored in this regard, this PhD research will comprehensively and interdisciplinarily investigate the spatial and temporal distribution of ARGs as well as the structural diversity of marine bacterial communities as their carriers and/or vectors along a trophic gradient of the central Adriatic Sea. The simultaneous application of culture-dependent and state-of-the-art culture-independent techniques such as next-generation sequencing (NGS) and RT-qPCR will provide new insights into the composition of the marine microbiome and the associated ARGs repertoire revealing how biological and non-biological factors influence the dynamics of microbial communities as ARGs carriers and, consequently, the diversity and distribution of the antibiotic resistome on a seasonal and spatial scale.

In light of the foregoing, the objectives and hypotheses of this research are as follows:

O1 To investigate spatial and temporal variations of marine bacterial community structure along the trophic gradient of central Adriatic.
O2 To explore resistome diversity and the distribution of selected antibiotic resistance genes at seasonal and spatial scales in Adriatic.
O3 To identify which bacterial genera are the main carriers of antibiotic resistance.
O4 To characterize the multidrug-resistant bacterial isolates in terms of antibiotic resistance genes carriage and clonal relatedness.
O5 To determine the structure and transferability of plasmids carrying carbapenemase-encoding genes.

H1 Biological and physicochemical factors diversely influence the bacterial community composition along the trophic gradient, and consequently, lead to shifts in resistome structure at seasonal and spatial scales.
H2 In coastal waters under anthropogenic influence resistome will be more diverse and supported predominantly by pathogenic bacteria of human and terrestrial origin.
H3 Multidrug-resistant isolates will harbour clinically relevant antibiotic resistance genes, among which carbapenemase-encoding genes located on diverse and conjugative plasmids.

1.2 Origin and burden of bacterial resistance to antibiotics

With the discovery of the first antibiotic penicillin, in 1928, an era began in which medicine and therapeutic treatment experienced great progress eventually saving many human lives. In the following decades, novel and more potent antibiotic classes were developed at an ever-increasing rate. However, the overuse of antimicrobial drugs in human and veterinary medicine, agriculture and aquaculture caused the emergence of AR in a substantial number of human pathogens, significantly limiting the possibilities of successful treatment. Out of 3,57 million deaths associated with antibiotic resistance in 2019, six major pathogens including *Escherichia coli*, followed by *Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii*, and *Pseudomonas aeruginosa* were responsible for a total of 929,000 deaths[1]. Considering these numbers and the limited investment of pharmaceutical industry in the research and development of antimicrobial drugs, WHO listed "global priority pathogens" against which novel antibiotics are urgently needed, placing the carbapenem-resistant Enterobacteriaceae (CRE), carbapenem-resistant *P. aeruginosa* and carbapenem-resistant *A. baumannii* in the most important, critical-priority category[30]. Moreover, strategy to combat AR emergence and spread are also a request of Council of European Union to its member states.

The scientists have discovered that bacteria had developed ability to tolerate antibiotics long before their mass production and overuse[31]. For instance, it was found that bacterial β-lactamases that inactivate the most widely used antibiotics in clinical medicine today, the β-lactams, are in fact ancient enzymes more than two billion years old, among which some serine β-lactamases were present on plasmids for millions of years before the 'age of antibiotics'[32,33] . In addition, the identification of ARGs in ancient microbial communities from permafrost, caves and deep soils point to a reservoir of genetic diversity that has survived through natural processes of selection and mutation[34]. Without any doubt, introduction of antibiotics in clinics, veterine and agriculture fasten the spread of AR by providing unprecedent selection pressure on microbiota in humans, animals and natural environment, that further promoted the mobilization and horizontal transfer of a wide range of ARGs among many bacterial species, particularly pathogenic bacteria which are now responsible for high morbidity and mortality rates.

The most important feature of the environmental microbiome is the immense diversity of ARGs that could potentially be acquired by pathogens to overcome the effect of antibiotics[35,36]. It was

found that the most ARGs evolved gradually from genes with other functions[37]. Some ARGs originated from chromosomal, non-transferable genes, which further gain ability to move within the bacterial genome, and then associated with insertion sequences or gene cassettes were incorporated into integrons[38,39]. This was followed by the ARGs relocation to a mobile genetic element such as plasmid which can be finally passed directly to pathogen through horizontal transfer. Notably, elevated antibiotic concentrations providing selection pressure across microbial communities are found to favorize such outcomes[39].

Antimicrobial drugs are being continuously introduced into environment by various ways, such as through the effluents of treated and/or untreated wastewater that contain human and animal faecal material, improper disposal of medications, direct contamination of the environment by aquaculture or by plant cultivation[40–42]. All this in the end impacts the microbial communities enhancing the propagation and further dissemination of ARGs and ARB.

It is therefore important to note that the AR is a complex problem that requires a coordinated approach across human health, food production, animal, and environmental sectors. As such, One Health perspective recognized that the health of humans, domestic and wild animals, plants, and the environment are intricately linked and inter-dependent. More to it, One Health-based research of phenomenon of antibiotic resistance is needed to achieve optimal and sustainable health outcomes for people, animals, and ecosystems.

1.2.1 Genetic background of ARGs dissemination

Bacterial resistance to antibiotics can be intrinsic or acquired feature. Bacteria can be intrinsically resistant to some antibiotic(s) due to their specific structural and/or functional characteristics. For example, Gram-negative bacteria are intrinsically resistant to vancomycin, because its molecule is too large to cross the bacterial outer membrane. On the other hand, environmental bacterium but also emerging opportunistic pathogen *Stenotrophomonas maltophilia* is naturally resistant to a variety of antibiotic classes including sulfonamides, β-lactams, macrolides, cephalosporins, fluoroquinolones, aminoglycosides, carbapenems, chloramphenicol, tetracyclines, and polymyxins due to its low membrane permeability and activation of chromosomally encoded multidrug resistance (MDR) efflux pumps, β-lactamases as well as antibiotic-modifying enzymes[43].

Acquired resistance occurs through point mutations or by acquisition of novel ARGs. Point mutations can result from errors occurring during replication or can be induced by harsh environmental conditions such as strong ionizing radiation or oxidizing and alkylating chemicals. Mutations can lead to a change in the protein sequence that can result in a loss of protein activity but can also enable novel bacterial functions giving selective advantage for their survival in the presence of an antibiotic [44].

1.2.1.1 Transposons

Bacterial ARGs can be located on the chromosome, or on plasmids, as a part of transposons or integrons. Transposons are small DNA segments that can be inserted into a chromosome, often randomly but sometimes at specific locations. They can also be removed from the chromosome and reinserted elsewhere. Transposons have inverted repeat (IR) sequences at their ends and contain a gene for the enzyme transposase, which facilitates their movement because it cleaves DNA at the IR sequences, allowing the transposon to be inserted into the target DNA, carrying various genes, including those coding for AR, between the repeats. If a transposon carries an ARG, its insertion into the chromosome results in the expression of that gene, making the bacterium resistant to the antibiotic. Also, when the transposon lacks ARG, its insertion into a gene that is crucial for the activity of antibiotic can make the bacterium resistant [45]. For example, insertion of transposon into genes coding for porins, which are essential for the uptake of antibiotics into the bacterial cell, can result in reduced susceptibility or even resistance to some

antibiotic classes[46]. It was found that many natural plasmids and some transposons contain genes that confer resistance to multiple antibiotics. In such cases, any of these antibiotics can lead to selection of the entire plasmid or transposon, which automatically confers resistance to several antibiotics[47].

1.2.1.2 Integrons

Integrons are genetic elements that facilitate the acquisition, rearrangement, and expression of gene cassettes in bacteria. They are found to play a crucial role in bacterial evolution and adaptation by allowing bacteria to capture and incorporate very diverse gene cassettes containing beneficial genetic determinats, such as ARGs, into their genome.

Although they are not mobile on their own, they are transferred among bacteria as parts of conjugative plasmids or transposons[49]. Integrons consist of three main components: the integrase gene (*intI*), an attachment site (*attI*), and a promoter (*Pc*) (Figure 1.). The integrase gene encodes an enzyme integrase, which catalyzes the site-specific recombination of gene cassettes into the integron at primary recombination site *attI* [48]. The gene cassettes as variable part of the integron can be inserted at the attachment site, afterwhich the promoter drives the expression of the captured genes. Gene cassettes are generally constituted by a single open reading frame (ORF) immediately followed by a recombination site termed *attC* (formerly called 59-base element) which is specifically recognized by *IntI* [48]. The excision of cassettes by the IntI integrase results in the formation of a nonreplicative, covalently closed circular intermediate.

Chromosomal integrons are thought to be an evolutionary precursors of mobile integrons (MIs) that are often located on mobile genetic elements such as plasmids or transposons. MIs played a significant role in the emergence of multiple antibiotic resistance in Gram-negative pathogens. The longest array of cassettes identified contains eight cassettes, showcasing heterogeneity in their encoded ORFs and *attC* sites[50]. Despite their varied origins, cassettes in mobile integrons (MIs) are predominantly associated with antibiotic resistance. Over 130 different cassettes containing various antibiotic resistance genes have been identified in MIs, providing resistance to a wide range of antibiotics, including β-lactams, aminoglycosides, chloramphenicol, trimethoprim, rifampin, and others [50,51].

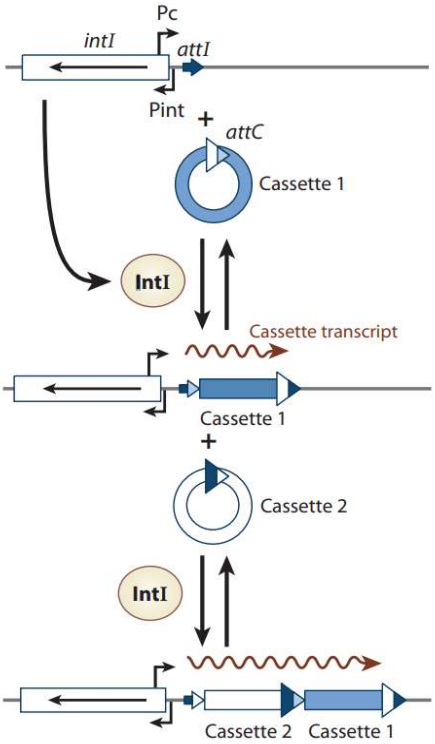

Figure 1. Integron-mediated gene capture and model for cassette exchange: the process involves repeated insertion of circular gene cassettes at the specific *attI* site downstream of the strong promoter Pc. *intI,* the integrase encoding gene; IntI, integrase. Credit: Cambray and colleagues [48].

Integrons are classified according to the amino acid sequences of their IntI integrases, with IntI1, IntI2, and IntI3 representing 'class 1,' 'class 2,' and 'class 3,' respectively[52]. Environmental studies revealed diverse *intI*1 genes, whereas clinical *intI*1 are remarkably uniform, suggesting a shared ancestor responsible for AR spread among Gram-negative pathogens. The clinical class 1 integrons originated from the capture of a chromosomal integron by a Tn402 transposon, initially carrying a disinfectant resistance gene (*qacE*), later acquiring a sulphonamide resistance gene (*sul1*) and o*rf5*, an open reading frame with unknown function (Figure 2a). Gene cassettes are embedded within these conserved sequences forming the variable region primarily encoding resistance to trimethoprim (*dfrA*) and aminoglycoside antibiotics (*aadA*). The Tn402 transposon has ability to target the res sites of plasmids, allowing the Tn402-class 1 integron hybrid to transpose into diverse plasmids, thereby facilitating lateral transfer into a broad range of bacterial species. Variation in resistance cassettes identified encoding metallo-β-lactamases IMP and VIM as well as acquisition of over 130 different ARGs cassettes, providing resistance to the majority of antibiotics employed against Gram-negative

pathogens[53,54]. Among others, these integrons have been previously identified in *Acinetobacter*, *Citrobacter*, *Escherichia*, *Klebsiella* and *Enterobacter*[55,56].

Importantly, the *intI1* gene has emerged as a potential biomarker for assessing anthropogenic pollution in natural environment[57], and it has been previously detected in various habitats, including wastewater[58], activated sludge[59], manure[60]as well as rivers, lakes, and sediments[61]. Increased levels of *intI1* gene have also been observed in aquatic environments contamined by antibiotics discharged from clinical sources and wastewater treatment plants highlighting its utility in tracking pollution sources[28,62].

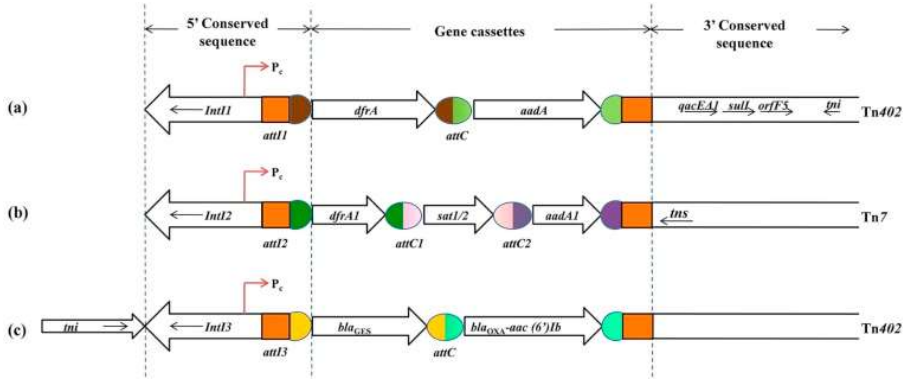

Figure 2. General features of various classes of integrons in *Enterobacteraceae.* (a) Class 1 integrons – consist of transposon Tn402, *intI1* gene, Pc promoter and *attI1* site *qacEΔ1* gene, a *sul1* gene and *orf5*; (b) Class 2 integrons - consist of transposon Tn7, *intI2* gene, Pc promoter and *attI2* site, gene cassettes *dfrA1–sat1/2–aadA1*; (c) Class 3 integrons consist of transposon oppositely directed within the Tn402 transposon, *intI3* gene, Pc promoter and *attI3* site. Credit: Kaushik and colleagues [63].

Similar to class 1 integrons, class 2 integrons are often linked to the Tn7 transposon family, including Tn1825, Tn1826, and Tn4132, housing both the recombination site *attI2* and the promoter Pc[64]. Structurally, class 2 integrons typically contain gene cassettes such as dihydrofolate reductase (*dfrA1*), streptothricin acetyltransferase (*sat1*), and aminoglycoside adenyltransferase (*aadA1*) (Figure 2b), conferring resistance to trimethoprim, streptothricin, and streptomycin, respectively[65]. Although not as prevalent as class 1 integrons, class 2 integrons still significantly contribute to the distribution of AR and are commonly reported many Gram-negative pathogens including *Acinetobacter*, Enterobacteriaceae, *Salmonella*, and *Pseudomonas* [66].

Class 3 integrons, although less prevalent, are highly adaptable for acquiring novel resistance cassettes. These integrons are incorporated in Tn402 transposon in reverse orientation compared to class 1 integrons (Figure 2c) and have gene cassettes forming the variable region with bla_{GES} gene and a fused gene cassette of bla_{OXA} and *aac(6')*-Ib encoding resistance to β-lactams and aminoglycosides. They have been identified in various Enterobacterales, including *Serratia*, *Klebsiella*, *Escherichia*, *Enterobacter* and *Citrobacter*[63] .

1.2.1.3 Plasmids

Plasmids are extrachromosomal circular DNA that can replicate independently of the bacterial chromosomal DNA and often carry genes for resistance to multiple antibiotics[54]. These genetic elements play a crucial role in the spread of ARGs among Gram-negative and Gram-positive bacteria. Within the resistance plasmids, accessory regions usually harbor one or more resistance genes together with associated mobile genetic elements such as insertion sequences, transposons and integrons. Plasmids serve as the primary agents for transferring genetic material in bacterial conjugation, and are categorised into conjugative, mobilizable, or non-mobilizable plasmid groups.

Conjugative plasmids encode their own membrane-associated pairing complex, enabling them to be self-transmissible. On the other hand, mobilizable plasmids do not contain all the genes required for conjugation transfer but can be transferred in the presence of a conjugative plasmid, which acts as an auxiliary plasmid that provides the necessary machinery for the transfer of a mobilizable plasmid. During conjugational transfer of plasmid, one strand of plasmid DNA is cut at oriT site, transferred and then joined to make it circular, and then the second strand is

synthesized in both the donor and recipient strains. Non-mobilizable plasmids are neither conjugative nor mobilizable and can be spread by transduction or transformation[67].

Identifying and categorizing plasmids relies on their stability during conjugation, known as plasmid incompatibility. Incompatibility refers to the inability of closely related plasmids to coexist stably within the same cell line because they share similar replication origins or partition mechanisms This occurs when plasmids interfere with each other's replication or partitioning systems. Only compatible plasmids can be successfully transferred to transconjugants[68]. The original incompatibility (Inc) groups were classified as follows: IncI for plasmids producing type I pili susceptible to phage Ifl; IncN for N3-related plasmids susceptible to phage IKe; IncF for plasmids producing type F pili susceptible to phage Ff; and IncP for RP4-related plasmids susceptible to the PRR1 phage[68].

Since 2005, a PCR-based replicon typing (PBRT) method has been developed, targeting the replicons of major plasmid families found in Enterobacteriaceae, including IncF, IncH, IncP, IncL/M, IncN, IncR, and IncW. Plasmid multilocus sequence typing (pMLST) has been introduced to further distinguish plasmids within incompatibility groups and help confirm their epidemiological and evolutionary relationships[69]. In this regard, pMLST schemes have been specifically developed for IncA/C, IncI, and IncN plasmids.

It has been found that some ARGs are associated with a specific Inc group of plasmids. For instance, IncF resistance plasmids, which are mainly found in *E. coli,* are commonly associated with $bla_{\text{CTX-M-14}}$ spread in Korea and France[70,71], and with $bla_{\text{CTX-M-1}}$ in animal sources in Spain[72]. IncF plasmids also harbor $bla_{\text{TEM-1}}$, which is prevalent in *E. coli* of human origin[73]. In addition, these plasmids can confer resistance to various antibiotics, including β-lactams, sulphonamides, aminoglycosides, and tetracyclines.

Plasmids belonging to IncP group can carry *mcr-1* and its variants, along with *dfrA1*, *tetA*, and *sul1*, thus conferring nonsusceptibility to colistin, tetracyclines, trimethoprim and sulfonamides[74]. In fact, IncP-1 plasmids are commonly associated with resistance to aminoglycosides, β-lactams, sulfonamides, tetracyclines, and trimethoprim, promoting the spread of multidrug resistance[75,76]. These plasmids, as well as those from the IncA/C and IncN groups, are conjugative, playing a major role in disseminating AR in the environment[77].

Moreover, IncL plasmids are found to be associated with *bla*$_{\text{OXA-48}}$ as well as *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-1}}$ and *bla*$_{\text{SHV-1}}$ and implicated in hospital infections[78]. Other groups of plasmids also harbour various clinically important *bla* genes, such as IncN plasmids and *bla*$_{\text{CTX-M-1}}$ in *E. coli* or IncR plasmids and *bla*$_{\text{VIM-1}}$[79].

1.2.2 Horizontal transfer of ARGs between bacteria

The main mechanisms of horizontal gene transfer encompass bacterial conjugation, transformation, and transduction/transfection. Bacterial transformation is the process through which bacteria uptake DNA from their environment (Figure 3a). This can occur naturally or artificially induced through the addition of chemicals such as calcium chloride or electroporation. Transformation can be nonspecific, allowing the uptake of any DNA from the environment, or specific, permitting the uptake of DNA only from the same species[80].

On the other hand, in transduction process the DNA is transferred between bacteria using bacteriophages as mediators (Figure 3b). Viruses act as vectors that infect bacteria, replicate, and leave the bacteria to infect other bacteria. During the replication process, viruses can incorporate some of the bacterial DNA into their own and transfer the DNA to other infected bacteria[80].

Bacterial conjugation is the contact-dependent, unidirectional transfer of DNA from a donor to a recipient cell (Figure 3c), which is facilitated by a mating apparatus expressed in the donor. It is a replicative process that results in both the donor and recipient cells having a copy of the plasmid [81,82]

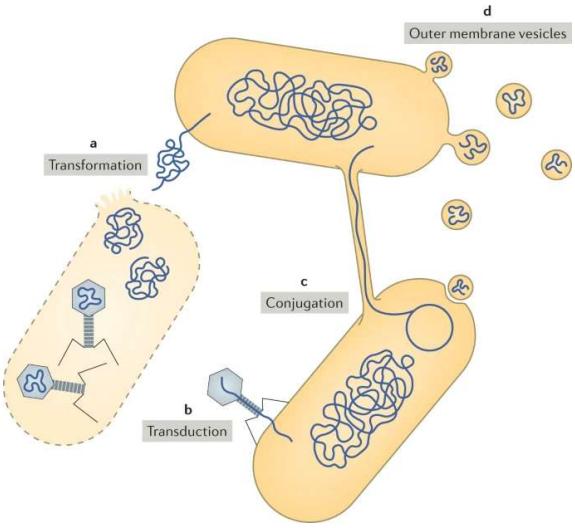

Figure 3. Mechanisms of horizontal gene transfer. a) transformation; b) transduction; c) conjugation; d) additional mechanisms involve outer membrane vesicles and DNA packaged into virus-like particles (not shown). Credit: Brito [80].

1.3 The role of aquatic environment in the propagation and spread of AR

The natural environment encompasses diverse ecosystems such as terrestrial, marine and freshwater ecosystems, which include interconnected biotic and abiotic factors such as soil, plants, animals and water. Coastal areas, as the intersection of marine and terrestrial environments and home to more than 45% of the world's population, are considered the most diverse and complex ecosystems. They are among the most vulnerable ecosystems, emphasizing their importance for studying the transmission pathways of resistance determinants [83,84]. Within the One Health concept, aquatic ecosystems are one of the most important natural environments that require comprehensive surveillance, prevention and protection regarding antibiotic resistance.

In the last decade, it has become clear that the environmental microbiota possesses an enormous and diverse collection of resistance genes, called the resistome [31], some of which are very ancient and similar to genes circulating in the pathogenic microbiota. Anthropogenic sources are increasingly contributing to the environmental resistome [85]. Wastewater discharges from hospitals and municipalities, as well as waste from veterinary sources and aquaculture, contribute to the spread of ARGs in the environment as they often contain ARGs, ARB and the residues of antibiotics and other antimicrobial agents, which can exert a selection pressure on bacteria favoring the survival and spread of antibiotic-resistant strains [86]. Notably, coastal microbiomes have been found to be significantly more enriched in ARGs than those of the deep sea and Antarctic [87], which is related to the extent of anthropogenic influence as ARGs abundance correlates with the pollution gradient [88]. Furthermore, the bacterial community has been shown to be an important determinant of the aquatic resistome, even in environments rarely impacted by human activities such as the High Arctic [24,87].

It has been found that environmental pollution influences the structure and diversity of bacterial communities forcing them to change constantly. Even sub-inhibitory concentrations of antibiotics, such as those found in various aquatic environments [85], can promote the selection of ARB through horizontal transfer of resistance plasmids [89–91]. In addition to antibiotics, environmental contamination with heavy metals, even at relatively low concentrations, promotes the co-selection of AR due to shared genetic or physiological mechanisms[92,93]. Without any doubt, the natural process of resistance selection driven by antibiotic production by environmental bacteria, mixing with antibiotic-resistant terrestrial bacteria and

environmental pollution with commercially used antibiotics plays a key role in the emergence of ARGs in the aquatic environment[10].

Nevertheless, indigenous bacterial communities in aquatic environments are also shaped by constant selection pressure from diverse physicochemical factors such as temperature, salinity, dissolved oxygen, pH, dissolved organic matter and available nutrients [94]. Namely, the ocean microbiome greatly differs from microbiomes of animals, plants, and soil due to several distinctive characteristics. Firstly, the primary producers are mostly microbial, contributing significantly to the ocean microbiome [95]. Microbial photosynthesis in surface waters and chemosynthesis in deeper regions is responsible for half of all primary production on Earth[96]. Secondly, engaging various mechanisms like proteorhodopsin, anoxygenic phototrophy, and chemolithotrophy that disrupt traditional trophic distinctions enable marine microbiota the adaptability in meeting the optimal metabolic needs in nonfavourable environmental conditions. Lastly, heterogeneity in the composition of organic matter in marine environments contributes to the diversity of marine microbiomes. For instance, planktonic and surface-attached bacteria and archaea dwelling individually in seawater are found to differ from those inhabiting marine networks and organic surfaces in terms of their genetic diversity, metabolism, motility, chemotaxis, and defense capabilities. While individual bacteria and archaea dominate in terms of numbers, those residing near surfaces exhibit higher rates of metabolism and growth per cell[97].

1.3.1 The mechanisms of AR in Gram-negative bacteria

Generally, the bacteria can become nonsusceptible to the antibiotic by two main ways. The first arises from alterations in the target molecules of the antibiotic, making it insensitive to its effect, whereas the second can result from the decreased dose of the antibiotic inside the cell due to reduction of its minimum inhibitory concentration (MIC)[98].

There are four molecular mechanisms that bacteria can use to evade the effect of antibiotic(s), and these include reduced permeability of the bacterial cell for the intake of antibiotics, modification of drug target, overexpression of efflux pumps that eject the drug from the cell, and the enzymatic degradation of antibiotic (Figure 4.).

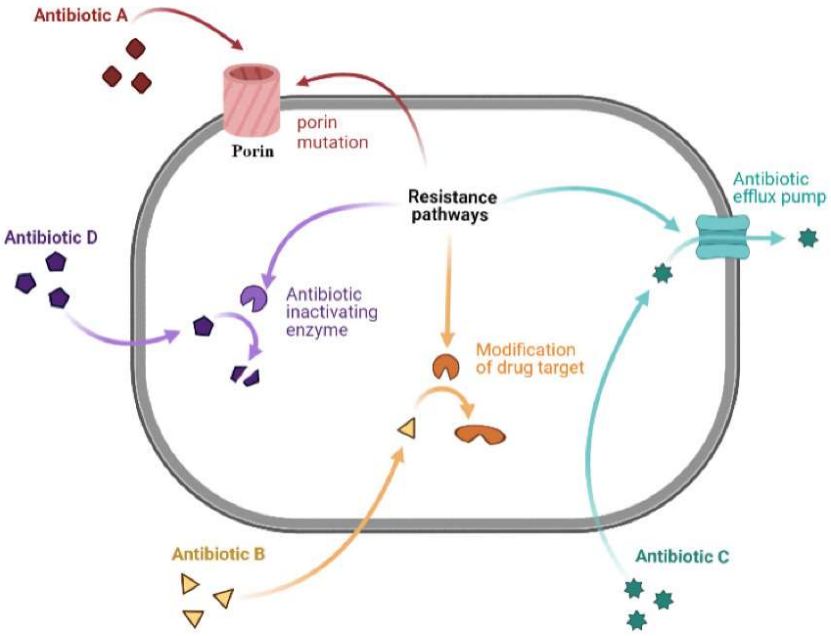

Figure 4. Mechanisms of AR. (a) restricting drug uptake (porins), (b) drug target modification, (c) antibiotic removed from the cell by the efflux pump mechanism through active transport and (d) drug inactivation by bacterial enzymes. Credits: Iskandar and colleagues [99].

Importantly, resistance to some antibiotics may be expressed through multiple mechanisms[44,100]. Nevertheless, there are some specific resistance mechanisms, such as active efflux for tetracyclines, enzymatic degradation by β-lactamases for β-lactam antibiotics, drug modification for aminoglycosides, and target modification for quinolones. Resistance mechanism is dependent on the bacterial species, the type of antibiotic and can be influenced by the differences in cell structure between Gram-positive and Gram-negative bacteria. Notably, Gram-negative bacteria possess an inherent resistance to many antibiotics by this structural difference, as penetration of the antibiotic can be inhibited by one or both cell membranes. Molecular transport through membranes is facilitated by specialized pores, which can demonstrate selectivity for positively or negatively charged molecules. Decreased membrane permeability caused by porin mutations in resistant strains, such as in case of OprD porin, cause resistance to carbapenems in *Pseudomonas aeruginosa*[101,102]. In addition, reduced permeability of the outer membrane in Gram-negative bacteria can also significantly contribute to resistance against quinolones and aminoglycosides[46].

Moreover, penicillin-binding proteins (PBPs) are found to play a crucial role in catalyzing the formation of peptide cross-links during peptidoglycan synthesis and are therefore vital to provide the strength and rigidity to the bacterial cell wall. β-lactam antibiotics, including penicillins and cephalosporins, mimic the structure of the PBPs peptide and therefore bind to and inhibit the activity of PBPs, ultimately leading to cell lysis and death due to the weakened cell wall. Structural changes in PBP molecules lead to reduced affinity for β-lactam antibiotics or even complete inability to bind them[103]. This mechanism is primarily utilized by Gram-positive bacteria such as methicillin-resistant *S. aureus* (MRSA), which possesses the *mecA* gene responsible for the synthesis of PBP-2a, which lacks affinity for any β-lactam antibiotic. Similarly, alterations in the PBP-2b protein of *Streptococcus pneumoniae* lead to resistance to penicillin and cephalosporins[104].

On the other hand, modifications of enzymes such as topoisomerases, the primary target of quinolones, lead to a reduction in quinolone affinity without affecting the function of the enzyme[105]. Enzymatic inactivation facilitated by acetyltransferase and phosphotransferase (derived from plasmids or transposons) also contribute to aminoglycoside resistance in aerobic Gram-positive bacteria (*Enterococcus faecium*)[104]. Notably, mutations in the 30S ribosomal subunit of enterococci disrupt their association with the streptomycin target, leading to aminoglycoside resistance. This mechanism is also associated with tetracycline resistance.

Genes like *tetM, tetO, tetQ* and *tetS* hinder drug efficacy by altering the ribosomes in a way to inhibit tetracycline binding. These genes are widely distributed in various genera, including *Campylobacter* and *Bacteroides*, and originate from both plasmids and chromosomes[106].

Active efflux is a crucial mechanism that Gram-negative bacteria use to remove variety of toxic substances from their cell, including antibiotics, heavy metals, detergents, and hydrocarbons. They operate through membrane protein pumps, actively transporting harmful substances from the periplasmic space through the outer membrane outside of the cell. Efflux pumps are categorized as primary and secondary transporters, each with distinct mechanisms and energy sources. Primary transporters, represented by the ATP-binding cassette (ABC) family, rely on the energy derived from ATP binding and hydrolysis for efflux. Secondary transporters, including the major facilitator superfamily (MFS), small multidrug resistance (SMR) family, resistance nodulation division (RND) family, and multidrug and toxic compound extrusion (MATE) family, utilize the energy of the membrane's electrochemical potential[107,108]. Among these, the most significant efflux for AR in Gram-negative bacteria is by the RND family pumps. They form a tripartite system consisting of an RND pump on the inner membrane, a periplasmic adapter protein (PAP), and a channel on the outer membrane[109]. These pumps exhibit versatility, transporting various compounds, including antibiotics from multiple classes, contributing significantly to the MDR phenotype[110]. *P. aeruginosa* possesses several genes encoding for RND pumps across its genome (12), several of which are associated with MDR. Moreover, comparative genomics revealed a high degree of homology among RND systems in different species, such as *E. coli* (AcrB), *P. aeruginosa* (MexB), *Campylobacter jejuni* (CmeB), *A. baumannii* (AdeB), and *Neisseria gonorrhoeae* (MtrD)[108]. In addition, intrinsic MDR attributed to efflux systems has been documented in various bacterial species, including *E. coli*[111], *C. jejuni*[112], *Haemophilus influenzae*[113], and *Salmonella enterica* serovar Typhimurium[114]. While RND complexes are generally chromosomally encoded, there are also reports of plasmid-based carriage of their encoding genes [115]. Namely, strains of *S. Typhimurium*, *E. coli* and *K. pneumoniae* are found to have OqxAB RND pump, which is plasmid-mediated and associated with quinolone resistance[116], whereas RND efflux pumps encoding genes have also been found on bacterial plasmids that carry other resistance determinants such as $bla_{\text{NDM-1}}$ gene [117].

1.3.2 Production of β-lactamases

The production of β-lactamases is the predominant mechanism of resistance to β-lactams in Gram-negative bacteria[44]. These enzymes break down the structure of the β-lactam ring, making it ineffective. First report of β-lactamases was before clinical use of penicilin, suggesting these enzymes existed before discovery of β-lactam antibiotics[118]. To overcome resistance, combination therapies with β-lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam, are often used to inhibit the action of β-lactamases and restore the effectiveness of β-lactam antibiotics.

The Ambler system of classification categorizes β-lactamases into four groups based on their amino acid sequences and functional characteristics: A, B, C, and D[119] (Figure 5.). Enzymes of the A, C, and D classes contain an active serine residue, while B class are metalloenzymes that contain one or two zinc ions at the active site to facilitate β-lactam hydrolysis. Group A includes extended-spectrum β-lactamases (ESBLs) among which the predominant types are TEM, SHV, CTX-M which confer resistance to penicillins, monobactams and 3rd generation cephalosporins but are mostly sensitive to inhibitors such as clavulanic acid[120]. They are disseminated worldwide because their encoding genes are located on plasmids and other mobile genetic elements across a range of Gram-negative pathogens, particularly the Enterobacteriaceae.

Infections caused by ESBL-producing bacteria have been effectively managed using carbapenems. However, the widespread usage of carbapenems has fastened the spread of another significant category of β-lactamases, the carbapenemases. These enzymes belong to Ambler classes A, B and D. Class A includes the most clinically significant *Klebsiella pneumoniae* carbapenemase (KPC) enzyme involved in the majority of opportunistic infections of immunocompromised patients in the healthcare units, which is successfully transmitted via plasmid through horizontal gene transfer[121]. Although the KPC β-lactamases are predominantly found in *K. pneumoniae*, there have been reports of these enzymes in *Enterobacter* spp. and in *Salmonella* spp.[122]. These enzymes are inhibited by clavulanic acid and tazobactam[123]. Class B carbapenemases are capable of hydrolyzing all carbapenem antibiotics except monobactams, and include NDM, VIM and IMP enzymes[124]. The class D carbapenemases consist of plasmid-mediated oxacillin-hydrolyzing (OXA) β-lactamases frequently detected in *Acinetobacter baumannii* [125]. On the other hand, AmpC cephalosporinases are members of class C β-lactamases and mediate resistance to cephalothin, cefazolin, cefoxitin, and most penicillins[126].

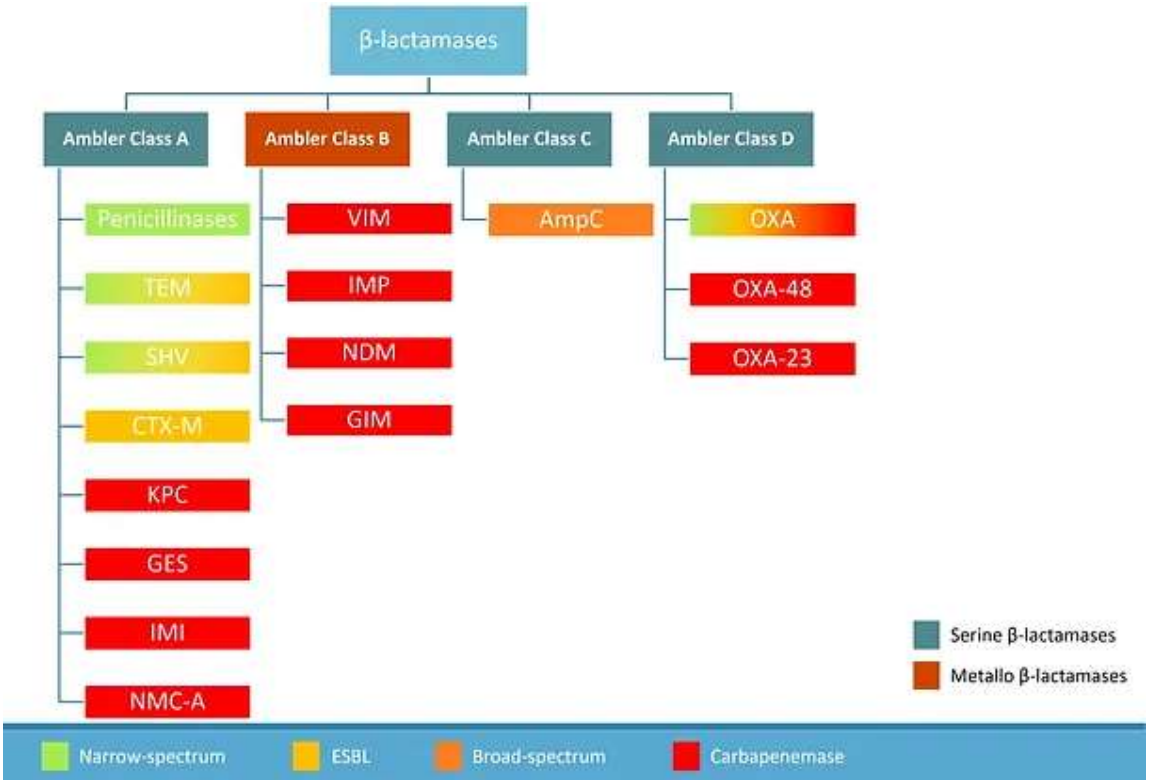

Figure 5. Classification of β-lactamases in Enterobacteriaceae. Credit: ICUreach (https://www.icureach.com/)

The recent reports evidence that the resistance to carbapenems among clinical isolates is increasing worldwide [127,128]. In Croatia, carbapenem resistance is particularly worrisome in clinical *K. pneumoniae*, that showed increased rates from 2% in 2018 to 12% in 2019 (ECDC, 2020) [129]. These rates were mostly due to the dissemination of KPC-2 carbapenemase, which initially occurred in clinics in the northern part of the country before spreading to the southern areas. In a nationwide study conducted by Jelić and colleagues [231], KPC isolates carrying the $bla_{\text{KPC-2}}$ gene were identified in 9 out of 40 centers across Croatia[130].

Moreover, ESBL-producing enterobacteriaceae were also found to be spread in Croatia, in both hospital settings and the environment. A study by Maravić and colleagues[26]. investigated human-impacted coastal waters of the eastern Adriatic Sea and detected ESBL encoding genes in 4.2% of Enterobacteriaceae, primarily in *E. coli*, *E. cloacae*, and *K. pneumoniae*. The most

prevalent genotype was *bla*$_{\text{TEM-1+SHV-12}}$, followed by *bla*$_{\text{CTX-M-15}}$ [26]. Krilanovic and colleagues confirmed the presence of β-lactamase genes, including *bla*$_{\text{VIM}}$, *bla*$_{\text{CTX-M}}$, and *bla*$_{\text{TEM}}$, in the river Neretva [131]. Moreover, *Klebsiella* carrying *bla*$_{\text{CTX-M}}$ genes were detected in animal isolates in Croatia[132]. Puljko and colleagues showed that inadequate wastewater treatment led to a marginal reduction of ESBL genes (particularly *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M-32}}$) whereas carbapenemase genes (including *bla*$_{\text{KPC-3}}$, *bla*$_{\text{NDM}}$, and *bla*$_{\text{OXA-48}}$) were occasionally detected, which was associated with several factors such as hospital size and wastewater concentration[133].

Despite extensive research on β-lactamases and CRE in hospitals and wastewater, their distribution along marine trophic gradients is still poorly understood. With increasing CRE pressure in healthcare facilities and wastewater treatment plants, the potential for further spread via marine microbial communities through runoff needs to be urgently investigated.

1.4 Metagenomic studies in the environment

Oceanographers began studying the ocean microbiome more than forty years ago when it was realized that microbes play a crucial role in energy transfer in the ocean. Thus, the marine microbiome is essential for maintaining the balance of life in the Earth's largest and least concentrated biological environment. When the protein proteorhodopsin was found to be widely distributed in the marine environment, enabling bacteria to survive in oligotrophic environments[134], this was the first exciting discovery resulting from the application of "meta-omics" methods in the ocean. Metaomics methods represent a comprehensive suite of high-throughput techniques used to analyze the collective genomes, transcripts, proteins, and metabolites of microbial communities. These approaches are integral to understanding the functional capabilities, diversity, and dynamics of complex microbial ecosystems. Metagenomics involves the sequencing and analysis of DNA from environmental samples to elucidate the genetic material of entire microbial communities, revealing their diversity, structure, and potential functional capabilities. Today, these methods are fundamental in transforming lists of organisms and their functions into clear relationships. Significant progress has been made in understanding the role and position of microbes in ocean biogeochemical processes. Metagenomics has also provided insights into novel cooperative relationships between microbes, how they exchange substances such as nitrogen, vitamins, hormones and antibiotics without exploiting each other.

Over the past decade, there has been a significant shift towards NGS in global research focused on environmental diversity. This shift has been driven by the emergence of highly efficient sequencing platforms that can process large amounts of data simultaneously. Some of the best-known platforms in this field include Illumina (formerly known as Solexa), Ion Torrent, Roche 454, SOLiD and others.

Amplicon sequencing and whole genome sequencing (WGS) are two different methods used in genome studies, each with specific applications and advantages. Amplicon sequencing is a targeted approach that focuses on sequencing specific genomic regions, usually by PCR amplification of marker genes. Different marker genes or sequences are used for different groups of organisms to enable accurate identification and classification. For example, the 16S rRNA gene is usually used for bacteria, the Internal Transcribed Spacer (ITS) region for fungi and the Cytochrome c Oxidase I (COI) gene for animals. This method is cost-effective and generates smaller amounts of data compared to WGS, making it suitable for studies that require

high-resolution taxonomic identification without the need for comprehensive genomic information. In contrast, whole genome sequencing sequences the entire genome of an organism, providing a comprehensive overview of the genetic composition, including coding and non-coding regions, regulatory elements and structural variants. WGS provides detailed genomic insights and enables in-depth studies of gene function, genetic variation and evolutionary biology. However, it is more expensive and generates large amounts of data that require extensive computational resources for data analysis and storage. Each method has its own merits, with amplicon sequencing being ideal for targeted taxonomic studies and WGS for comprehensive genomic analyses.

In 16S rRNA amplicon sequencing, a specific 16S rDNA fragment is amplified, sequenced and used for taxonomic identification. NGS technology, such as Illumina Sequencing (HiSeq and MiSeq systems) and Ion Torrent Sequencing generates short reads of approximately 150–300 base pairs (bp) and restricts sequencing to specific regions of the 16S rRNA gene, which is approximately 1.5 kilobases (kb) long, in a single run. The conserved regions within the gene allow the development of universal primers suitable for amplification of 16S rRNA gene of most bacteria and archea. The variable regions V3 and V4 are among the most frequently sequenced regions in different habitats (Figure 6).

This is a significant challenge in all microbiology, not only in resistome research as only a small fraction of microbial species can be cultivated and grown in laboratory conditions[135]. Moreover, some microbial species that harbor ARGs may be viable but cannot be cultured.

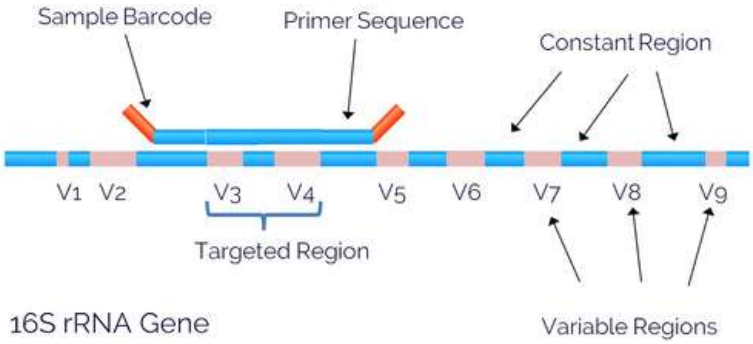

Figure 6. Schematic representation of the distribution of hypervariable regions V1-V9 of 16S rRNA gene. Locus-specific primers that bind to the conserved regions are used to amplify the V3 and V4 region of the 16S rRNA gene from genomic DNA. Credit: (source: http://www.lcsciences.com/discovery/applications/genomics/16s-mobile/).

In computational analyses, 16S rRNA genes serve as markers for taxonomic identification, allowing researchers to link ARG profiles to microbial populations. ARG profiles refer to the comprehensive characterization and identification of genes within a microbial community that confer resistance to antibiotics. The DNA of the microbial community is sequenced to identify and quantify the presence of ARGs. Sequences are compared to known resistance gene databases (ARDB (Antibiotic Resistance Genes Database, https://ardb.cbcb.umd.edu/), CARD (Comprehensive Antibiotic Resistance Database, https://card.mcmaster.ca/), ResFinder (http://genepi.food.dtu.dk/resfinder), ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation, https://www.mediterranee-infection.com/)), allowing the resistance genes in the sample to be profiled. Although 16S rRNA gene sequence data is useful in defining the core microbial community, it alone may not provide comprehensive information. The core microbial community refers to the set of microbial species or strains that are consistently found in a particular environment across different samples or individuals, regardless of temporal or spatial variations. These core members are thought to play essential roles in maintaining the stability and functionality of the microbial ecosystem. Integrated approaches that combine 16S rRNA sequencing and other tools like PICRUSt and qPCR can reveal bacterial interactions and dynamics within resistome communities. PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) is a bioinformatics tool that predicts the functional potential of microbial communities from 16S rRNA gene sequence data by inferring the presence of functional genes based on phylogenetic relationships and known genomic information from related organisms. Quantitative Polymerase Chain Reaction can provide

accurate and sensitive detection of nucleic acids, commonly employed in gene expression analysis, pathogen detection, and quantification of DNA sequences. For instance, Wang and colleagues studied the seasonal and spatial proliferation of ARGs under the influences of land-ocean interaction in the Sishili Bay using high-throughput qPCR. Ten ARGs (coding for resistance to tetracyclines, sulphonamides and macrolides) and integrase gene (*intI1*) were detected and quantified [137]. Similarly, Kvesić and colleagues utilized a combination of 16S rRNA sequencing and PICRUSt to investigate the resistome of wastewater-receiving coastal waters in Croatia, showing a dominance of phylum Proteobacteria, Bacteroidetes and Firmicutes, and the functional prediction of their gene repertoire[28]. Picrust prediction was used to associate bacterial species with resistance genes, the exact representation of these genes was additionally verified by qPCR, and this method served as an additional in situ confirmatory factor.

Nevertheless, the use of metagenomics to study the environmental resistome is still in its early stages and is constantly evolving. New sequencing technologies, such as Oxford Nanopore Techology (ONT) sequencing, are continually being refined for resistome research[138,139]. While metagenomics provides a robust approach to study the resistome, a major limitation is that the mere presence of a gene doesn't guarantee its functionality or expression by cells. To close this data gap, it's essential to integrate metagenomics tools with complementary approaches such as functional metagenomics. Functional metagenomics entails the extraction of DNA from environmental samples, its subsequent cloning into suitable host organisms, and the screening of these clones for specific functions or activities, thereby enabling the study of microbial gene functions beyond mere sequence analysis. This methodology facilitates the discovery of novel enzymes, antibiotics, and other bioactive compounds, overcoming the limitations inherent in traditional culture-based approaches.

2. SCIENTIFIC PAPERS

2.1 Microbiome profiling and characterization of virulent and vancomycin-resistant *Enterococcus faecium* from treated and untreated wastewater, beach water and clinical sources

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Microbiome profiling and characterization of virulent and vancomycin-resistant *Enterococcus faecium* from treated and untreated wastewater, beach water and clinical sources

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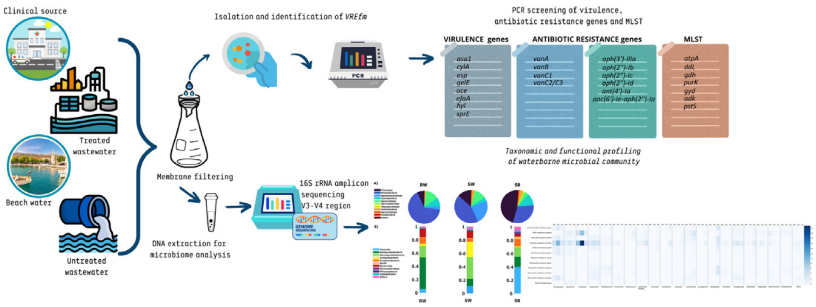
HIGHLIGHTS

- Hospital and environmental vancomycin-resistant *Enterococcus faecium* were studied.
- VREfm enter marine environment by outfalls of treated and untreated wastewater.
- *VanA* genotype predominates among hospital and waterborne isolates in Croatia.
- Hospital and environmental VREfm carry similar virulence and resistance genes.
- Microbiome analysis revealed accompanying pathogenic taxa and resistance genes.

GRAPHICAL ABSTRACT



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ABSTRACT

Vancomycin-resistant *Enterococcus faecium* (VREfm) is an opportunistic pathogen among the highest global priorities regarding public and environmental health. Following One Health approach, we determined for the first time the antibiotic resistance and virulence genes, and sequence types (STs) affiliation of VREfm recovered simultaneously from marine beach waters, submarine outfall of a wastewater treatment plant and an offshore discharge of untreated sewage, and compared them with the surveillance VREfm from regional university hospital in Croatia to assess the hazard of their transmission and routes of introduction into the natural environment. Importantly, VREfm recovered from wastewater, coastal bathing waters and hospital shared similar virulence, multidrug resistance, and ST profiles, posing a major public health threat. All isolates carried the *vanA* gene, while one clinical isolate also possessed the *vanC2/C3* gene. The hospital strains largely carried the aminoglycoside-resistance genes *aac(6′)-Ie-aph(2″)-Ia*, and *aph(2″)-Ib* and *aph(2″)-Id*, which were also predominant in the environmental isolates. The *hyl* gene was the most prevalent virulence gene. The isolates belonged to 10 STs of the clonal complex CC17, a major epidemic lineage associated with hospital infections and outbreaks, with ST117 and ST889 common to waterborne and hospital isolates, pointing to their sewage-driven dissemination.
To gain better insight into the diversity of accompanying taxons in the surveyed water matrices, microbiome taxonomic profiling was carried out using Illumina-based 16S rDNA sequencing and their resistome features predicted

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using the PICRUSt2 bioinformatics tool. An additional 60 pathogenic bacterial genera were identified, among which *Arcobacter, Acinetobacter, Escherichia-Shigella, Bacteroides* and *Pseudomonas* were the most abundant and associated with a plethora of antibiotic resistance genes and modules, providing further evidence of the hazardous effects of wastewater discharges, including the treated ones, on the natural aquatic environment that should be adequately addressed from a sanitary and technological perspective.

1. Introduction

The most recent data on the global burden of antimicrobial resistance evidence that multidrug-resistant bacterial infections are among the major causes of death for people of all ages, resulting in a total of 4.95 million deaths in 2019 (Murray et al., 2022). Given the limited number of effective therapeutic options and the paucity of novel antibacterial drugs in the research and development pipelines, a struggle to rein the antibiotic resistance phenomenon in all aspects of human life has never been more straightforward. In this regard, pathogens from the WHO global priority list are of most importance, with vancomycin-resistant *Enterococcus faecium* (VREfm) placed in the high-priority category (WHO, 2017).

E. faecium is a commensal of the human and animal gut microbiota, but VREfm strains have emerged as particularly challenging pathogens in the healthcare context due to their intrinsic and acquired resistance to multiple classes of antibiotics and global dissemination. Notably, this microorganism is associated with a range of infections, urinary tract and bloodstream in particular, and is the eighth most common pathogen based on the summary of global deaths attributable to and associated with antibiotic resistance in 2019 (Murray et al., 2022). This is linked with its increasing trends for resistance to vancomycin (VAN), once a drug of choice for treatment, which rose steadily to 16.8 % in Europe from 2016 to 2020, with no distinct geographic pattern between countries (WHO, 2021). Croatia is no exception, reporting a VREfm rate among nosocomial isolates of 17 % (Tambić Andrašević et al., 2018). Of the nine genotypes related to VAN resistance (*vanA, vanB, vanC, vanD, vanE, vanG, vanM, vanN,* and v*anL*), *vanA*, encoding acquired inducible resistance to VAN and teicoplanin (TEC), and *vanB*, responsible for variable resistance to VAN and susceptibility to TEC, are the two most commonly reported (Lebreton et al., 2011). In addition, vancomycin-resistant enterococci (VRE) can acquire genes encoding several aminoglycoside-modifying enzymes (AMEs), including aminoglycoside phosphoryl transferase (APH), aminoglycoside nucleotidyl transferase (ANT), and aminoglycoside acetyl transferase (AAC), resulting in high levels of gentamicin resistance and the characteristic high-level aminoglycoside-resistant (HLAR) phenotype (Haghi et al., 2019). The *aac (6′)-Ie-aph (2″)-Ia* gene, which encodes the enzyme AAC (6')-APH (2"), is frequently detected in such isolates (Haghi et al., 2019). In addition, *E. faecium* possesses a variety of virulence factors such as enterococcal surface protein (Esp), and different enzymes that enhance host tissue colonization and damage (Haghi et al., 2019), contributing to the clinical importance of this opportunistic pathogen.

As faecal bacteria, multidrug-resistant enterococci, including VREfm, can enter the aquatic environment, especially river water, via hospital and municipal wastewater (Ekwanzala et al., 2020; Gotkowska-Płachta, 2021). Indeed, *E. faecium* has been found to predominate in hospital and municipal wastewater compared to other enterococci (Gotkowska-Płachta, 2021; Monticelli et al., 2019). Nevertheless, reports of VREfm in coastal marine waters remain very scarce. From the literature review, five *vanA*- or *vanB*-positive VREfm isolates have been found in seawater from public beaches and a fishing pier in Washington, USA (Roberts et al., 2009), while only a few isolates (*van* genes were not assayed) have been detected in coastal waters in Sicily, Italy (Monticelli et al., 2019), southeastern Brazil (de Oliveira and Pinhata, 2008), and northern Iran (Alipour et al., 2014). Importantly, no previous studies have comprehensively addressed this public health threat by linking the marine environment, sewage discharges, and hospital settings as reservoirs of VREfm, focusing on their virulence, antibiotic resistance determinants, and potential routes of dissemination between natural and clinical environments.

Accordingly, we molecularly characterized environmental VREfm isolates obtained simultaneously from the outfalls of untreated municipal wastewater, treated hospital and municipal wastewater, seawater from the public beach near the sewage outfall, and surveillance isolates from the regional university hospital in southern Croatia, for the presence of genes encoding virulence factors and resistance to VAN and aminoglycosides, and assigned them to sequence types (ST) groups. The aim was to test for the first time the hypothesis that VREfm of similar genotypes circulate in southern Croatia and enter coastal waters via treated and untreated sewage effluents. In addition, Illumina-based 16S rRNA amplicon sequencing and bioinformatics complemented by PICRUSt2 prediction tool were used to provide in-depth data on the taxonomic diversity of the microbiome and the overall resistome in surveyed water bodies, identifying which pathogenic taxa are most potent reservoirs of antibiotic resistance in the human-influenced marine environment.

2. Material and methods

2.1. Site description and sampling procedure

Water sampling was conducted from July to September 2020 at three sites located on the eastern coast of the central Adriatic Sea (Suppl Fig. S1). The study focused on the highly urbanized coast of Kaštela Bay, which is a conglomeration of the cities of Trogir, Kaštela, Solin and Split with a population of about 350,000. The area is still one of the most polluted areas on the eastern Adriatic coast due to the lack of fully functional urban infrastructure, especially in terms of wastewater collection and disposal. The wastewater system for the municipalities of Split and Solin has been built and operational since 2004, while the secondary systems for the other cities are still under construction. The first site (BW; 43°32′ 42.1"N, 16°25′41.0″E) is a public beach located in the city of Kaštela and is used for bathing and other recreational purposes, especially for the elderly people due to the sandy sediment and shallow water. This beach was previously identified as feacally polluted (Ordulj et al., 2022) and a potent reservoir of Gram-negative pathogens resistant to multiple antibiotics (Maravić et al., 2013, 2014, 2015, 2018; Šamanić et al., 2021). Namely, the public sewerage system of Kaštela includes a large number of small subsystems that collect wastewater and rainfall by gravity and discharge it into the bay without treatment through outlets located close to the coast (Knezić and Margeta, 2002). One of these outlets represents the second sampling site (SW; 43°32′41.2"N, 16°25′37.3″E), and is the closest outlet to the beach mentioned above. The third site is a submarine outfall of treated wastewater (SB; 43°28′53.6 "N, 16°31'04.3 "E), located at a depth of 37 m in the Brač Channel. The sampling and physico-chemical analysis of the wastewater from this site was conducted as a part of our previous study (Kvesić et al., 2022). The outfall is connected to the wastewater treatment plant Stobreč-Stupe, which treats municipal wastewater from part of the Split urban area (but not from Kaštela) by primary treatment (coarse and fine screening, oil and sand removal) with an average daily capacity of 37,500 m^3.

All water samples were collected in triplicate. Beach water (BW) was collected 1 m from shore and approximately 20 cm below the surface in sterile 1-L sterile borosilicate glass bottles. Untreated sewage effluent (SW) was sampled approximately 50 cm from the coastal outfall pipe and 20 cm below the sea surface. Effluent from the submarine sewer outfall Stobreč-Stupe (SB) was collected at the sea bottom from a boat using a Niskin sampler. All samples were stored in portable cooler during transport and analyzed within 4 h.

2.2. Determination of faecal pollution and physicochemical parameters

The level of faecal pollution at the studied sites was assessed by enumeration of *E. coli* (ISO 9308-1:2014) and faecal enterococci (ISO 7899-2:2000). Physico-chemical parameters of the SB samples were determined by Kvesić et al. (2022). Temperature, salinity, and pH of BW and SW samples were recorded with a handheld YSI Pro1030 pH and conductivity meter implemented with a pH 1002 sensor. Dissolved inorganic nutrient concentrations (nitrates, nitrites, ammonia, and orthophosphates) and total nitrogen and phosphorus concentrations (after UV oxidation) were determined colorimetrically in unfiltered samples using the AutoAnalyzer III colorimeter (Seal Analytical). Total nitrogen (NTOT) and phosphorus (PTOT) were analyzed in unfiltered samples after autoclaving in an acid potassium persulfate solution at 130 °C for 120 min (Mckelvie et al., 1995).

2.3. Isolation and identification of VREfm from water samples

Volumes of 100 mL were filtered through 0.45-μm cellulose nitrate membrane filters (Sartorius, Germany) and the membranes were placed onto chromogenic agar ChromID VRE (bioMerieux, France) for selection of VAN-resistant phenotypes. Plates were incubated aerobically at 37 °C and examined after 24 h and 48 h of incubation. Purple-stained colonies were selected as putative VREfm and identified to the species level using matrix-assisted laser desorption/ionization time-of-fight (MALDI-TOF) mass spectrometry and MALDI-Biotyper software version 4.1.80 (Bruker Daltonics, Bremen, Germany) according to the manufacturer's instructions.

2.4. Membrane filtration and DNA extraction for microbiome analysis

One liter of water samples was filtered in triplicate through fast-flow 0.22 mm MCE membranes (Whatman, UK) and the genomic DNA was extracted using DNeasy PowerWater Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA concentration was measured using a NanoDrop® Spectrophotometer 1000 (Thermo Scientific, USA) and the DNA stored at −20 °C until further analysis.

2.5. Clinical isolates

Clinical VREfm isolates were obtained during routine surveillance from rectal swabs of patients upon admission to intensive care unit or transfer to other hospital ward at University Hospital Centre Split from November 2020 to January 2021 (Table 1). Total of 299 swabs were screened for the presence of VREfm, out of which 65 (21.7 %) were found to be positive. The institution is a university teaching hospital with 1651 beds and the second largest clinical hospital centre in Croatia, serving as a referral hospital for most of southern Croatia with a total population of approximately 1 million. Strains were isolated using chromogenic CHROMagar VRE agar (CHROMagar, France) after 24 h of incubation at 37 °C, based on the characteristic pink to mauve appearance of the colonies (Goić-Barišić et al., 2020). Suspect colonies were identified by MALDI-TOF mass spectrometry as mentioned above. Fifteen VREfm were randomly selected for further molecular characterization.

2.6. Species identification and antibiotic susceptibility testing

Antibiotic susceptibility testing was carried out using the disc-diffusion method against ampicillin (AMP, 2 μg), gentamicin (GEN, 30 μg), VAN (5 μg), TEC (30 μg), tigecycline (TGC, 15 μg) and linezoid (LZD,10 μg) according to The European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2020). High-level aminoglycoside-resistant (HLAR) phenotype was defined if <8 mm of inhibition zone was produced with GEN disc (30 μg/mL) using disc-diffusion assay (EUCAST, 2020).

2.7. PCR screening of virulence and antibiotic resistance genes

Genomic DNA of the VREfm isolates was isolated using the NucleoSpin Microbial DNA Kit (Macherey-Nagel, UK) according to the manufacturer's instructions. Isolates were screened by multiplex and single PCR reactions for the presence of genes encoding for virulence factors, including aggregation substance (*asa1*), cytolysin (*cylA*), enterococcal surface protein (*esp*), gelatinase (*gelE*), collagen-binding protein (*ace*), endocarditis antigen (*efaA*), and hyaluronidase (*hyl*) using the primers and amplification protocols described by Golob et al. (2019) as well as serine protease (*sprE*) according to Stępień-Pyśniak et al. (2019).

Multiplex PCRs were performed to detect genes mediating resistance to VAN and aminoglycosides. The *vanA, vanB, vanC1* and *vanC2/C3* genes were searched according to the protocol of Said and Abdelmegeed (2019), while genes encoding for AMEs, including *aph(3′)-IIIa, ant(4′)-Ia, aac(6′)-Ie-aph(2″)-Ia, aph(2″)-Ib, aph(2″)-Ic,* and *aph(2″)-Id*) were screened as previously described Özdemir and Tuncer (2020).

Table 1
Origin and characteristics of hospital surveillance VREfm isolates included in this study.

Isolate no.	Isolation date[a]	Hospital ward	Patient's year of birth/sex	ST	CC	Resistance phenotype[b]	Virulence genes	VAN-resistance genes	Aminoglycoside-resistance genes	Coisolation
ND 5288	30/11/2020	H-K	1959/M	780	17	AMP-VAN-TEC	*hyl*	*vanA*	*aac(6′)-le-aph(2″)-la*	
ND 5286	30/11/2020	ICU-F	1935/M	721	17	AMP-GEN-VAN -TEC	*esp*	*vanA*	*aac(6′)-le-aph(2″)-la*	
ND 5287	30/11/2020	ICU-K	1956/M	117	17	AMP-GEN-VAN-TEC	*hyl*	*vanA*	*aac(6′)-le-aph(2″)-la, aph(2″)-Ib, aph(2″)Id*	
ND 189	11/01/2021	ICU-F	1944/F	117	17	AMP-GEN-VAN-TEC	*hyl*	*vanA*	*aac(6′)-le-aph(2″)-la, aph(2″)-Ib, aph(2″)Id*	Multidrug-resistant *Acinetobacter baumanii*
ND 5744	22/12/202	H-K	1983/M	192	17	AMP-VAN-TEC	*esp*	*vanA*	*aac(6′)-le-aph(2″)-la*	
ND 5658	17/12/2020	ICU-K	1948/M	117	17	AMP-GEN-VAN-TEC	*hyl*	*vanA*	*aac(6′)-le-aph(2″)-la, aph(2″)-Ib, aph(2″)Id*	
ND 5308	30/11/2020	R	1944/M	889	17	AMP-GEN-VAN-TEC	*hyl*	*vanA, van C2/C3*	*aph(2″)-Ib, aph(2″)Id*	
ND 138A	09/01/2021	N-K	1945/M	117	17	AMP-GEN-VAN-TEC	*hyl*	*vanA*	*aac(6′)-le-aph(2″)-la, aph(2″)-Ib*	KPC-producing *Klebsiella pneumoniae*
ND 5766	23/12/2020	R	1950/M	78	17	AMP-VAN-TEC	*esp*	*vanA*	*aac(6′)-le-aph(2″)-la*	
ND 144	09/01/2021	ICU-K	1952/M	889	17	AMP-VAN-TEC	*hyl*	*vanA*	*aac(6′)-le-aph(2″)-la*	
ND 80	07/01/2021	ICU-K	1942/M	117	17	AMP-VAN-TEC	*hyl*	*vanA*	*aac(6′)-le-aph(2″)-la, aph(2″)-Ib, aph(2″)Id*	
ND 5343	01/12/2020	ICU-K	1955/M	117	17	AMP-GEN-VAN-TEC	*hyl*	*vanA*	*aac(6′)-le-aph(2″)-la*	
ND 5266	27/11/2020	ICU-F	1958/M	117	17	AMP-GEN-VAN-TEC	*hyl*	*vanA*	*aac(6′)-le-aph(2″)-la*	
ND 5387	03/12/2020	H-K	1994/M	117	17	AMP-VAN-TEC	*esp,hyl*	*vanA*	*aac(6′)-le-aph(2″)-la*	
ND 5185	23/11/2020	ICU-K	1946/M	117	17	AMP-GEN-VAN-TEC		*vanA*	*aac(6′)-le-aph(2″)-la, aph(2″)-Ib, aph(2″)Id*	

Abbreviations: H—K, Department of Hematology, Križine; ICU-F, Intensive Care Unit Firule; ICU-K, Intensive Care Unit Križine; H—K, Department of Hematology, Križine; R, Clinic for Respiratory Disease; N—K, Department of Nephrology, Križine; AMP, ampicillin; GEN, gentamicin; VAN, vancomycin; TEC, teicoplanin.

[a] Isolation date is given as dd/mm/year.

[b] All GEN-resistant isolates displayed HLAR phenotype.

2.8. Multilocus sequence typing (MLST)

All VREfm isolates included in this study were characterized by MLST. Single PCR reactions were carried out using primers and conditions described by Homan et al. (2002) to amplify internal fragments of seven housekeeping genes (*atpA, ddl, gdh, purK, gyd, adk* and *pstS*). PCR products were purified and bidirectionally sequenced using the Sanger method in Macrogen Europe servis (Amsterdam, The Netherlands). The resulting sequences were alligned using Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) and compared with reference alleles available in the PubMLST database (https://pubmlst.org/organisms/enterococcus-faecium). Unique MLST allelic profiles were submitted to the database curator for assignment of a new sequence type (ST) and a clonal complex (CC).

2.9. Taxonomic and functional profiling of waterborne microbial community

Samples of genomic DNA were sent to Novogene Europe (Cambridge, UK) for 16S rRNA amplicon sequencing. As previously described (Kvesić et al., 2022), a hypervariable V3-V4 region of 470 bp was amplified using standard primers 341F and 806R and Phusion® High-Fidelity PCR Master Mix (New England Biolabs, UK). Libraries were prepared using the NEBNext® UltraTM DNA Library Prep Kit (Illumina, UK) and sequencing was performed on the NovaSeq platform (Illumina). Paired-end raw reads of 250 bp were merged using FLASH (V1.2.7) and clean tags were obtained after quality filtering (Caporaso et al., 2010) using the QIIME pipeline (V1.7.0). Sequences were analyzed using Uparse (v7.0.1001), and those showing an identity of ≥97 % were assigned to the same Operational Taxonomic Units (OTUs), which were then compared to the SILVA Database (Quast et al., 2013) using MUSCLE (White et al., 2009) to obtain a species annotation for each taxonomic rank (threshold: 0.8–1).

The PICRUSt2 tool (Douglas et al., 2020) was used to functionally annotate the 16S rRNA amplicon sequences in relation to antibiotic resistance mechanisms according to the KEGG Brite Antimicrobial Resistance Genes Database (KO01504). As described in our previous study (Šamanić et al., 2021), this approach was used to search for the hypothesized repertoire of antibiotic resistance genes by evaluating the variants and sets of genes associated with antibiotic resistance, including the β-lactam, aminoglycoside, tetracycline, macrolide, phenicol, fosfomycin, sulfonamide, trimethoprim, rifamycin, quinolone, and VAN resistance genes, as well as VAN, multidrug, and cationic antimicrobial peptide (CAMP) resistance modules. Heatmap was created to show the relationship of the most abundant pathogenic genera in microbiomes with resistance gene sets and gene variants using reads from trimmed OTUs table and Python 3.9 scripts. Graphical design was done using Inkscape (v1.0.1).

2.10. Data analysis

Relative abundance of bacteria at phylum, class, order, family, genus, and species ranks were calculated by dividing an absolute abundance with a total abundance of species. Alpha diversity indices (Observed-species, Chao1, Shannon, Simpson, ACE, Good-coverage), as well as beta diversity on weighted and unweighted UniFrac were calculated using QIIME software (v1.7.0). The principal coordinate analysis (PCoA) was done using the WGCNA, stat and ggplot2 packages in R software (v2.15.3). Analysis of similarities (ANOSIM) and multi-response permutation procedure (MRPP) analysis were performed by R software (Vegan package: anosim function, mrpp function) to evaluate the differences between and within the structure of bacterial communities. The linear discriminant analysis (LDA) effect size (LEfSe) algorithm was conducted (Segata et al., 2011) to determine the significantly different OTUs (threshold: LDA score [log10] > 2). Metastat was calculated by R software and *p*-value using a permutation test ($p < 0.05$ was statistically significant). Hierarchical clustering analysis and heatmaps were done in MATLAB R2020b.

3. Results

3.1. Environmental parameters

The physicochemical and microbiological parameters of the beach water and the sewage effluent-receiving seawater are shown in Table S1 of the Supplemental material. As expected, the highest concentrations of nutrients, as well as faecal indicator bacteria (FIB; ≥ 10^5 CFU/100 mL), were recorded at SW, depicting the untreated wastewater. Consequently, faecal pollution was detected in samples from the nearby public beach (BW), but FIB counts were within the values defined for the excellent quality of bathing waters according to the Croatian legislation (Regulation on the Quality of Marine Bathing Waters; OG 73/08).

3.2. Antibiotic susceptibility of hospital and environmental VREfm

A total of 35 waterborne VREfm isolates were recovered, including 4 from public beach (BW) and 31 from marine outfalls of treated (SB) and untreated sewage (SW) (Table 2). Fifteen hospital surveillance isolates were collected from patients at six wards of University Hospital Centre Split (Table 1). Apart from the uniform resistance to VAN, all isolates were resistant to AMP and TEC, whereas almost all environmental (34/35) and majority of hospital (9/15) isolates were GEN-resistant, displaying HLAR phenotype (Tables 1 and 2). Resistance to TGC and LZD was not observed.

3.3. Antibiotic resistance and virulence genes

The molecular determinants of antibiotic resistance and virulence detected in environmental and hospital VREfm isolates are summarized in Tables 1 and 2. The *vanA* gene was identified in all hospital and environmental isolates, while one hospital isolate additionally carried *vanC2/C3*. Regarding the aminoglycoside-resistance genes, *aac(6′)-Ie-aph(2′′)-Ia* was the most common among hospital isolates (14/15), while it was not detected in waterborne isolates. However, six hospital isolates additionally possessed *aph(2′′)-Ib* and *aph(2′′)-Id* genes, which were predominant in the environmental isolates (32/35) and were simultaneously present in 19 isolates. None of the isolates contained *aph(3′)-IIIa* or *ant(4′)-Ia* genes.

Regarding the virulence factors, *hyl* gene, encoding the hyaluronidase, was the most prevalent, being detected in all waterborne and majority of hospital (11/15) VREfm isolates. *Esp* gene, coding for enterococcal surface protein (Esp), was detected in four hospital isolates.

3.4. MLST profiles

MLST analysis assigned the isolates to 10 STs, all belonging to the CC17 (Tables 1 and 2). ST1888, ST1889, and ST1890 were newly identified STs in this study and were detected only among isolates from effluent-receiving seawater (SW and SB). ST177 and ST889 were the most common and were associated with hospital and environmental VREfm, regardless of their origin. Isolates from beach water and untreated wastewater discharge were mostly associated with these two STs. Overall, the majority of hospital isolates (9/15) and nearly half (15/35) of environmental isolates, including 14 from wastewater discharges and 1 from the beach belonged to ST117, while 13 isolates (9 from wastewater discharges, 2 from the beach, and 2 hospital isolates) each belonged to ST889.

3.5. Composition of bacterial community at marine sewage outfalls and public beach

A total of 1,060,185 filtered reads were generated on the Illumina platform. After quality filtering, 852,545 effective tags with a medium length of 412 bp were clustered into 11,685 OTUs. An average of 1298 OTUs were identified per sample, with 1486, 1568, and 842 OTUs found in BW, SW, and SB samples, respectively. Of these, 285 OTUs were found to be common to all three samples (Fig. S2). The largest overlap was detected between the SW and BW samples, which shared a total of 127 OTUs.

Table 2
General information and characteristics of virulence and antibiotic resistance of environmental VREfm isolates collected in this study.

PubMLST isolate id	Isolate no.	Origin	Sampling date[a]	ST[b]	CC	Resistance phenotype[c]	Virulence genes	VAN-resistance genes	Aminoglycoside-resistance genes
4158	X1	SW	06/07/2020	889	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4333	X2	SW	06/07/2020	889	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4334	X3	SW	06/07/2020	117	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4335	X4	SW	06/07/2020	889	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib*
4336	X5	SW	06/07/2020	117	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4337	X6	SW	06/07/2020	117	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4338	X7	SW	06/07/2020	117	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4339	X8	SW	06/07/2020	117	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4340	X9	SW	06/07/2020	889	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4341	X10	SW	06/07/2020	117	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4342	X11	BW	28/07/2020	117	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4343	X12	BW	28/07/2020	889	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4344	X13	BW	28/07/2020	1385	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4159	X14	SW	28/07/2020	1888	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Id*
4345	X15	SW	28/07/2020	117	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4346	X16	SW	28/07/2020	1888	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Id*
4347	X17	SW	28/07/2020	117	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Id*
4348	X18	SW	28/07/2020	117	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4349	X19	SW	28/07/2020	117	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Id*
4350	X20	SW	28/07/2020	117	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4351	X21	SW	28/07/2020	117	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	
4352	X22	SW	28/07/2020	889	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib*
4353	X23	SW	28/07/2020	889	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4354	X24	SW	28/07/2020	889	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4355	X25	SW	28/07/2020	889	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4160	X26	SW	28/07/2020	1889	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Id*
4356	X33	BW	28/07/2020	889	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Ib, aph(2″)-Id*
4357	X51	SW	28/07/2020	117	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Id*
4161	C3	SB	22/07/2020	1890	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Id*
4173	C4	SB	22/07/2020	889	17	AMP-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Id*
4174	C5	SB	22/07/2020	1890	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Id*
4175	C6	SB	22/07/2020	1890	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Id*
4176	C7	SB	22/07/2020	1890	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	*aph(2″)-Id*
4162	R9	SB	21/09/2020	1890	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	
4177	R10	SB	21/09/2020	117	17	AMP-GEN-TEC-VAN	*hyl*	*vanA*	

Abbreviations: BW, beach water; SW, untreated sewage water from outlet near the beach; SB, treated sewage water from submarine outfall; AMP, ampicillin; GEN, gentamicin; VAN, vancomycin; TEC, teicoplanin.

[a] Sampling date is given as dd/mm/year.

[b] Underlined numbers indicate new STs reported in this study.

[c] All GEN-resistant isolates displayed high-level aminoglycoside-resistant (HLAR) phenotype.

A total of 33 bacterial phyla were identified in the metagenomes of the analyzed water samples. The highest number of phyla was observed in SB (n = 28), while 20 were identified in BW and SW. *Firmicutes* was the most abundant phylum at the submarine outfall of the WWTP effluent SB (45.6 %), while *Proteobacteria* prevailed in the untreated wastewater (SW) and beach water (BW) adjacent to the untreated wastewater outlet (Fig. 1). A comparison of microbial diversity at the class level was more conclusive and showed a distinctive pattern for each microbiome (Fig. 1). A total of 57 bacterial classes were identified in this study. *Alphaproteobacteria* was the most abundant class in BW (47.6 %), followed by *Gammaproteobacteria* and *Bacteroidia.* SW microbiome was mainly composed of *Gammaproteobacteria* (32.84 %), *Campylobacteria* and *Clostridia*, while *Clostridia* (40.1 %) predominated in SB microbial community and was followed by the classes *Alphaproteobacteria* and *Gammaproteobacteria*, respectively.

The differences in the relative abundance of the ten most common bacterial orders, families, and genera in the samples are shown in Fig. S3. *Clostridiales* (48.1 %) was the most abundant order in SB microbiome from the submarine outfall and consisted mainly of the bacteria of the family *Lachnospiraceae* (34.5 %), among which the genera *Agathobacter, Blauthia* and *Fusicatenibacter* predominated. On the other hand, *Campylobacterales* (23.1 %) were the most abundant order in the microbiome of untreated wastewater SW, mainly due to the prevalence of the genus *Arcobacter* (22.7 %) and the family *Arcobacteraceae. Rhodobacteraceae* (39.3 %) from the order *Rhodobacterales* was the most abundant family in beach water microbiome BW, with *Ascidiaceihabitans* and *Nereida* being the most abundant bacterial genera (9.5 and 8.2 %), respectively. The relative abundances of bacteria from genus to class level in nine analyzed microbiomes can be found in Table S2 in the Supplementary Material. In addition, a more detailed inspection of the 35 most abundant genera in microbiomes is presented in Fig. S4. Comparison between BW and SW microbial communities revealed an impact of the faecal pollution on beach water and clustered these two microbiomes together, although the microbiological parameters for the beach water corresponded to the excellent quality according to the Croatian legislation (Regulation on the Quality of Marine Bathing Waters; OG 73/08). Notably, some taxa typical for faecal material, like enterococci, were present at the similar abundance in both SW (avg 0.05 %) and BW (avg 0.03 %). This is also evident for bacteria from the genera *Arcobacter* (avg 22.7 % in SW and 3.3 % in BW) and *Acinetobacter* (avg 6.3 % in SW and 1.2 % in BW), respectively. On the other hand, most abundant genera in SB, among which *Agathobacter* and *Bifidobacterium* (avg 9.7 %, and 2.9 %) were far less common in BW and SW (Fig. S4, Table S2).

Overall, the bacterial community from untreated sewage water (SW) showed higher richness and diversity compared to that from other sites and was significantly different ($p < 0.05$; Wilcox test) from SB (Table S3; Fig. S5). This was somewhat expected as the wastewater treatment affected the microbiome structure in submarine effluent.

3.6. Spatial variations of microbiome composition

Beta diversity of the bacterial communities was assessed using Bray–Curtis distance and visualized by the PCoA biplot (Fig. 2). The samples of the same origin clustered tightly, except for the sample obtained near the submarine effluent in September (SB.9), which was composed of less

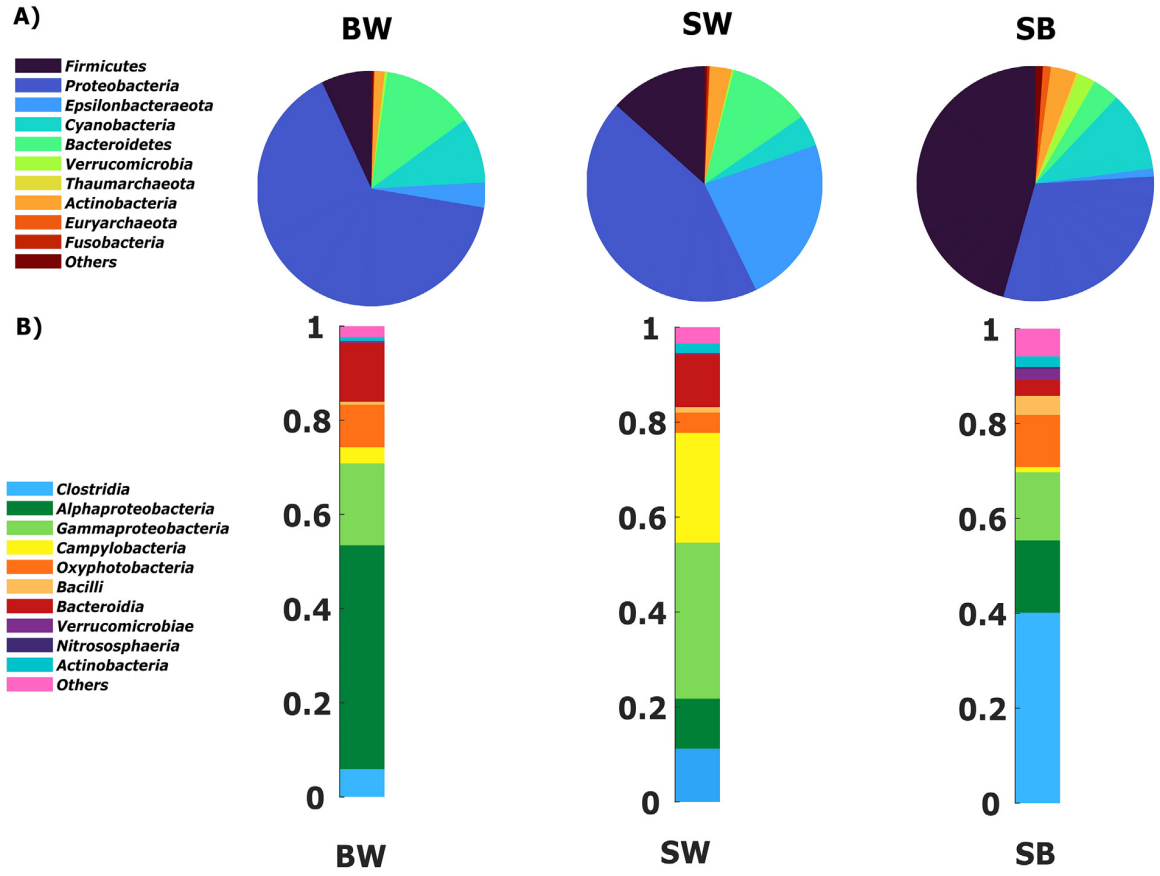

Fig. 1. Relative abundance of the ten most common bacterial phyla (A) and classes (B) in water samples from marine outfalls of treated (SB) and untreated (SW) wastewater and nearby public beach (BW).

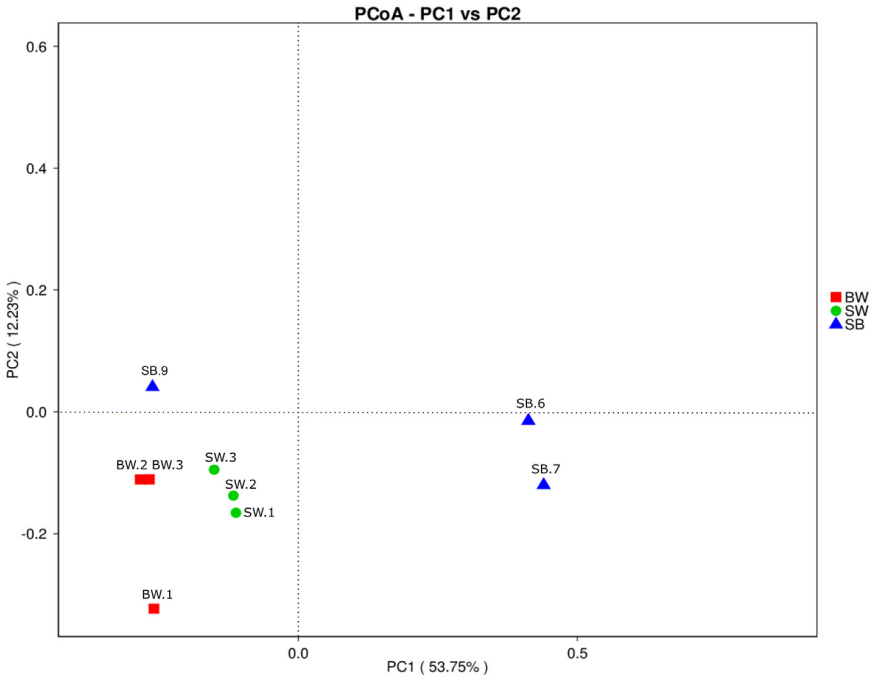

Fig. 2. Beta diversity of the analyzed microbiomes at studied sites. PCoA biplots were constructed applying the weighted Unifrac distance matrix on the CSS normalized OTU table data.

Clostridia (only 7.3 %) but was enriched with *Alphaproteobacteria* (28.3 %), *Gammaproteobacteria* (21.7 %) and *Oxyphotobacteria* (30.8 %) (Table S2). Overall, BW and SW microbiomes plotted more closely, segregating over axis 1 when compared to the SB, and pointing again to the contamination of the beach water with raw sewage material which was probably facilitated by the proximity of the outfall and the public beach. Of note, both microbial communities were enriched with the phyla *Proteobacteria* and *Bacteroidetes* (Fig. 1). On the other hand, structure differences were confirmed to be statistically significant ($p < 0.05$, Wilcox test) in the case of SW and SB by the weighted UniFrac distance boxplot of beta diversity indices (Fig. S6) and MRPP test (Table S4).

Moreover, LEfSe analysis identified which taxa were differentially abundant when comparing the three waterborne microbiomes mutually (Fig. 3), as well as between the more similar SW and BW (Fig. S7), and the more distant SW and SB microbiomes (Fig. S8). A number of bacteria were pointed as biomarkers discriminative for SW in contrast to SB and BW, among which those from the phyla *Campylobacterota* (f_*Arcobacteraceae,* g_*Arcobacter),* *Proteobacteria* (o_*Aeromonadales,* g_*Comamonas*), *Bacteroidetes* (species *B. graminisolvens* and *B. vulgatus,* g_*Macellibacteroides*) and *Firmicutes* (genera *Coprococcus* and *Dialister*) (Fig. 3). However, when comparing SW to SB solely, only the genus *Arcobacter* from the previous list remained, and was further accompanied with genera *Acinetobacter* (mostly *A. johnsonii*), *Prevotella* and *Rhodopherax* (Fig. S8). Moreover, fewer taxa were characteristic to SB microbiome in comparison to SW and BW; most important include the genus *Salmonella,* followed by the genera *Cutibacterium* and *Gordonibacter* of the phylum *Actinobacteria,* and genus *Butyricicoccus* from the phylum *Firmicutes* (Fig. 3). Of note, only *Salmonella* was significantly more abundant in SB when compared to SW (LDA score [log10] > 3) (Fig. S8). On the other hand, BW biomarkers mostly included fastidious or unculturable marine bacteria of the class *Alphaproteobacteria,* such as the genera *Nereida* and *Marinibacterium,* as well as the families *Cryomorphaceae* and particularly *Flavobacteriaceae* that encompasses genera *Polaribacter, Flavobacterium* and *Ulvibacter* (Fig. 3).

3.7. Presence of bacterial genera involved in pathogenesis

Of the 572 bacterial genera identified in the water samples analyzed in this study, 60 are associated with human infections. As expected, their relative abundance was highest in the SW microbiome (42.2 %) and further decreased to 17.8 % in SB and 9.3 % in BW (Table S3). *Arcobacter, Acinetobacter, Escherichia-Shigella, Salmonella, Bacteroides, Paenibacillus, Bifidobacterium,* and *Prevotella* were the most common, presented by >2 % in at least one of the analyzed microbiomes (Fig. 4). SW was particularly abundant with bacteria of the genera *Arcobacter* (22.7 %) and *Acinetobacter* (3.3 %). Consequently, the microbial community at the adjacent public beach (BW) was also enriched in these taxa (6.3 % and 1.1 %). Enterococci accounted for 0.05 % in SW and 0.03 % in BW and SB microbiomes.

3.8. Resistome prediction

Using the PICRUSt2 tool, we were able to infer the repertoire of ARGs and modules responsible for resistance to VAN, β-lactams, tetracyclines, macrolides, phenicols, aminoglycosides, sulfonamides, and quinolones, as well as multiple resistance in each microbiome (Fig. 5; Table S5). As expected, the highest absolute abundance of antibiotic resistance markers was identified in the SW community which originated from the untreated wastewater. However, we found that in all three waterborne communities, the most abundant genes were those encoding multidrug resistance efflux systems, particularly MexAB-OprM (max 3446.4 in SW, avg. 2639.4), MexJK-OprM (max 1421.07 in SW, avg. 1322), and MexXY-OprM (max 757.1 in SW, avg. 842), followed by AdeABC, AcrEF-TolC, and MdtEF-TolC (mostly in SW, avg. 823 to 782.6). Nevertheless, it should be noted that these pumps were significantly more present in SW compared with BW and SB ($p < 0.05$ or less). The same refers to beta-lactam resistance markers, including the genes encoding class A, C, and D beta-lactamases and those involved in the repression of OprD porin, which mediates resistance to imipenem. Interestingly, microbial communities were also found to be enriched in genes associated with resistance to CAMP, such as the *dltABCD* operon (avg abundance of 343.3 in SB, 432.8 in BW and 2488.4 in SW), but these were still significantly ($p < 0.05$) more abundant in SW than in other microbiomes.

On the other hand, SB microbiome that originated from the WWTP submarine effluent, was more enriched in the genes responsible for glycopeptide (VAN and TEC) resistance (*vanA, vanB, vanD* and *vanM* resistance modules), multidrug resistance (AbcA efflux pump) and tetracycline resistance (Tet38 efflux pump) in Gram-positive bacteria, as

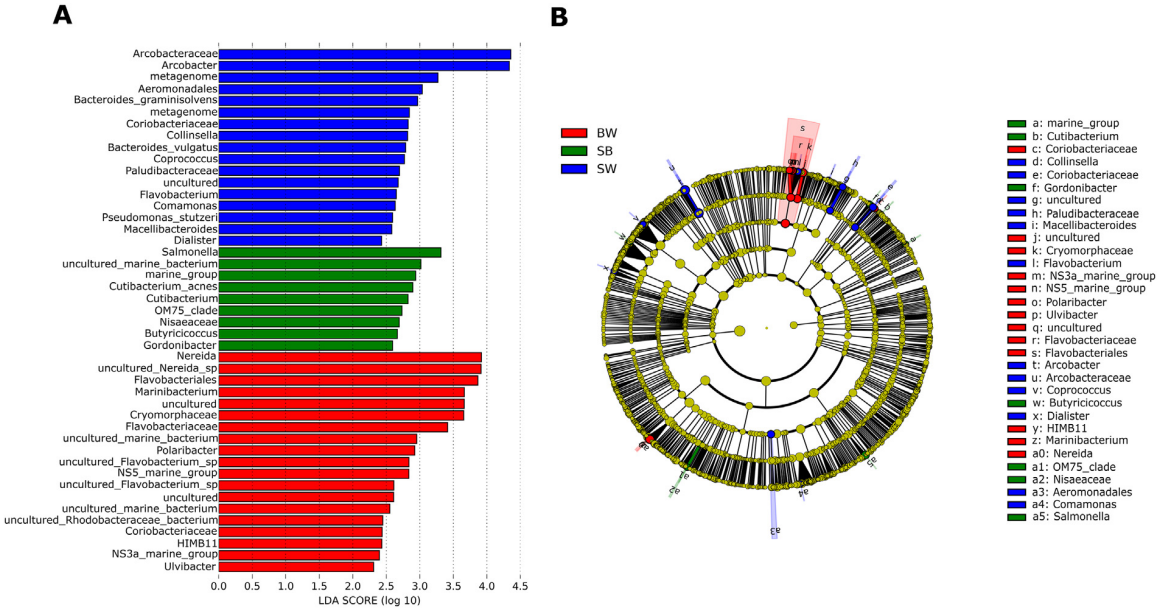

Fig. 3. Histogram of the LDA scores (A) and cladogram (B) showing the phylogenetic distribution of differentially enriched taxa in the BW (red), SB (green) and SW (blue) microbiomes with LDA values of 2.0 or higher based on LEfSe analysis. The diameter of each circle is proportional to a taxon's abundance. Circles from inner region to outer region represent the phylogenetic levels from class to genus. Yellow represents insignificant difference.

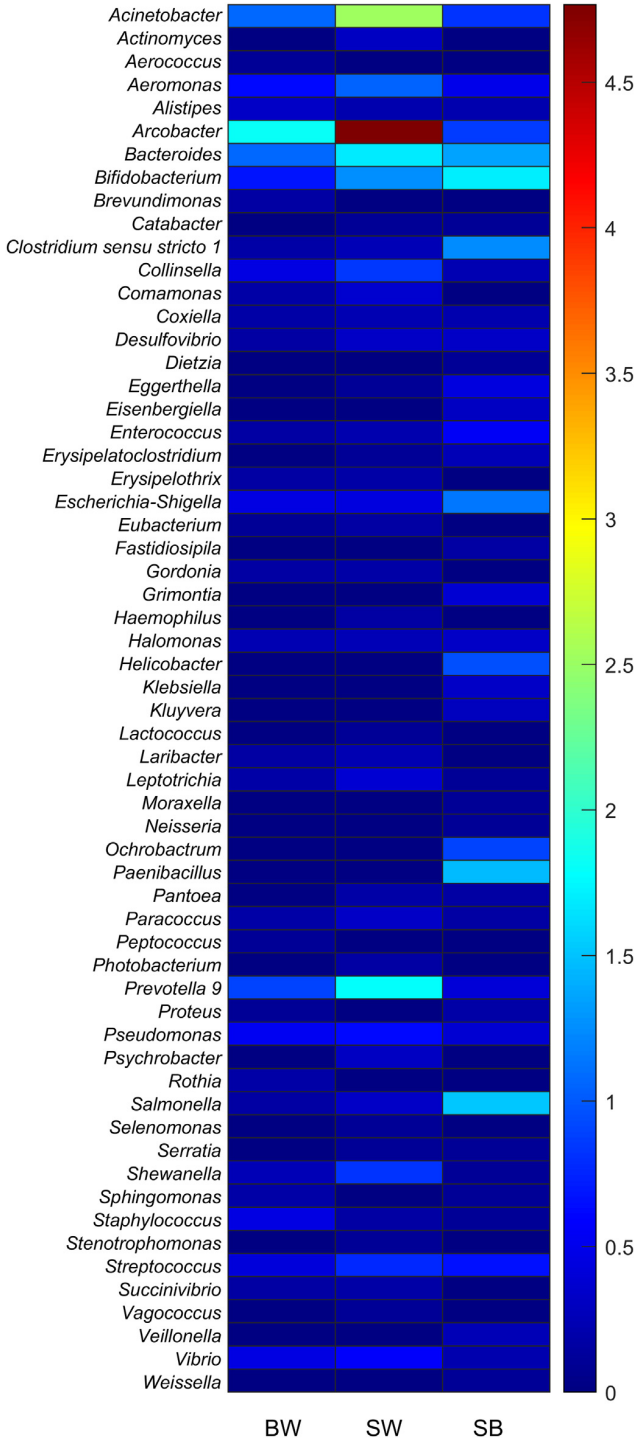

Fig. 4. Heatmap depicting relative abundance of 62 pathogenic bacterial genera in water samples collected near the submarine WWTP outfall (SB), the untreated sewage discharge (SW), and the adjacent public beach (BW). Abundances are expressed as square roots of the percentage values.

well as the aminoglycoside resistance genes encoding for a variety of *N*-acetyltransferases (Fig. 5; Table S5).

Furthermore, Fig. 6 and Table S6 show more specifically how the most common pathogenic genera are associated with the aforementioned ARGs and modules in each microbiome. For instance, genus *Acinetobacter* greatly contributed to aminoglycoside, macrolide, phenicol, quinolone, β-lactam and particularly, the multidrug resistance in SW and BW microbiomes. On the other hand, *Arcobacter* followed by a number of genera, including *Bacteroides, Escherichia-Shigella, Aromonas* and *Pseudomonas* were found to most often contribute to multidrug resistance in analyzed microbiomes, regardless of their origin. Taken together, *Acinetobacter, Bacteroides* and *Aeromonas* are the genera that contributed to the resistance to the most of antibiotic classes in analyzed bacterial communities (Fig. 6).

4. Discussion

The aim of this study was to determine, for the first time and extensively, the molecular characteristics of antibiotic resistance and virulence of VREfm persisting in hospital and coastal marine environments in Croatia. VREfm were collected from public beach water, a WWTP submarine outfall at 37 m depth, and from offshore discharge of untreated wastewater, and compared with the surveillance VREfm from the regional university hospital to assess the epidemiological hazard for their transmission and routes of introduction into the natural environment. This study is of particular importance as it provides the first data on antibiotic resistance determinants and ST affiliations of clinical VREfm isolates from Croatia. In addition, taxonomic and antibiotic resistance profiling of microbial communities was performed to learn more about the diversity and pathogenic potential of the accompanying taxons in sewage and beach water matrices.

4.1. Characteristics of the hospital and environmental VREfm analyzed in this study

Importantly, there is a lack of studies that have comprehensively addressed this growing health problem in the marine environment and in the clinic, as authors have mainly focused on the detection of VRE either in seawater (Roberts et al., 2009; de Oliveira and Pinhata, 2008; Alipour et al., 2014; Monticelli et al., 2019) or wastewater (Ekwanzala et al., 2020; Gotkowska-Płachta, 2021) or in hospitals (Freitas et al., 2016; Jakovac et al., 2017; Said and Abdelmegeed, 2019; Fioriti et al., 2020). More importantly, only a few studies have identified the *van* genotype of marine VREfm isolates, which is relevant from an epidemiological perspective. The *vanA* or *vanB* genes have been identified in VREfm from seawater from public beaches in the United States (Roberts et al., 2009) and in VRE from beach water in Puerto Rico (Santiago-Rodriguez et al., 2013), but the isolates have not been further molecularly characterized or compared with hospital strains.

The *vanA* gene was uniformly detected in all environmental and hospital isolates from this study. The predominance of *vanA*-mediated resistance in VREfm is consistent with studies from several other countries (Freitas et al., 2016; Fioriti et al., 2020). This gene was identified in a surveillance isolate from a hematologic patient at the same university hospital (Goić-Barišić et al., 2020), indicating the spread of this resistance genotype in southern Croatia in recent years. Jakovac et al. (2017) found that *vanB*-mediated resistance predominated in clinical VREfm isolates from Croatia, followed by *vanA*; however, this study did not include isolates from the Split University Hospital. Moreover, the aminoglycoside resistance gene *aac(6′)-Ie-aph(2″)-Ia* was prevalent among hospital isolates from this study, which is consistent with reports from Italy (Zarrilli et al., 2005), China (Li et al., 2015), and India (Padmasini et al., 2014). However, this gene was not detected in the waterborne isolates from this study. Interestingly, the hospital isolate ND80, which had the non-HLAR phenotype, carried the *aac(6′)-Ie-aph(2″)-Ia.* This could be related to the genetic environment of this gene, as in the case of the insertion of the *IS1216V* sequence into the 5′-end of *aac(6′)-Ie-aph(2″)-Ia*, which resulted in the loss of GEN resistance in hospital *E. faecium* from Taiwan (Chen et al., 2021). Nevertheless, this should be carefully investigated in future studies.

Importantly, six hospital *vanA*-positive VREfm isolates, affiliated to ST117, additionally harboured the aminoglycoside resistance genes *aph(2″)-Ib* and *aph(2″)-Id*, which were found predominantly in waterborne isolates, including the 13 isolates (1 from beach water and 12 from untreated

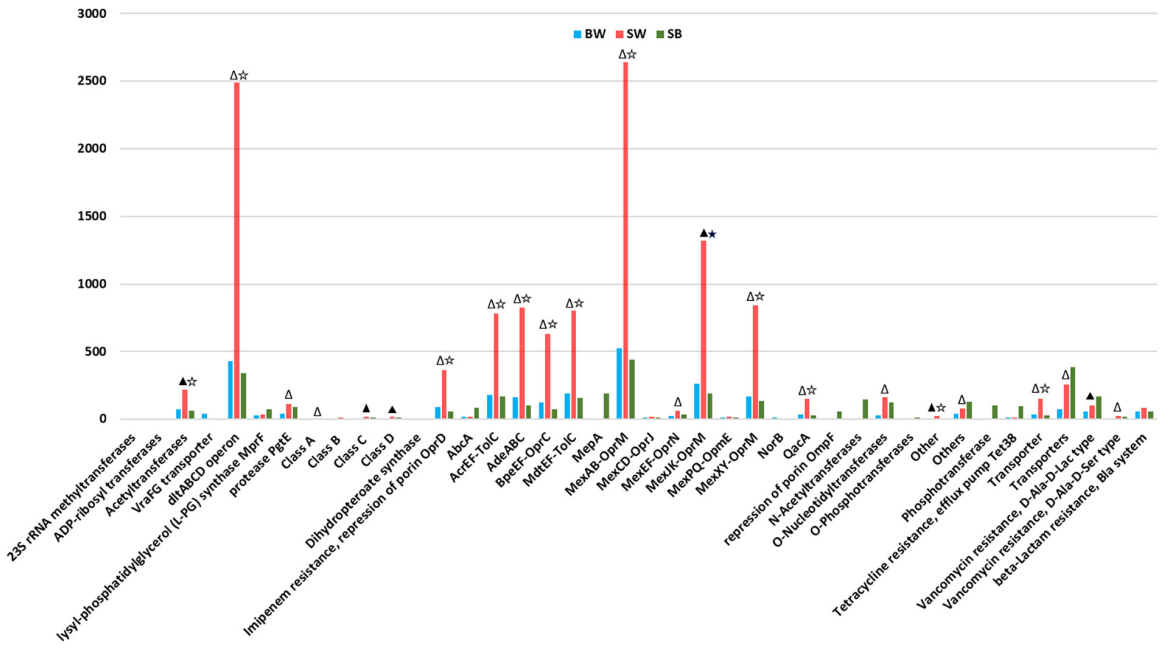

Fig. 5. PICRUSt2-predicted ARGs content associated with the microbiomes originating from the submarine WWTP outfall (SB), the untreated sewage discharge (SW), and the adjacent public beach (BW), represented as the average values of the absolute abundances of sets and variants of ARGs from KEGG Brite Antimicrobial Resistance Genes database. Using Student's two-sample *t*-test significant increasement of abundance is denoted as follows: Δ for $p < 0.05$ and ▲ for $p < 0.005$ between SW and BW microbiomes; ☆ for $p < 0.05$ and ★ for $p < 0.005$ when comparing SW and SB microbial communities.

sewage effluent) of this ST group. These waterborne and clinical isolates also carried the *hyl* virulence gene, which further points to their relatedness. However, the strain clonality should be discarded because the hospital isolates also carried the *aac(6′)-Ie-aph(2″)-Ia* gene. Nevertheless, the fact that the majority (9/15) of hospital surveillance isolates from this study belonged to this ST and that this lineage was also the most prevalent among waterborne isolates (15/35) strongly suggests that the ST117 lineage is of particular importance for the spread of VREfm in Croatia. In addition, the hospital and waterborne ST117 isolates shared the same virulence and similar antibiotic resistance features, suggesting possible undetected circulation between the hospital and the natural environment. The *vanA*-type ST117 VREfm are globally disseminated (https://pubmlst.org/organisms/enterococcus-faecium) and are frequently detected in European hospitals both as colonizing isolates and as causative agents of nosocomial outbreaks (Freitas et al., 2016; Tedim et al., 2017). Fioriti et al. (2020) found that *vanA*-type VREfm predominates in Italian hospitals and these mainly belong to ST78, ST80, and ST117. To our knowledge, ST117 VREfm has not been previously identified in the marine environment. Also, this study represents the first detection of ST117 VREfm in Croatian hospitals.

Moreover, 22 environmental and 2 hospital isolates from this study showed association with ST889. It appears that the *vanA-aph(2″)-Ib-aph (2″)-Id* resistance genotype is common to this lineage; however, two hospital strains additionally carried the *aac(6′)-Ie-aph(2″)-Ia* and *vanC2/C3* genes. Importantly, the latter has never been detected in Croatian hospitals (Jakovac et al., 2017). Overall, the *vanA*-type ST889 VREfm have been identified less frequently, for instance, in rectal swab specimens of immunosuppressed patients in Germany (Both et al., 2022). On the other hand, two hospital isolates from this study belonged to the ST78 and ST192, which are among the most common high-risk clones of hospital-associated VREfm worldwide (Fioriti et al., 2020; Freitas et al., 2021). These lineages have already been reported in neighbouring countries such as Italy, Hungary, and Austria (Fioriti et al., 2020; https://pubmlst.org/organisms/enterococcus-faecium), but this is the first evidence of their silent dissemination in the hospital setting in Croatia. The non-HLAR phenotype of these isolates may have contributed to their scarce report.

4.2. Diversity and antibiotic resistance markers of bacterial communities at marine wastewater outfalls and public beach

We performed an extensive bioinformatic analysis of the data obtained by 16S rRNA amplicon sequencing and profiled the microbial taxonomy structure and resistome of the water samples from which VREfm were

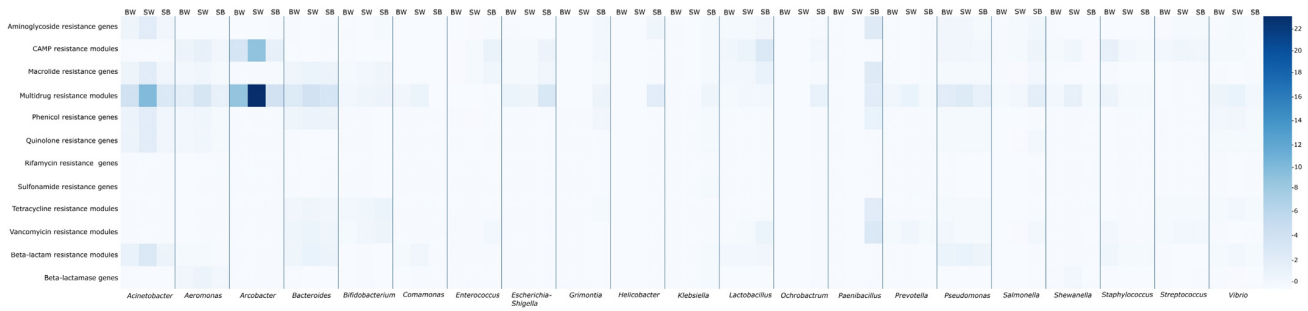

Fig. 6. Heatmap showing the relative abundance of 21 most common pathogenic bacterial genera regarding their annotation in the KEGG Brite Antimicrobial Resistance Genes database. Values are expressed as square roots of the percentage values.

isolated to gain insight into their full repertoire of pathogenic genera and antibiotic resistance markers.

As expected, the wastewater microbiomes were rich in phyla typical of human gastrointestinal tract, such as *Firmicutes, Proteobacteria,* and *Epsilonbacteraeota* (*Campylobacterota*), with the predominance of the classes *Clostridia, Alpha-, Gammaproteobacteria* and *Campylobacteria*, reflecting the composition of the human gut microbiota (Wang et al., 2022). Consequently, these taxa represent major components of WWTPs effluents (Narciso-da-Rocha et al., 2018) as well as the receiving coastal water (Zheng et al., 2019; Kvesić et al., 2022). Nevertheless, an apparent difference between the untreated and treated wastewater-derived microbiomes from this study can be seen even by the relative abundance of the most represented phyla, with *Firmicutes* being predominant in SB (45.6 %) and *Proteobacteria* in SW (43.8 %) (Fig. 1), which was also corroborated by the LEfSE analysis discerning a list of highly abundant taxa (Fig. S8). Interestingly, although *Firmicutes* prevailed in the SB microbiome, *Salmonella* of the phylum *Proteobacteria* was pointed as SB microbial marker in comparison to SW. Notably, it was shown previously that *Salmonella* spp. can survive wastewater treatments (Sahlstrom et al., 2006), even with a higher ability than *E. coli* (Wery et al., 2008). Giving that SB sample undergone only the primary wastewater treatment, our results corroborate previous studies (Espigares et al., 2006; El Boulani et al., 2017), highlighting the importance of efficient tertiary treatment of wastewater for the adequate removal of these enteric pathogens (Koivunen et al., 2003).

On the other hand, bacterial genera *Dialister* and *Coprococcus* from *Firmicutes,* which are relevant for a healthy human gut microbiome (Valles-Colomer et al., 2019), were pointed as discriminative to SW in contrast to SB, while *Arcobacter* of the phyla *Campylobacterota* (*Epsilonbacterota*) was found to be the most relevant microbial marker of SW and was followed by *Acinetobacter* (mostly *A. johnsonii*) (Fig. S8). The genus *Arcobacter* comprises diverse environmental and enteric bacteria, which are getting more scientific attention as some species are recognised as antibiotic-resistant human opportunistic pathogens (Shrestha et al., 2022) that can be found in a variety of different habitats, particularly wastewaters (Kvesić et al., 2022; Shrestha et al., 2022). Similarly, bacteria from the genus *Acinetobacter* were also commonly detected in wastewaters, including the treated WWTP effluent (Kvesić et al., 2022). *A. johnsonii* is known to cause a range of opportunistic infections and has been isolated from various environments, including wastewater (Liu et al., 2022). Notably, this bacterium was the predominant *Acinetobacter* species in faecal samples from healthy individuals in the Netherlands (Dijkshoorn et al., 2005).

It is particularly worrisome, however, that we detected negative shifts in the diversity of beach water microbiome and associated resistome due to the wastewater leakage from the adjacent outfall, even though the beach water was of excellent quality based on microbiological parameters and legislation currently in force in Croatia. Notably, several analyses carried out in this study, among which OTUs diversity represented by Venn diagrams, PcoA and clustering of the relative abundance of the most abundant genera in samples, evidenced the similarity between the SW and BW microbiomes in comparison to SB. PCoA of beta diversity among the bacterial communities plotted SW and BW more closely (Fig. 2), while beta diversity indices were found to be statistically significant in the case of SW and SB by weighted UniFrac distance (Fig. S6). Moreover, the heatmap of the most abundant pathogenic genera in the samples (Fig. 4) clearly depicted that the abundance of *Arcobacter, Acinetobacter, Bacteroides, Prevotella, Pseudomonas* and *Streptococcus* in BW microbiome was a direct consequence of the wastewater inflow to the analyzed beach water. Consequently, these genera, particularly *Arcobacter, Acinetobacter, Bacteroides* and *Pseudomonas* substantially contributed to the BW resistome, regarding the resistance to specific antibiotic classes as well as the multiple classes. Indeed, *Arcobacter* spp. are found to be resistant to various classes of antibiotics, including penicillins, cephalosporins, macrolides, fluoroquinolones, aminoglycosides, and tetracyclines (Shrestha et al., 2022). This is in concordance with our results, as this genus is the main ARG host of multidrug resistance in both, SW and BW, microbiomes (Fig. 6). Moreover, the second most abundant pathogenic genus in these samples, *Acinetobacter* (Fig. 4), was also the second major ARG host in these microbiomes and was predominantly associated with the multidrug resistance (Fig. 6). This is consistent with results from similar studies (Kvesić et al., 2022; Liu et al., 2022), as it is known that a resistance-nodulation-cell division (RND) efflux pump AdeABC expels various drug classes out of the bacterial cell, such as aminoglicosides, tetracyclines, phenicols, fluoroquinolones, and some beta-lactams, leading to the multidrug resistance phenotype (Bonomo and Szabo, 2006). Several other chromosomally encoded RND efflux systems were also found to be more abundant in SW and BW microbiomes than in SB, particularly MexAB-OprM, MexJK-OprM and MexXY-OprM whose overexpression in *Pseudomonas aeruginosa* results by nonsusceptibility to multiple classes of antibiotics (Dey et al., 2020). This was also a case for AcrEF-TolC pump, which is one of the main instruments of intrinsic resistance in *E. coli* (Chetri et al., 2018) as well as MdtEF-TolC (also known as YhiUV)-TolC), whose increased activity in *E. coli* leads to active export of cationic biocides, including glycopeptide antibiotics (Novoa and Conroy-Ben, 2019).

Moreover, it is interesting to note that the beach water microbiome was more enriched in VraFG transporter genes, which are involved in the formation of a five-component system that mediates resistance of *Staphylococcus aureus* to CAMP (Falord et al., 2012). Indeed, the BW microbiome had more OTUs related to *Staphylococcus*, which could be explained by the fact that the beach water samples were collected in July, and the staphylococci, as a skin commensals, could have originated from the bathers.

On the other hand, we found that the SB microbiome was more enriched with VAN and TEC resistance genes, among which *vanA* and *vanB* ARGs. The presence of enterococci, even VRE, and associated genes, in WWTP effluent in a higher abundance than in untreated sewage has been previously reported. Namely, several studies evidenced that the wastewater treatment may even contribute to the enrichment of VRE and *vanA* (Rosenberg Goldstein et al., 2014; Makowska et al., 2021), while VRE have been detected at various stages of the wastewater treatment process (Araujo et al., 2010), suggesting that the discharge of wastewater may be partially responsible for the spread of VRE into the environment and further into the human community (Guardabassi et al., 2004; Caplin et al., 2008).

5. Conclusions

This paper reports the first comprehensive study of the high-priority pathogen VREfm recovered from the hospital and coastal marine environment, focusing on the antibiotic resistance, virulence, and ST affiliation to depict the diversity of STs and routes of introduction of high-risk clones into the natural environment. We also present the first data on the diversity of VREfm STs in Croatia. The *vanA* genotype predominated among hospital and waterborne isolates from this study, highlighting the relevance of ST117 and ST889 lineages in the circulation of hospital and environmental VREfm isolates of the same virulence, and similar multidrug resistance traits. Offshore discharges of untreated municipal sewage, as well as the submarine WWTP outfalls, are evidenced as continuous routes for the introduction of several major healthcare-associated STs of VREfm as well as other pathogenic and antibiotic-resilient bacterial populations in coastal marine environment, including the public beach waters, creating a serious hazard for opportunistic infections and an objective risk for the further dissemination of strains and associated resistance genes in the natural environment.

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CRediT authorship contribution statement

Mia Dželalija: Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Marija Kvesić:** Investigation, Formal analysis, Visualization, Writing – review & editing. **Anita Novak:** Investigation, Validation, Writing – review & editing. **Željana Fredotović:** Investigation, Writing – review & editing. **Hrvoje Kalinić:** Formal analysis, Data curation, Writing – review & editing. **Ivica Šamanić:** Validation, Writing – review & editing. **Marin Ordulj:** Investigation, Writing – review & editing. **Slaven Jozić:** Investigation, Writing – review & editing. **Ivana Goić Barišić:** Validation, Writing – review & editing. **Marija Tonkić:** Validation, Writing – review & editing. **Ana Maravić:** Conceptualization, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing.

Data availability

The raw data from 16S rRNA amplicon sequencing of SW (SW1–3) and BW (BW1-3) samples obtained in this study were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under Bioproject no. PRJEB48800. The following 16S rRNA sequences of the SB samples, deposited in ENA under Bioproject no. PRJEB45742 by Kvesić et al. (2022), were retrieved and used for bioinformatics: SB6 (accession no. ERS6603494), SB7 (ERS6603495) and SB9 (ERS6603496).

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159720.

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2.2 Marine resistome of a temperate zone: Distribution, diversity, and driving factors across the trophic gradient

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Marine resistome of a temperate zone: Distribution, diversity, and driving factors across the trophic gradient

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ABSTRACT

Marine and ocean environments are the most widespread habitats in the world but are still the least studied from the aspect of antibiotic resistance. The indigenous and tetracycline (TET)- and sulfamethoxazole (SXT)-resistant planktonic bacterial communities were simultaneously investigated for the first time along a trophic gradient of a temperate zone, regarding their taxonomic and functional structures as well as biotic and abiotic factors affecting their dynamics as vehicles of antibiotic resistance genes (ARGs), thus impacting the ARGs distribution at seasonal and spatial scales.

A total of 80 microbiomes, recovered seasonally from bottom layer and surface waters along a 68-km transect from wastewater-impacted estuary to coastal and pristine open sea in the central Adriatic (Mediterranean Sea), were analysed using 16S rRNA amplicon sequencing, PICRUSt2 bioinformatics and extensive biostatistics. Eighty-one bacterial phyla were identified, with majority ($n = 49$) in summer when communities were found to be more species enriched across the gradient. Microbial diversity was more site-specific and pronounced in surface microbiomes in winter. Nevertheless, both richness and community diversity decreased with distance from the coast. Although the microbiomes from human-influenced sites significantly differed from those in oligotrophic offshore area, *Proteobacteria* were still the most abundant phylum during both seasons at the surface and seabed along the gradient, and the major contributors to the marine resistome regarding native and TET- and SXT-resistant microbial communities. Resistome structure was more diverse in winter, whereas peptide, vancomycin and multidrug resistance modules predominated regardless of season, trophic status, or antibiotic. However, multidrug, beta-lactam resistance modules as well as macrolide, phenicol, aminoglycoside, and particularly imipenem resistance genes were much more frequent in winter, suggesting that the diversity of indigenous resistomes is highly dependent on seasonal variations of the water column, driven by thermohaline stratification and nutrients. Moreover, several pathogenic genera stood out as important carriers of multiple resistance traits in TET- and SXT-related resistomes in both seasons, particularly *Acinetobacter, Vibrio, Bacillus* and *Pseudomonas*, beside which *Proteus, Serratia* and *Bacteroides* prevailed in native resistomes.

This study evidenced seasonal and spatial variations of the marine microbiome and resistome and their driving forces along the trophic gradient, providing a comprehensive insight into the diversity and distribution of antibiotic resistance in the marine ecosystem of a temperate zone.

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1. Introduction

Coastal areas, as the intersection of marine and terrestrial environments, are considered the most diverse and complex ecosystems while being the most densely populated areas in the world at the same time, underscoring their importance for studying the global phenomenon of antibiotic resistance. Antibiotic resistance genes (ARGs) are ancient in origin and predate the antibiotic era (D'Costa et al., 2011), but human activities (e.g., urbanization and discharge of pollutants) accelerated the evolution of novel variants and facilitated their dissemination in the natural environment, especially in coastal areas (Guillén-Chable et al., 2022; Mukhejri et al., 2022). Although the resistomes of the world's oceans and coastal areas are diverse (Cuadrat et al., 2020), some patterns were recognized. First, coastal microbiomes have been found to be significantly more enriched in ARGs than those of the deep ocean and Antarctica (Yang et al., 2019), which is related to the extent of anthropogenic influence as the ARGs abundance correlates with the pollution gradient (Chen et al., 2019). Second, the bacterial community has been shown to be a major determinant of the composition of ARGs in aquatic environments, even in the seldom human-impacted environments such as the High Arctic (Zhang et al., 2022a). Thus, we hypothesize that the certain geographical and seasonal-associated environmental factors will engage structural variations in the microbial communities, which will produce shifts in antibiotic resistome in the ecosystem. Moreover, we observed a distinct lack of comprehensive research to provide large-scale and in-depth analyses of the microbial community in relation to different trophic states and the available ARG repertoire in marine environment.

Led by this, we investigated the level of impact of biological and nonbiological factors affecting the native bacterial communities and, accordingly, shaping the tetracycline (TET)- and sulfamethoxazole (SXT)-resistant communities in the Adriatic Sea (Mediterranean), Croatia, over summer and winter period. The antibiotics were selected as essential first or second choice for empirical treatment of infections for which were classified as 'access antimicrobials' by WHO (2021). Using 16S rRNA amplicon sequencing and extensive functional gene annotation, this study is the first to provide a comprehensive analysis of seasonal and spatial shifts in the structure of indigenous and TET- and SXT-resistant cultured bacterial communities and associated resistomes from the surface water and bottom layer sampled along the trophic gradient of the marine ecosystem.

2. Materials and methods

2.1. Study area

Surface (extension S) and bottom (extension B) layer samples were recovered seasonally in March (winter) and August (summer) 2021 from the sites in southeastern Adriatic (Croatia): Jadro (JS), Kaštel Sućurac (KSS), Vranjic Bay (VBS/VBB), Kaštela Bay (KBS/KBB), Split Chanel (SCS/SCB), and Stončica (STS/STB) (Supplementary Fig. S1, Table S1). The sites were selected due to geographical location and anthropogenic influence following the trophic gradient from the human-impacted river mouth and coastal beach waters to open sea. The eutrophic JS is in the estuary of the river Jadro, the main karstic river of the Kaštela Bay, deteriorated by uncontrolled domestic wastewater discharge (Maravić et al., 2016). Moreover, KSS site is also eutrophic and located near the public beach which is under significant anthropogenic influence due to an inadequate sewage system that discharges municipal sewage, opportunistic pathogens, and ARGs into coastal waters (Maravić et al., 2015). The station VS/VB in Vranjic Bay is strongly influenced by the freshwater of the Jadro River. Mesotrophic KBS/KBB site is in the middle of Kaštela Bay, but influenced by the mainland, while the oligotrophic SCS/SCB further follows the trophic gradient from the coast to the open sea. Finally, the STS/STB is an oligotrophic and an open sea site located near Vis Island. A total of 80 water samples were collected in triplicates, of which half was collected during summer and others during winter period. Estuarine samples were collected during the low tide to decrease the dilution effect by the incoming seawater. Seawater samples were collected with a Niskin sampler 10–20 cm below the surface and above the seafloor and were filtered through 0.22 μm pore-size mixed celulose ester (MCE) membranes within 1 h.

2.2. Environmental parameters

Temperature (TEMP), salinity (SAL) and pH were recorded using SeaBird 25 CTD profiler and a handheld YSI Pro1030 pH and conductivity meter implemented with a pH 1002 sensor. Dissolved inorganic nutrient concentrations (NO_3^-, NO_2^-, NH_4^+, and PO_4^{3-}) were determined on a Bran+Luebbe AutoAnalyser (II and III models) colorimetrically in unfiltered samples using standard colorimetric method (Grasshoff et al., 2007).

2.3. Biological parameters

Chlorophyll a (Chl *a*) was determined from 500-mL sub-samples, filtered through GF/F glass-fiber filters with 0.7 μm pore size (Whatman, UK) and stored at −20 °C. Filters were homogenized, extracted in 90 % acetone and subsequently analysed fluorometrically with a Turner TD-700 Laboratory Fluorometer calibrated with pure Chl *a* (Strickland and Parsons, 1972).

Abundances of *Synechococcus,* (SYN), *Prochlorococcus* (PRO), picoeukaryotes (PE), heterotrophic bacteria (HB), heterotrophic nanoflagellates (HNF), and viruses were determined using flow cytometry with a fast flow rate of 60 μL/min (Gasol and Morán, 2016).

Samples of autotrophic cells (2 mL) were preserved in 0.5 % glutaraldehyde, flash-frozen and stored at −80 °C. Samples for the analysis of the HB and HNF were preserved in 2 % formaldehyde and stored at 4 °C. Samples for determination of viral abundance were fixed for 15 min at room temperature in the dark with electron microscopy-grade glutaraldehyde at final concentration of 0.5 % (Sigma-Aldrich), followed by flash-freezing in liquid nitrogen and stored at −80 °C.

Bacterial cell production was measured from DNA synthesis based on incorporation rates of ^{3}H-thymidine (Fuhrman and Azam, 1982). Methyl-^{3}H-thymidine was added to 10 ml samples to a final concentration of 10 nmol. Triplicate samples and a formaldehyde-killed adsorption control were incubated for 1 h at room temperature and incubations were stopped by adding formaldehyde. The thymidine samples were extracted with ice-cold trichloroacetic acid (TCA). The TCA-insoluble fraction was collected by filtering the samples through a 0.2-μm pore size filters.

2.4. Enumeration of viable bacteria

Membrane filtration method was used for enumeration of *E. coli* (ISO 9308–1:2014) and intestinal enterococci (ISO 7899–2:2000). The total viable heterotrophic bacterial counts were determined using spread plate technique in triplicates. The SXT- and TET-resistant heterotrophic bacteria were isolated on Marine agar (BD Difco, USA) infused with SXT (512 μg/ml) or TET (16 μg/ml), representing the minimal inhibitory concentration breakpoints interpretive for resistance to these antimicrobials based on CLSI (2021). The percentage of resistant bacteria was calculated as the ratio of the number of colonies grown on agar supplemented with antibiotic per total number of colonies grown on medium without it, multiplied with 100.

2.5. Filtration and DNA extraction

Two liters of water samples were filtered in triplicate through fast-flow 0.22 μm MCE membranes (Whatman, UK) for extraction of metagenomic DNA. To cultivate antibiotic-resistant communities, aliquots of 10 to 100 ml of water were filtered and membranes placed on SXT- and

TET-supplemented Marine agar (BD Difco). After one week of incubation at room temperature, colonies were scraped from the triplicate plates, representing samples S.SXT and B.SXT for all-sites surface and bottom SXT-resistant bacterial community, and S.TET and B.TET for all-sites surface and bottom TET-resistant bacterial community, respectively. Metagenomic DNA was extracted using DNeasy PowerWater Kit (Qiagen, Germany). DNA concentration was measured using Qubit Fluorometer 3.0 (Thermo Fisher Scientific, USA) and stored at −20 °C until further analysis.

2.6. Illumina sequencing, taxonomic annotation, and functional prediction

Metagenomic DNA undergone 16S rRNA amplicon sequencing on the NovaSeq platform (Illumina, UK). A 470 bp hypervariable V3-V4 region was amplified using 515F-806R primers and Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR products were purified, and libraries generated using NEBNext® UltraTM DNA Library Prep Kit (Illumina). 250 bp paired-end raw reads were merged using FLASH (v1.2.7) and high-quality clean tags obtained after quality filtering using QIIME2 (v2022.2). Tags were compared with the reference using uchime and vsearch algorithm to detect and remove chimera sequences. A total of 6.186.038 effective read tags with an average of 77.325 reads per sample and length of 419 bp were generated. Sequences with ≥97 % similarity were assigned by Closed Reference clustering method to the same Operational Taxonomic Units (OTUs). OTU sequences were classified by vsearch method and SILVA database (v138) for relative taxa abundances and for generating a phylogenetic tree. Diversity analysis and functional prediction was done using QIIME2 (v2022.2) and PICRUSt2 on the Nephele platform (Weber et al., 2018; Douglas et al., 2020). Filtering of metagenomic data for visualization was done by in-house program MiaViz (https://github.com/apavlinovic/mia-viz), Python 3.9.0 (library: Pandas, SciPy, Seaborn) and Circos (http://mkweb.bcgsc.ca/tableviewer/).

2.7. Statistical analysis

Alpha diversity measures inspected taxa richness and evenness by calculating Observed species, Gini-Simpson, Shannon, Chao1, Evenness-Pielou, and Rarity rare abundance. Detailed description is given in Suppl. Text S1. Wilcoxon test was performed for analysis of significance of difference between groups. Comparison and visualizing group-based differences or similarities calculations were done using microbiome (Lahti and Shetty, 2018), phyloseq (McMurdie and Holmes, 2013), ggpubr (Kassambara, 2020), and dplyr (Wickham et al., 2017) packages in R software (v4.2.4) (R Core Team, 2022). Relative abundance of bacterial taxa was calculated by dividing the absolute abundance with the total abundance of species at each taxonomic rank. MATLAB (2021) was used for plotting the most abundant taxa in the microbiomes and creation of heatmap depicting the relative abundance of pathogenic genera. Beta diversity measures were done using packages in R software: vegan (Oksanen et al., 2022), phyloseq (McMurdie and Holmes, 2013), tidyverse (Wickham et al., 2019), patchwork (Pedersen, 2022), agricolae (de Mendiburu, 2021) for Principal Coordinate Analysis (PCoA). It was performed to get principal coordinates and visualize from complex, multidimensional data based on Bray Curtis matrix. UPGMA Tree was performed as a type of hierarchical clustering method to interpret the distance matrix using average linkage and was conducted by QIIME (v1.7.0) on weighted UniFrac matrix. To compare relative abundances of taxa between sites, we used the non-parametric two-sided Wilcoxon signed rank based on weighted UniFrac distances. Multiple Response Permutation Procedure (MRPP) analysis was performed by R (Vegan package) to determine whether community structure significantly differs between groups (Supple. Text S1). Linear discriminant analysis Effect Size (LEfSe) analysis was conducted by LEfSe software (v1.1.2) (Segata et al., 2011) to detect differentially enriched taxa as biomarkers in marine microbiomes during winter and summer seasons (Suppl. Text S1).

3. Results

3.1. Environmental parameters and bacterial counts during winter and summer seasons

The detailed review of physicochemical and biological factors is provided in the Supplementary material (Tables S1 and S2). TEMP varied seasonally, ranging from a winter minimum of 12.22 °C to a summer maximum (27.01 °C) at the surface. The surface TEMP at the deeper sites (≥ 35 m depth) in summer was higher than at the seafloor, indicating water column stratification, which was not observed at the shallow Vranjic station (18 m depth). *Chl a* concentrations were highest at this station and decreased away from shore. SAL did not vary in the water column and, comparing to other marine sites, was lowest at KS, where the effluent outlet is located. Overall, nutrient loads were high at the nearshore sites, particularly concentrations of nitrates, ammonia, and phosphates at Vranjic, and in the lower layers of the open sea sites.

Abundance of microbial community members decreased along the coastal-open sea transect (with increasing distance from the coast), except PRO and PE (Table S2). The HB peaked in summer at Vranjic (1.27×10^6 cells/mL) and had its lowest abundance at open-sea ST (0.26×10^6 cells/mL). Abundance of autotrophic picoplankton peaked in winter at coastal stations, while PRO was most abundant in summer at ST. Viral abundance doubled in summer at all marine stations, but decreased with increasing distance from the coast, ranging from 16.68×10^6 to 2.47×10^6 VLP/mL. The HNF abundance also decreased from coastal to offshore stations, being highest at JS (3.53×10^3 cells/mL) and lowest at ST (0.30×10^3 cells/mL) in winter. HNA bacteria dominated at JS (55 %), while LNA bacteria were more abundant at other sites, except at KB in summer (83 %).

A substantial level of fecal pollution was detected in the Jadro River (JS) following the public beach (KSS) (Table S2). The highest percentage of TET- and SXT-resistant bacteria was also found at JS (39.55 % and 20 %, respectively). In general, the prevalence of SXT-resistant bacteria increased across the trophic gradient in summer, while the abundance of TET-resistant bacteria was more site-specific and followed less the trophic gradient (Table S2).

3.2. 16S rRNA sequencing overview

A total of 8404,830 high-quality paired-end reads were generated, giving an average of 105,060 reads per sample. A total of 35,240 OTUs were obtained in summer giving an average of 906 per sample, while total of 24,080 OTUs with an average of 602 were identified from winter samples. Rarefaction curves were presented in Fig. S2.

3.3. Bacterial community structure along the trophic gradient

A total of 81 unique bacterial phyla were identified, with most of them ($n = 49$) detected in summer.

The total number of phyla was also increased in summer compared to winter regardless of trophic status. *Proteobacteria* were the most abundant phylum during both seasons at the surface and bottom layer (Fig. 1A). This was particularly evident for surface samples, which were even more abundant by *Proteobacteria* in summer (their abundance doubled to 63.03 % at KSS compared to winter). Noteworthy, the microbiome from the subsequent offshore station KBB displayed a different pattern; *Proteobacteria* abundance decreased in summer at 24.54 %, resulting in *Firmicutes* as the most prevalent phylum (41.53 %). This was also observed for its surface microbiome (KBS). On the contrary, at other sites, the abundance of *Firmicutes* decreased to about 0 % in summer. Of other phyla, *Actinobacteriota* were highly abundant at the open sea sites (STS, STB and SCS microbiomes), while *Bacteroidota* were

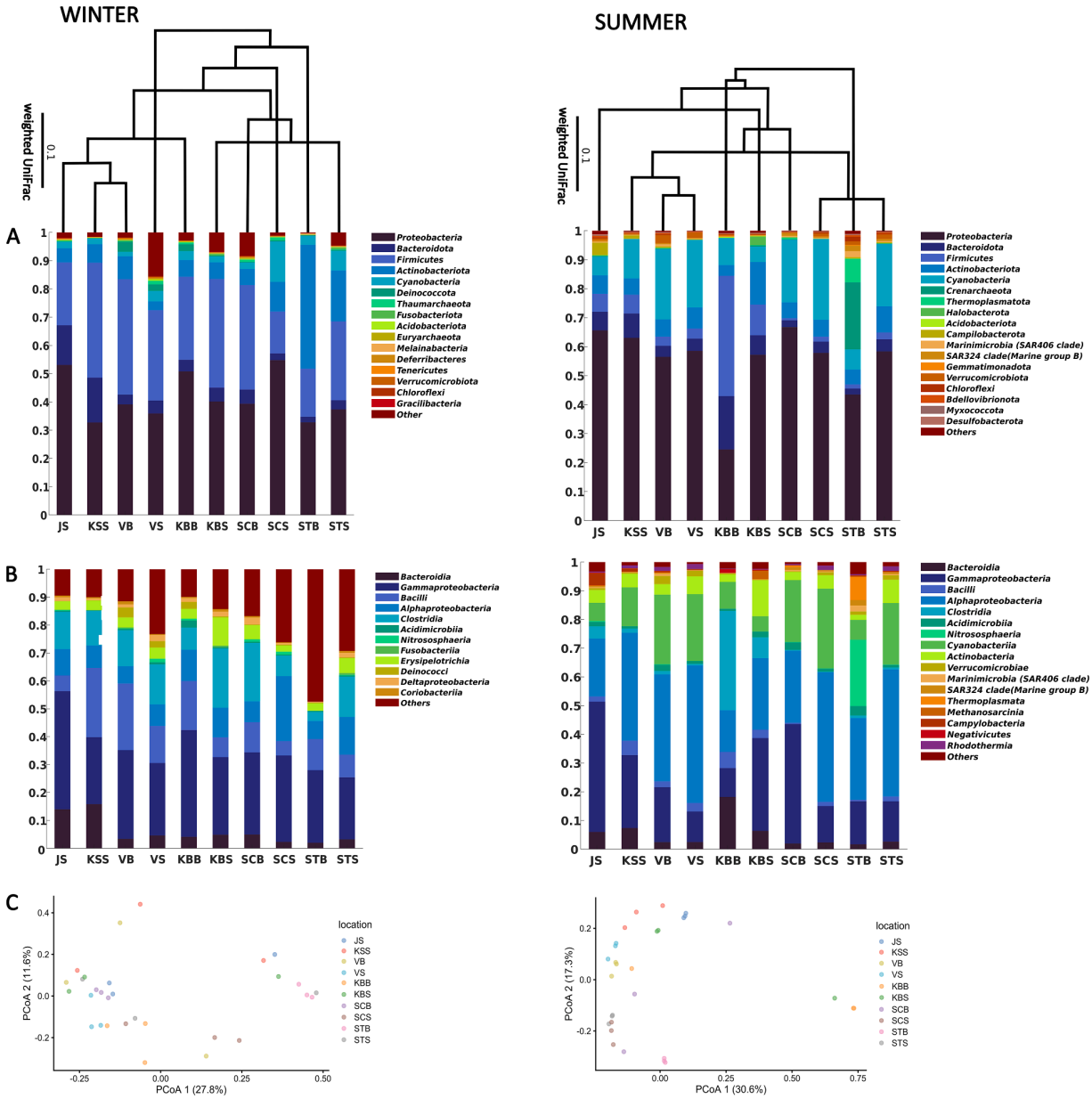

Fig. 1. Relative abundance of the 16 most abundant taxa at phylum (A) and class (B) levels comprising the bacterial communities from sea surface and sea bottom during winter and summer. The relative abundance was calculated by dividing the absolute abundance by total abundance of species at each taxonomic rank. The most abundant taxa at phylum and class levels were plotted using MATLAB (2021). Beta diversity was assessed by PCoA (C) based on the Bray–Curtis and Weighted Unifrac distance matrix was used for UPGMA cluster analysis.

commonly presented at KBB, particularly at summer (18.41 %). *Cyanobacteria* increased during summer at most sites along the trophic gradient (SCS, VB, VS, SCB and STS) ranging from 27.75 % to 21.53 %. Overall, due to the formation of the thermocline, the microbiomes from the surface and the bottom layer show greater structural differences in summer than in winter, when the water column is not stratified.

Moreover, a total of 62 and 158 bacterial classes were identified during winter and summer season (Fig. 1B). In winter, *Gammaproteobacteria, Bacilli, Alphaproteobacteria, Bacteroidia* and *Clostridia* were the most abundant. In contrast, *Alphaproteobacteria, Gammaproteobacteria, Cyanobacteria, Bacteroidia*, and *Bacilli* were more prevalent in summer. Only at river site (JS) *Gammaproteobacteria* were the most abundant during both seasons. KSS microbiome was the most enriched with *Bacilli* during the winter, and *Alphaproteobacteria* in the summer. At all other sites, both surface and bottom layer microbiomes, were most abundant by *Gammaproteobacteria* in winter, while in summer the composition shifted as *Alphaproteobacteria* took the lead, except for surface microbiomes SCB and KBS, where *Gammaproteobacteria* were still the most prevalent class.

Clostridiales were the most abundant order in wastewater-impacted marine microbiomes in winter, while *Enterobacteriales* prevailed in estuary JS (Fig. S3). In summer, *Rhodobacterales* were most abundant along the trophic gradient, except in SCB, KBS, and KBB, whose communities were dominated by *Pseudomonadales, Alteromonadales*, and *Lachnospirales*. In addition, the communities also displayed seasonal variation in the top 35 most abundant genera (Fig. 2). We observed the predominance of gut-associated bacteria in winter in the estuary (*Serratia, Bacteroides*) and impacted coastal sites (*Lactobacillus* and

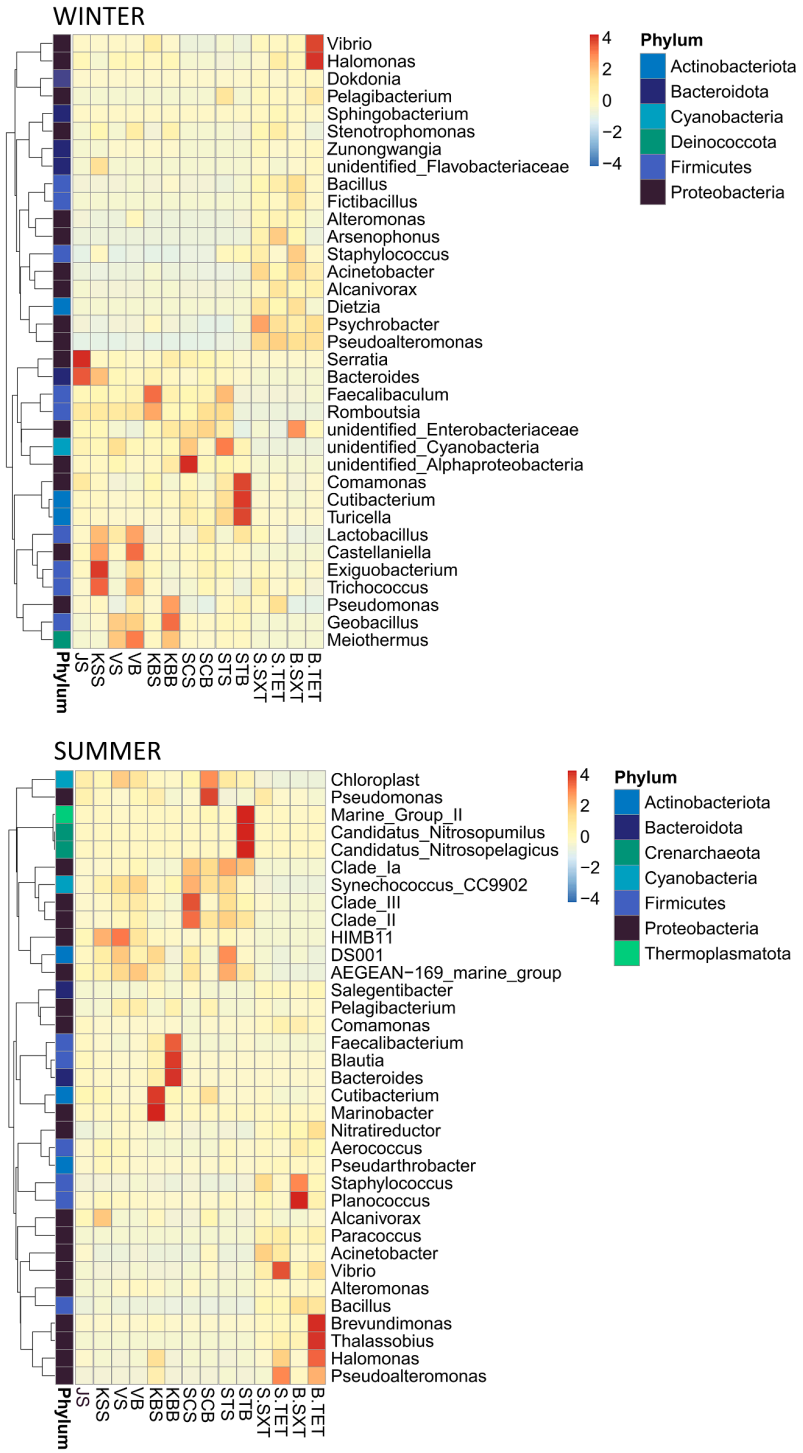

Fig. 2. Taxonomic abundance cluster heatmap plotted by names of microbiomes and the 35 most common sequences detected in indigenous and SXT- and TET-resistant bacterial communities. The scale displays the row Z score (Z score = [relative abundance of bacterial taxa in a specific community - mean of relative abundance of the same taxa in all analyzed communities]/standard deviation). The positive Z scores indicate values above the mean, while negative Z scores values are below the mean in units of standard deviation.

Bacteroides in KSS; *Faecalibaculum* and *Romboutsia* in KBS), which declined sharply in summer when marine autochthonous bacteria such as *Alphaproteobacteria* HIMB11, AEGEAN-169 marine group, *Pelagibacterium, Marinobacter* and *Synechococcus* sp. CC9902 prevailed. The surface microbiome of the open sea (STS) was predominantly colonised by members of clades Ia, II and III, while the Marine_group II, Candidatus_*Nitrosopumilus* and Candidatus_*Nitrosopelagicus* were predominant at a depth of 100 m (Fig. 2).

The structure of TET- and SXT-resistant communities showed fewer differences in the most abundant orders (Fig. S4) and genera (Fig. 2).

TET-resistant communities in winter were largely composed of *Oceanospirillales* (genus *Halomonas*), *Alteromonadales (Pseudoalteromonas), Pseudomonadales (Pseudomonas, Psychrobacter),* and *Bacillales (Bacillus),* regardless of site origin, whereas *Vibrionales (Vibrio)* were ahead of the previously listed orders in summer.

Similarly, the most represented bacterial orders in the SXT-resistant communities in winter were *Alteromonadales, Pseudomonadales, Bacillales,* and *Oceanospirillales*, while *Staphylococcales, Pseudomonadales*, and *Vibrionales* predominated in the summer season. We highlight the predominance of the genus *Psychrobacter* in the SXT-resistant surface communities in winter, while its abundance decreased sharply in summer, when *Acinetobacter* and *Vibrio* predominated. In contrast, *Planococcus* predominated in the SXT-resistant bottom layer community in summer (Fig. 2).

3.4. Biogeographical shifts in microbial community structure are driven by the seasonality

Alpha diversity of indigenous microbial community was assessed, revealing heterogeneity in community structure (Table S3). Higher species richness was consistently observed in summer compared to winter across the gradient. Microbial diversity was less dependent on seasonality, more site-specific, and more pronounced in surface microbiomes in winter. Nevertheless, both community richness and diversity decreased across the gradient (Fig. 3).

Bray-Curtis dissimilarity calculated from OTUs counts was used to determine the differences among the sites (Fig. 1C). Microbiomes from human-influenced sites (VB, KS, JS, KBB) clustered together as did those from unpolluted offshore areas (STS, SCB) in summer, whereas this was less evident in winter. In addition, Wilcoxon test on weighted UniFrac distances and MRPP analysis calculated the differences in relative abundances and phylogenetic lineages among microbiomes (Tables S4 and S5), also showing that most significant ($p \leq 0.001$) differences occurred more frequently when surface microbiomes were compared to seafloor microbiomes, regardless of location and trophic status.

In this context, LefSE analysis identified a higher number of differentially enriched taxa in surface summer microbiomes, discerning biomarkers for different levels across the gradient (Fig. S5). Human gut and soil-associated bacteria belonging to *Acinetobacter, Arcobacter, Sphaerotilus* and *Aeromonas* were statistically significant in the polluted estuary (JS), while *Oceanospirillales* were predominant in the surface waters along the coast (KSS). Surface waters at the site VS, which is strongly influenced by river water, were rich in *Rhodobacterales*, while the following site (KBS), which is less but still influenced by river inflow, was characterized by a mixture of terrestrial and marine taxa (*Rhisobiaceae, Pseudomonas, Halomonas, Alteromonadales*). However, the sea bottom microbiome at this site (KBB) was represented by the class *Bacteroidia* and the genus *Enterococcus*, indicating fecal contamination due to sewage discharges into the bay. Notably, the coastal surface microbiome SCS in summer consisted considerably of typical marine bacteria belonging to *Halomonas, Synechococcus*, clades II and III, whereas this was even more evident at the open-sea site STS, whose surface waters were significantly enriched by the clades Ia, SAR116 and SAR86, AEGEAN-169 marine group and *Litoricola* (Fig. S5).

3.5. Diversity of pathogenic genera across the trophic gradient

The relative abundances of pathogenic genera within the native and antibiotic-resistant microbial communities were represented by heatmaps (Fig. 4). Human gut-associated *Serratia* and *Bacteroides* prevailed in the river microbiome JS in winter, while decreasing away from the coast. Similarly, this was observed for *Acinetobacter* and *Arcobacter* in summer. In contrast, *Pseudomonas* was among the most common pathogenic genera in native communities across the trophic gradient,

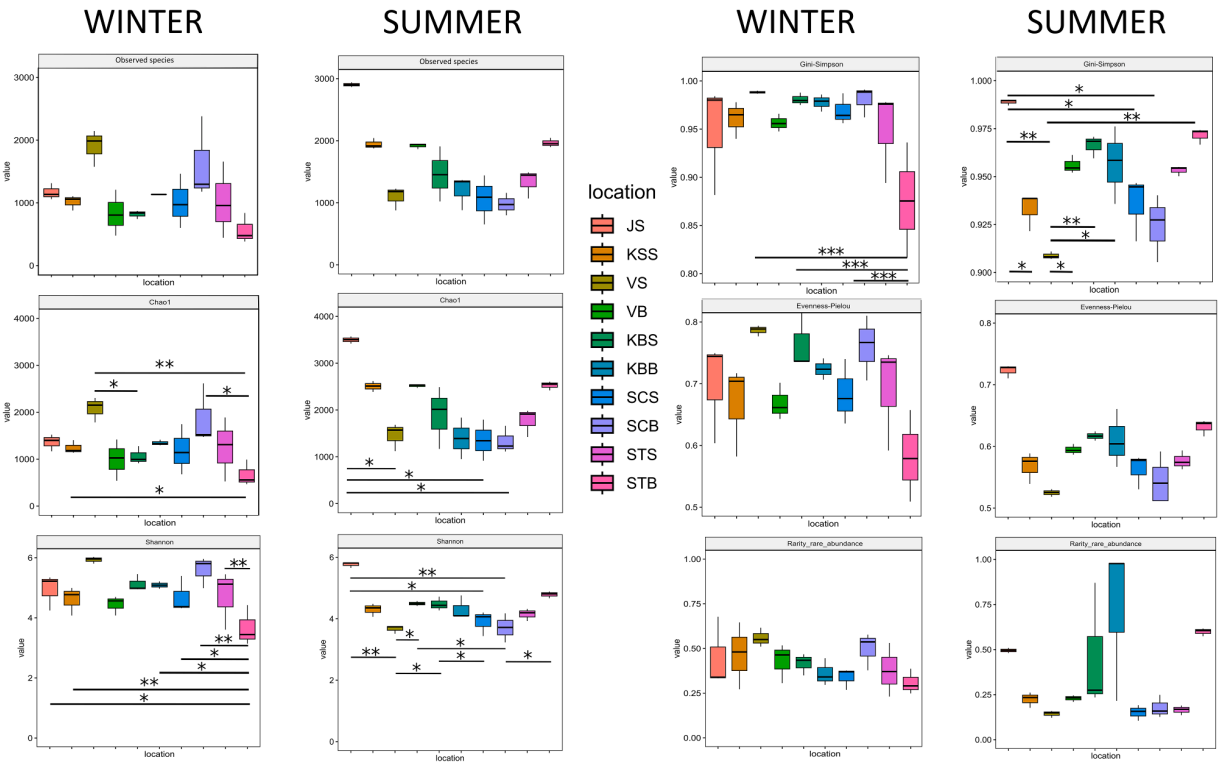

Fig. 3. Boxplots representing alpha diversity by community richness (Observed species and Chao1 indices), diversity (Shannon and Gini-Simpson indices) and abundance (Evenness Pielou and Rarity rare abundance) between communities along the trophic gradient during winter and summer. Alpha indices significantly different between the groups are marked with an asterisk (Wilcoxon signed-rank test; *, $p < 0.05$; **, $p<0.01$; ***, $p<0.001$).

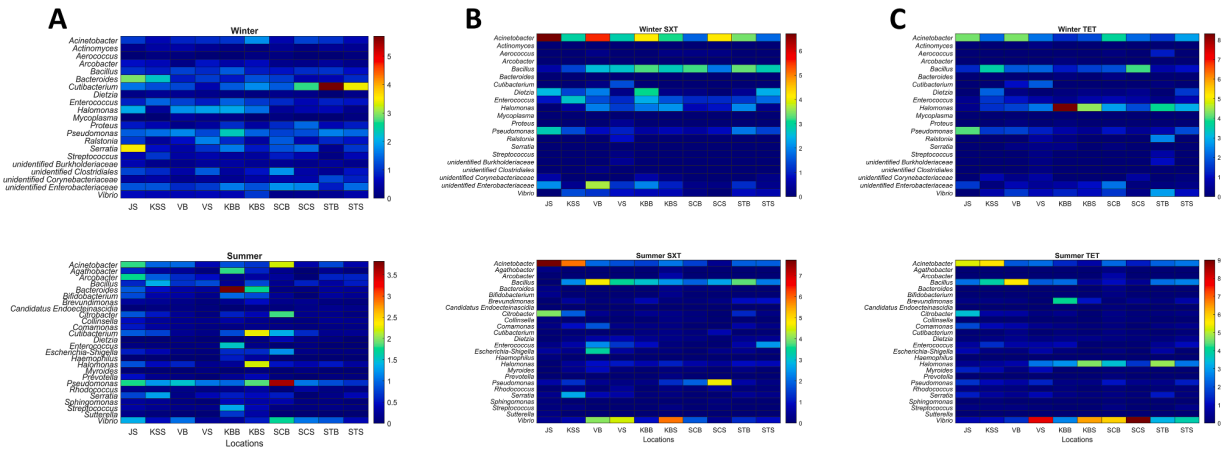

Fig. 4. Heatmap of the most abundant pathogenic genera in native (A) and antibiotic-resistant (B and C) bacterial communities during winter and summer, showing variations in relative abundance (expressed as square roots of the percentage values).

predominating at human-influenced sites and offshore (SCB), especially in summer (Fig. 4-A). Marine-borne genera such as *Vibrio* and *Halomonas* were also more abundant in summer.

Moreover, we found that *Acinetobacter, Enterococcus, Bacillus, Dietzia, Vibrio* and *Pseudomonas* build up the large part of pathogenic SXT-resistant communities in both seasons. This was particularly evident in winter, whereas only *Vibrio* and several enterobacterial genera increased along the gradient in summer (Fig. 4-B). Very similar distribution of pathogenic genera was also observed in case of TET-resistant bacterial communities in both seasons. However, the exception was *Halomonas* that was highly abundant across the gradient regardless of season when compared to SXT-resistant community (Fig. 4-C).

3.6. Resistome profiling

The contribution of a particular type of antibiotic resistance in indigenous and antibiotic-resistant communities was further investigated by network analysis (Fig. 5) while the detailed list of bacterial genera and functional genes contributing by ≥ 2 % to certain resistance per site is presented in Tables S6-S8. CAMP and vancomycin resistance modules predominated in resistome structure regardless of season, trophic status, or antibiotic (Fig. 5) as these were associated primarily to the most abundant taxa (Fig. 2) as well as those differentially enriched in individual microbiome (Fig. S5) such as *Bacteroides,* HIMB11, *Synechococcus, Pseudomonas, Halomonas, Comamonas,* SAR11 (subclades Ia, II, III) and SAR116 clades, *Vibrio, Arcobacter, Blautia, Romboutsia, Serratia, Lactobacillus* and *Castellaniella*. Multidrug resistance (MDR) modules were the third most common resistance mechanisms, attributed mostly to HIMB11, *Pseudomonas, Comamonas* and *Vibrio,* but also to a variety of a less frequently represented genera such as *Bacillus, Ralstonia, Acinetobacter, Klebsiella* and *Psychrobacter* (Fig. 5, Table S6). However, some resistomes displayed distinctive features. For instance, trimetoprim resistance genes (*dfrA1, drfD*) accounted for nearly 22 % of the bottom layer resistome in the middle Kaštela Bay (KBB), in contrast to the surface resistome (0.8 %) (Fig. 5), and were largely hosted by gut-associated *Proteus* spp. (Table S6). Moreover, imipenem resistance was highest (6.9 %) during summer in microbiome from 50 m depth (SCB) and related mostly to *Pseudomonas, Ralstonia,* and *Alcaligenes* while KBB microbiome from 35 m-deep seafloor had the highest abundance (2.7 %) of phenicol resistance genes (*catA, catB*) due to *Romboutsia, Klebsiella, Proteus* and *Alcaligenes* (Fig. 5, Table S6).

Nevertheless, seasonality-driven changes in resistome structure were observed; MDR modules as well as macrolide, phenicol, aminoglycoside, and especially imipenem resistance genes were much more frequent in all resistomes in winter (Fig. 5). Based on the excessive bioinformatics on functional prediction pooled by the PICRUSt2, these shifts appear to be related to the seasonal structure of a total community rather than to a specific taxon. However, some interesting facts were found. For instance, *Proteus* abundance in the surface microbiome SCS was differentially increased in winter (Fig. S5), which enriched the resistome with aminoglycoside (*aac6, aadK, aph3* genes by up to 12 %), phenicol (*catA*, 11 %), beta-lactamase (bla_{CMY}, 21 %), sulfonamide (*sul2*, 36 %), TET (*tetA*, 33 %), and rifamycin resistance genes (*arr* as much as 87 %) (Table S6). Moreover, differentially abundant *Turicella,* and *Comamonas* in the deepest seafloor STB microbiome in winter (Fig. S5), were found to contribute 35 % and 40 % to quinolone resistance. On contrary, in summer this resistance was mostly attributed to *Vibrio* (35 % of the contribution) and *Alcanivorax* (23 %), respectively.

As for pathogen-function relationship, we found that several pathogenic genera simultaneously accounted for a variety of resistances across the trophic gradient in winter, among which rifamycin, sulfonamide, TET, trimethoprim, and beta-lactamase resistance genes due to *Proteus*, trimethoprim, beta-lactam, and macrolide resistance due to *Bacillus*, and imipenem and MDR resistance due to *Pseudomonas* (Fig. 6). Notably, several major genera were found to be more prevalent in summer resistomes than in winter, including *Pseudomonas* which highly contributed by multiple resistances, *Acinetobacter* that accounted for MDR, macrolide and particularly beta-lactam and phenicol resistance, while *Vibrio* was responsible for various resistances, especially to imipenem, phenicol, quinolones, tetracycline, and β-lactams (Fig. 6).

Moreover, the differences between the summer and winter resistomes of the SXT- and TET-resistant communities were less evident (Fig. 5), probably due to the low bacterial cultivation on antibiotic-supplemented media. Namely, observed species index for SXT- and TET-resistant communities was only 243.5 and 229, while diversity (Shannon index) was about 4.5. Both resistant communities were less abundant in CAMP and vancomycin resistance modules when compared to native resistomes, while increased the abundance of MDR modules (as much as from 9.6 % to 24.5 % in estuarine resistome JS). In addition, abundance of genes encoding phenicol resistance (highest increase from 0.3 % to 4 % in VS) and beta-lactamases (max. from 0.1 % to 2.2 % in SCS resistome) was increased in TET-resistant communities (Fig. 5). CAMP resistance was mainly associated to the *dlt*ABCD operon in Gram-positive bacteria (*Bacillus* contributed by up to 21 %, and was followed by *Lactobacillus*), and protease PgtE production in Gram-negative (particularly *Pseudoalteromonas* across the gradient while contribution of *Enterobacteriaceae* enhanced by the proximity of the coast and sewage inflow).

In general, TET-resistant communities were enriched by genes mediating MDR via efflux pumps, among which MdeF-TolC contributed

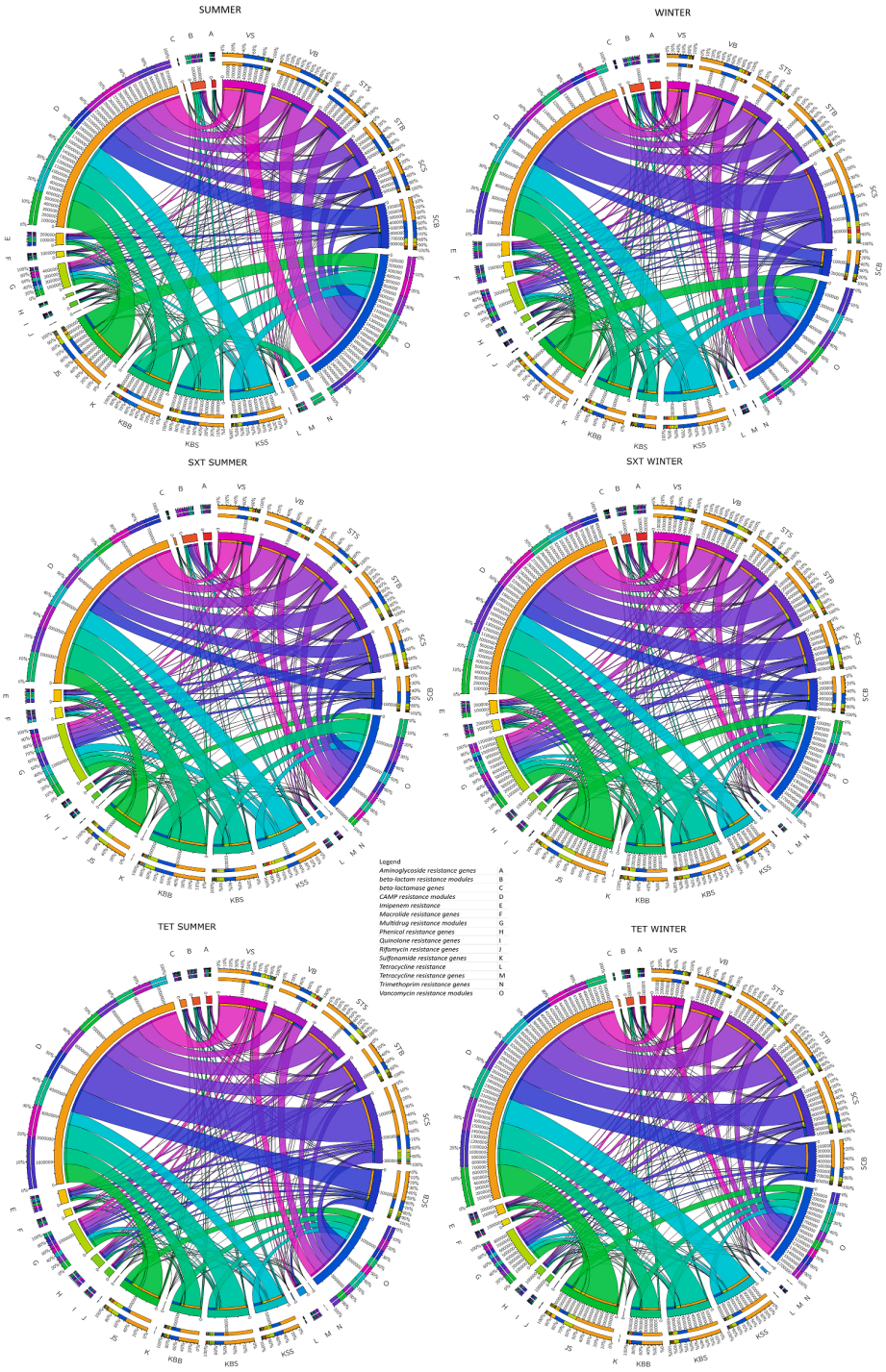

Fig. 5. Pathways identified by PICRUSt2 tool from KEGG base as functional prediction relationships between obtained microbiomes and antibiotic resistance mechanisms. The plot was computed using Circos.

most and was more abundant towards the open sea (28 %, 42 %, and 72 % in surface resistomes VS, KBS, and SCS, respectively). Other pumps were associated to bacteria not native to the marine environment, were less abundant and their abundance decreased away from the shore, including NorB and NorC (in *Bacillus* and *Staphylococcus*), AdeABC and BpeEF-OprC in *Acinetobacter* (up to 3 % in surface resistomes JS and KSS), and AcrEF-TolC in *Citrobacter* (2 % in estuarine resistomes JS). Phenicol resistance was mostly attributed to *catA* and *catB* genes carried by autochthonous marine bacteria such as *Pseudoalteromonas* (33 % in STS) and *Vibrio* (98 % in SCS), but also to *Acinetobacter* (high 48 % in KSS compared to 9 % at surface of open sea STS) and *Aeromonas* in estuarine waters (12 % compared to 0.03 % at STS), respectively. Moreover, beta-lactamase genes were also largely associated to *Vibrio* (bla_{CARB} genes), *Acinetobacter* and *Aeromonas* (bla_{OXA}) and *Citrobacter* (bla_{CMY}). We found that TET resistance genes were also increased in this resistome, particularly in summer, and were predominantly associated to *Vibrio* whose

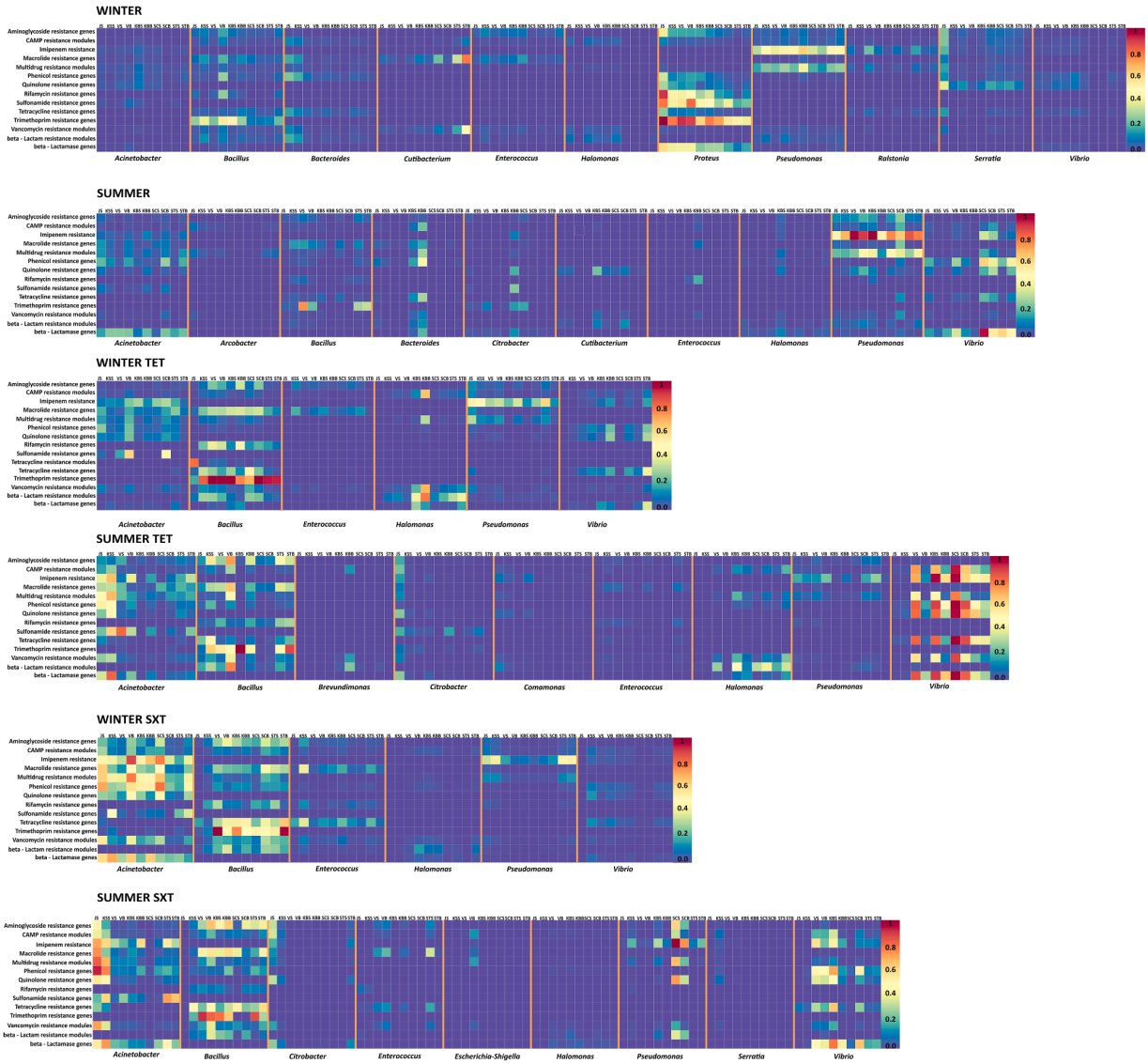

Fig. 6. Heatmap showing the average abundance (%) of antibiotic resistance genes or modules possessed by the most abundant pathogenic genera (≥ 2 %) in native and antibiotic-resistant communities based on their annotation in the KEGG Database.

tet35 gene contributed by 86 %, and *Bacillus* (*tetB* and *tetA* contributed up to 11 %) (Table S7), respectively.

On the other hand, the SXT-related resistome was enriched only by MDR compared to the native resistome (Fig. 5). This was due to a variety of efflux pumps, including AbcA of *Planococcus* (36 % in SCB), MexPQ, MexCD, MexEF, and MexJK of *Pseudomonas* (ranging from 2 % to 7 % depending on the site), followed by those common in estuarine and coastal resistomes such as AdeABC of *Acinetobacter* (4 % in estuarine resistomes JS, while only 0.4 % in STS) and AcrEF-TolC carried by *Enterobacteriaceae*, which accounted for about 2 % of the total MDR pathway (Table S8). *Sul1* and *sul2* genes, coding for the SXT-resistant enzymes such as dihydropteroate synthetase, were carried by *Acinetobacter* and *Aeromonas* across the gradient. Additionally, *Enterobacter* and PS1 clade highly contributed by *sul1* in estuarine SXT-resistome (30.3 %) and *sul2* gene in the seafloor and surface SXT-resistome SCS and SCB (96 %), respectively.

Taken together, several pathogenic genera stood out as important carriers of resistance traits in TET- and SXT-related resistomes in both seasons, particularly *Acinetobacter, Bacillus Vibrio* and *Pseudomonas,* while *Enterococcus* highly contributed during winter (Fig. 6). Opposed to native resistomes, *Proteus* and *Serratia* were not among the most abundant pathogenic genera.

4. Discussion

The main objective of this study was to identify seasonal and spatial variations in the taxonomic structure and ARG repertoire of indigenous, and TET- and SXT-resistant marine bacterial communities inhabiting the bottom layer and surface marine waters along the trophic gradient of a temperate region. We hypothesize that specific physicochemical and biological factors influence the dynamics of marine bacteria as carriers of resistance determinants, thus impacting the marine resistome at seasonal and spatial scales.

4.1. Microbial diversity

Importantly, there is a lack of studies that have comprehensively addressed the problem of antibiotic resistance in the marine

environment, as most authors have focused on particular types of marine ecosystems such as estuarine (Mukhejri et al., 2022), coastal (Guillén-Chable et al., 2022), or deep-sea areas (Yang et al., 2019). To the best of our knowledge, the structure of the bottom layer and surface marine microbiome and resistome have not yet been studied simultaneously in terms of seasonality and relationship to trophic status. More importantly, there are no available data on the taxonomic and functional profiles of TET- and SXT-resistant bacterial communities from marine environment obtained by metagenomics.

The central Adriatic has long been anthropogenically influenced particularly by wastewater discharges, leading to the emergence of clinically relevant pathogens and ARGs in the Jadro River estuary (Maravić et al., 2016) and in the coastal waters (Maravić et al., 2015; Kvesić et al., 2022). FIB counts confirmed wastewater pollution at the JS and KSS in this study. Its open waters, including Stončica (STS, STB), are not exposed to long-term human activities and are considered pristine environments. *Proteobacteria* were found to be the major constituents of the microbial community and the major contributors to the marine resistome across trophic gradient in this study. Consistent with this, *Proteobacteria* were previously found to predominate in seawater from pollution-prone environments (Mukherji et al., 2022) as well as those with no or scarce human impact (Zhang et al., 2022a; 2022b). Marine *Proteobacteria* (particularly *Alpha-*, followed by *Gammaproteobacteria*) were also previously identified as the most abundant phylum in deep-water (Šantić et al., 2021; 2023) as well as the shallow coastal Adriatic (Korlević et al., 2022). Similar to latter reports, *Alphaproteobacteria* mainly encompassed HIMB11 and AEGEAN-169 lineages from nearshore to open sea, while SAR11 (including Ia, II and III subclades), which dominates the ocean bacterioplankton (Giovannoni, 2017), were highly abundant in oligotrophic waters. This was particularly pronounced in summer (Fig. 1), as these heterotrophs can supply energy via other cell mechanisms (Pinhassi et al., 2016), enabling them to successfully overcome nutrient deprivation. Moreover, beside the alphaprotebacterial AEGEAN-169 bacteria, LEfSe analysis revealed that open surface waters (STS) in summer were differentially enriched by marine gammaproteobacterial *Litoricola,* SAR116 and SAR86 lineages, all of which were previously identified among the most common proteobacterial genera in similar environments (Šantić et al., 2021). The predomination of marine *Synechococcus* over other cyanobacteria in this study was also previously observed in Adriatic Sea (Šantić et al., 2021; 2023; Korlević et al., 2022).

Moreover, *Firmicutes*, as commensals of the human gut, are expected to be among the leading taxa in microbiomes emerging off the human-impacted coastline (Lu et al., 2019). Thus, they prevailed from eutrophic estuarine JS and coastal KS to mesotrophic coastal VS, VB, KBS, and KBB sites, demonstrating their spatial arrangement along the trophic gradient. On contrary, members of the phylum *Actinobacteriota*, known to withstand environments with extreme pressure, salinity, and temperature fluctuations (Shivlata and Satyanarayana, 2015), were particularly abundant in open-sea microbial communities during winter (43.8 % in STB, 18 % in STS), including *Turicella* which was previously found in deep-sea sediments of the Atlantic Ocean (Stach et al., 2003). It was, however, unexpected to find *Cutibacterium,* a skin commensal, to be enriched in such environment (Fig. 2). Nevertheless, this genus has been identified in several fish species from the eastern Mediterranean (Meron et al., 2020), and would be interesting to investigate its persistence in the deep sea and open surface waters through future metagenomic studies.

4.2. Seasonality is the main driving factor of microbial diversity along the trophic gradient, but it impacts less with the proximity to the coast

Microbiomes from the sites most impacted by anthropogenic activities such as river estuary and nearshore sites were enriched by human gut-associated bacteria, particularly *Clostridiales* and *Enterobacterales.* The PcoA plot revealed that open-sea site STB was the most different from the other sites, due to the high prevalence of classes native to this pristine area, such as marine *Alphaproteobacteria, Nitrososphaeria, Thermoplasmata* and *Cyanobacteria* (Fig. 1) The abundance of intestinal commensals (such as *Serratia, Lactobacillus, Bacteroides, Faecalibaculum, Romboutsia, Faecalibacterium, Blautia*) and soil bacteria (*Pseudomonas, Exiguobacterium, Castellaniella)* decreased away from the coast, resulting in a shift of resistome towards the intrinsic resistance mechanisms of native marine bacteria. In such habitats, effect of seasonality is strong and primarily related to the stratification of the water column, driving the differences between surface and bottom resistomes in summer compared to winter period (Fig. 5).

Of note, bottom layer microbiome KBB was the only coastal microbiome that was highly abundant by intestinal genera (*Blautia, Bacteroides, Faecalibacterium*) in summer, because of stratified water column. Far less differences were observed between resistomes in summer and winter along the coast, and these are almost exclusively due to the influx of allochthonous bacteria and their genes through sewage discharges and mainland runoff (Fig. 6). In winter, we observed the higher abundances of intestinal bacteria in coastal waters because the seawater is kept closer to the outlets due to the coastal relief and the winds. In addition, the lower insolation and surface temperature in winter may have facilitated bacterial stress, promoting their higher survival rate compared to summer (Hernroth et al., 2009).

Communities in summer had higher species richness than in winter and were represented by most of identified phyla ($n = 49$) and classes ($n = 158$), with considerable variations in the proportion of the 10 most abundant orders and families (Fig. S3). Increased human activities in the coastal Adriatic during summer (e.g., extensive tourist activities, nautical traffic, sewage discharges) may contribute to the influx of autochthonous bacteria into the ecosystem, while specific climatic features, such as winds, allow their further dispersal along the trophic gradient. In addition, seasonally driven factors contributed to the bloom of native marine bacteria characteristic for this ecosystem (e.g. HIMB11, AEGEAN-169 and SAR11 lineages) as previously observed by Korlević et al. (2022).

Microbial picoplankton communities in the Adriatic are known to rapidly respond to the changes to the environmental conditions (Šolić et al., 2010; 2022). Also, a top-down control, such as grazing and viral lysis may have contributed to community richness. The abundance of viruses doubled in summer, indicating their higher activity in this area as confirmed previously by Ordulj et al. (2017). Furthermore, non-selective grazing by HNF controls the number of bacterial population while species-specific viral lysis could determine the size of bacterial groups and thus affect the microbial diversity (Sandaa et al., 2009). Moreover, both community richness and diversity decreased with distance from the coast, following the decrease of Chl *a* and a shift from eutrophic to oligotrophic conditions in the studied area (Šolić et al., 2022). Moreover, most human-influenced sites had elevated richness and diversity indices while the deepest sampling point in our study, the bottom layer at the 100 m depth (STB), had the lowest taxa richness and diversity, supporting the hypothesis that human-derived pollution contributes to microbial diversity (Chen et al., 2019).

Surface communities differed from the bottom layer microbiomes across the trophic gradient, and this was more significant with increase of depth (Table S4). After the thermocline formation during summer, surface communities at mesotrophic (KBS) and oligotrophic sites (SCS, STS) became more influenced by temperature, depicting biomarkers for different trophic areas (Fig. 2). For instance, *Faecalibacterium, Blautia*, and *Bacteroides* were characteristic to KBB microbiome at the bottom layer at 35 m depth, in contrast to *Cutibacterium* and *Marinobacter* at the surface. *Pseudomonas* was differentially enriched at 50 m depth in SCB, while the alphaproteobacterial subclades II and III of the SAR11 lineage characterized its surface microbiome. Interestingly, several taxa within *Archaea* were most abundant in indigenous open sea microbial community (STB) at 100 m depth, including *Thermoplasmatota* Marine group II (8 %) and *Thaumarchaeota* "*Ca.* Nitrosopumilus" (7 %) and "*Ca.*

Nitrosopelagicus" (15.4 %). Apart from carbon, these bacteria can oxidize ammonia and nitrate as an energy source, allowing them to live heterotrophically in nutrient deficient environments, while their co-occurrence has been reported mainly in coastal waters (Kim et al., 2019; Korlević et al., 2022).

4.3. Pathogens that mostly contributed to marine resistome

Proteobacteria and *Firmicutes* contributed most to the pathogenic genera and the resistome structure. During winter pathogenic genera typical of sewage-influenced waters (e.g. *Bacteroides, Serratia, Streptococcus, Enterococcus, Arcobacter*) (Kvesić et al., 2022) persisted in estuary and nearshore while in summer were more dispersed along the gradient, which affected the resistome structure. On contrary, *Pseudomonas, Acinetobacter* and *Vibrio* were present along the gradient throughout the year. Similarly, Zeng et al. (2019) identified *Escherichia, Bacteroides*, and *Clostridium* as the predominant bacterial hosts of ARGs in gut-associated environments, whereas *Alteromonas, Vibrio,* and *Proteobacteria* in pristine environments. Moreover, SXT- and TET-resistant microbiomes were highly enriched by species from the *Pseudomonadales*, supporting their broad spectrum of antibiotic resistance (Begmatov et al., 2022). Notably, we found previously that the multidrug-resistant *Pseudomonas* and *Acinetobacter* isolates, recovered from estuarine and coastal waters studied herein, owe their resistance phenotype to the overexpression of intrinsic efflux pumps, and carriage of various acquired resistance genes including β-lactamase genes (Maravić et al., 2016; 2018).

The surface- and seafloor-derived TET- and SXT-resistant communities differed more in summer than in winter, as was the case for the native communities, demonstrating seasonality. Of note, there were genera that persisted in the resistant communities regardless of season and contributed significantly to the resistome. Namely, we found that TET-resistant community mediated resistance to this antibiotic through a combination of resistance mechanisms, including upregulation of multidrug efflux pumps (norB, norC, MexAB-OprM, AcrAB-TolC) and *tet*-like genes *(tet35, tetA, tetB*), which were mostly carried by staphylococci, *Pseudomonas, Enterobacteriaceae* as well as autochthonous marine genera such as *Pseudoalteromonas, Vibrio*, and *Halomonas* (Weston et al., 2018; Begmatov et al., 2022; Elmahdi et al., 2016; Dang et al., 2008; Teo et al., 2002). Similarly, SXT-related resistomes were predominated by the mechanisms that, among others, drive resistance to SXT, such as multidrug efflux pumps of *Pseudomonas* (MexAB and MexEF), *Acinetobacter* (AdeABC, AdeIJK) (Coyne et al., 2011), as well as *sul1* and *sul2* genes carried by multiple genera (Table S8).

In conclusion, we represented herein a complex pattern of antibiotic resistance persisting along a trophic gradient. Although the diversity and abundance of antibiotic resistance mechanisms and bacterial taxa as their carriers decreased with the decline of anthropogenic influence, we still detected pathogenic genera and a considerable arsenal of resistance determinants in native and TET- and SXT-related resistomes, which were reinforced by marine genera such as *Vibrio, Pseudoalteromonas* and *Alteromonas.* Of the various environmental parameters shown to shape microbial communities in this study, temperature contributed the most to both indigenous and antibiotic-resistant communities. Antibiotic resistance determinants were generally more abundant during summer along the trophic gradient but were more frequent nearshore during winter. *Proteobacteria,* followed by *Firmicutes* at human-influenced sites, predominated by *Acinetobacter, Vibrio, Bacillus* and *Pseudomonas* which were found to contribute most to TET- and SXT-related resistomes, resulting in concomitant persistence of peptide, MDR, beta-lactam, phenicol and quinolone resistance along the trophic gradient of a marine ecosystem in a temperate zone.

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Authors contributions

M.DŽ. and S.J. carried out sampling and filtration. M.DŽ., H.K., A.P. and M.K. performed extensive bioinformatics and statistical analyses. S. J., I.Š., M.O. and A.M. helped with data analyses. A.M and M.DŽ. designed the study, while all co-authors contributed to the preparation of the manuscript.

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.

Data availability

Raw 16S rRNA amplicon sequencing data of microbial communities (KS, JS, VS, VB, KBS, KBB, SCS, SCB, STS, and STB) were prepared in triplicate and enumerated (e.g., KS.1, KS.2, KS.3) An extension to the file name was added, e.g., KS.1.S, to distinguish summer (S) samples from the winter (W) samples. Sequences were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI as follows: autochthonous microbiomes from winter and summer under Bioproject no. PRJEB49384, SXT-resistant microbial community as PRJEB49628, and TET-resistant microbial community as PRJEB49630.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.120688.

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2.3 Large-Scale Biogeographical Shifts of Abundance of Antibiotic Resistance Genes and Marine Bacterial Communities as Their Carriers along a Trophic Gradient

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Large-Scale Biogeographical Shifts of Abundance of Antibiotic Resistance Genes and Marine Bacterial Communities as Their Carriers along a Trophic Gradient

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Abstract: The role of marine environments in the global spread of antibiotic resistance still remains poorly understood, leaving gaps in the One Health-based research framework. Antibiotic resistance genes (ARGs) encoding resistance to five major antibiotic classes, including sulfonamides (*sul1*, *sul2*), tetracyclines (*tetA*, *tetB*), β-lactams ($bla_{\text{CTX-M}}$, bla_{TEM} bla_{VIM}), macrolides (*ermB*, *mphA*), aminoglycosides (*aac3-2*), and integrase gene (*intl1*) were quantified by RT-qPCR, and their distribution was investigated in relation to environmental parameters and the total bacterial community in bottom layer and surface waters of the central Adriatic (Mediterranean), over a 68 km line from the wastewater-impacted estuary to coastal and pristine open sea. Seasonal changes (higher in winter) were observed for antibiotic resistance frequency and the relative abundances of ARGs, which were generally higher in eutrophic coastal areas. In particular, *intl1*, followed by bla_{TEM} and bla_{VIM}, were strongly associated with anthropogenic influence and *Gammaproteobacteria* as their predominant carriers. Water column stratification and geographic location had a significant influence on ARGs distribution in the oligotrophic zone, where the bacterial community exhibited a seasonal shift from *Gammaproteobacteria* in winter to Marine group II in summer.

Keywords: Adriatic Sea; antibiotic resistance; bottom layer; environmental factors; marine environment; Mediterranean; seasonal and spatial distribution; seawater; surface water; qPCR

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1. Introduction

The escalating levels of antibiotic resistance in human pathogens have caused a global public health emergency and raised significant apprehension [1]. Aquatic ecosystems are among the natural environments of the highest concern when it comes to the research of antibiotic resistance phenomenon within the One Health framework [2]. Namely, the coastal area is a highly diverse and densely populated ecosystem where the terrestrial environment overlaps with the marine, making it important but challenging to study the transmission pathways of resistance determinants.

Previous studies have shown that antibiotic resistance genes (ARGs) can be very widespread and diverse in the marine environment, especially in coastal areas [3,4]. These genes can originate from a variety of sources, including human-impacted rivers [5], submarine outfalls from wastewater treatment plants [6], as well as direct contamination from recreational water users [7]. However, much remains to be understood about the fate of ARGs and their bacterial reservoirs in the marine environment, particularly with regard

to their spatial and seasonal nature. It appears that the dynamics of ARGs are complex and influenced by multiple factors. Studies have shown that shifts in bacterial communities [8] and environmental conditions such as nitrogen and phosphorus concentrations, pH, salinity, and temperature play an important role in the accumulation of ARGs [9–12]. It is, therefore, crucial to investigate the comprehensive profiles of ARGs, as well as the main factors behind their spread.

In our previous study, we provided extensive taxonomic and functional profiles of the marine microbial communities obtained seasonally from bottom and surface waters in the central Adriatic (Mediterranean), covering a 68 km line from the wastewater-influenced river mouth to coastal and pristine open sea [8]. We found that the resistome was more diversified along the trophic gradient in winter, but cationic antimicrobial peptide (CAMP), vancomycin, and multidrug resistance modules prevailed regardless of trophic status or season. Nonetheless, resistance determinants for multidrug, beta-lactams (particularly imipenem), macrolides, aminoglycosides, and phenicols were much more common in winter, indicating that the diversity of native resistomes is greatly contingent on seasonal variations in the water column, caused by thermohaline stratification and nutrient availability [8].

In the present study, we aim to (i) quantify by RT-qPCR the clinically relevant ARGs associated with resistance to five major classes of antibiotics, including two sulfonamide resistance genes (*sul1*, *sul2*), two tetracycline resistance genes (*tetA*, *tetB*), three β-lactam resistance genes ($bla_{\text{CTX-M}}$, bla_{TEM}, bla_{VIM}), two macrolide resistance genes (*ermB*, *mphA*), one aminoglycoside resistance gene (*aac3-2*), and the class 1 integron integrase gene (*intl1*); (ii) determine how environmental factors, including physico-chemical and biological factors, influence the abundance of these genes along the trophic gradient; (iii) associate specific bacterial taxa with targeted ARGs to gain deeper insights into gene distribution and resistome structure in the marine environment.

2. Results

2.1. Frequency of Antibiotic Resistance along the Trophic Gradient

The proportion of antibiotic-resistant heterotrophic bacteria in the surface and bottom layers along the trophic gradient was evaluated against antimicrobial agents belonging to five major antibiotic classes, including macrolides, tetracyclines, aminoglycosides, β-lactams, and sulfonamides. In most cases, a higher percentage of antibiotic-resistant bacteria was detected in winter than in summer, although these rates differed seasonally along the trophic gradient (Figure 1; Table S2). These differences were statistically significant at open-sea oligotrophic site STS/STB (paired *t*-test, $p = 0.02$) and were primarily related to cefotaxime—(CTX)-resistant bacteria (2.5% of resistant bacteria at STB and 4.03% at STS in summer, compared to 0.7% and 2.0% at the same site in winter). In addition, tetracycline—(TET)-resistant bacteria also greatly contributed to these shifts at open sea; 8.23% and 4.03% of resistant bacteria were recorded in summer, compared to 2.12% and 3.1% in winter, respectively.

Gentamicin (GEN) resistance was found to be of particular importance for the bacterial community in the surface layer and led to the largest rate shifts in summer (paired *t*-test, $p < 0.01$). This was most evident at STS (4.6% in summer and 0.63% in winter) and KS (3.16% in summer and 0.02% in winter). In comparison, more bacteria resistant to GEN were found at the bottom layer in winter (up to 9.6% in KBB), and this was less seasonally influenced (Table S2).

In contrast to GEN, TET resistance in surface waters was among the highest measured regardless of season; it followed the trophic gradient and decreased from the coast to the open sea. Namely, in the winter, it ranged from 56.7% and 36.3% at the eutrophic sites KS and J to 3.1% at the oligotrophic STS, while in summer, the values at the same sites were 7.6%, 23.8%, and 4.03% (Figure 1; Table S2).

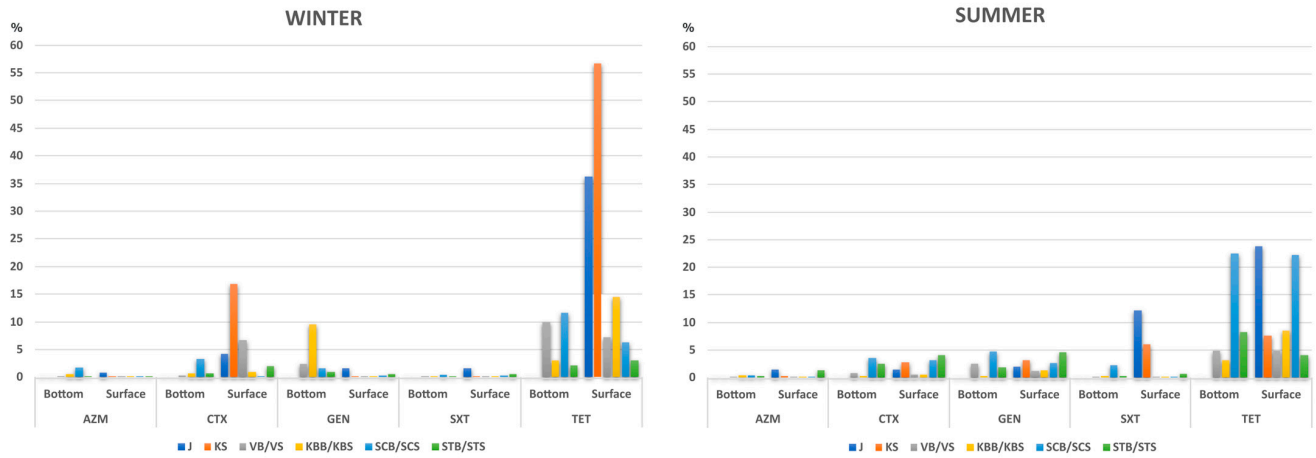

Figure 1. Frequency of resistance to five major classes of antibiotics in summer and winter along the trophic gradient encompassing eutrophic (JS, KS), mesotrophic (VBS/VBB, KBS/KBB), and oligotrophic (SCS/SCB, STS/STB) marine areas. The percentage was calculated as the ratio between the number of bacteria grown on agar infused with antibiotics per mL and the total heterotrophic bacteria.

Moreover, the highest sulfamethoxazole (SXT) resistance was recorded in the surface layer in summer, and it followed the trophic gradient regardless of season (from 12.14% and 6.03% at J and KS to 0.7% at STS in summer, with 1.67%, 0.01%, and 0.63% of SXT resistance at the same sites in winter) (Figure 1; Table S1). Seasonality also had no effect on the rate of resistance to this antibiotic in the bottom layer; however, this community appeared to be site-specific rather than driven by trophic status.

The percentage of CTX-resistant bacteria was found to be seasonal and highest in winter, while at the same time, it followed the trophic gradient and was more pronounced in the surface layer microbial community (16.81%, 6.73%, and 3.33% at KS, VS, and STS, respectively) (Figure 1).

Furthermore, we found that azithromycin (AZM) resistance in the surface bacterial community was not dependant on the seasonality but rather on trophic status. However, the bottom layer community exhibited similar rates from the nearshore to the open sea in summer (approximately 0.4% resistance from KBB to STB), whereas this pattern was somewhat different in the winter season, showing variations from 0.1% to 1.8% along the trophic gradient (Figure 1; Table S2).

2.2. Quantitative Analysis of ARGs and intl1

The abundance of ten ARGs (*sul1*, *sul2*, *tetA*, *tetB*, *mphA*, *ermB*, *aac3-2*, bla_{TEM}, $bla_{\text{CTX-M}}$, bla_{VIM}) and the integrase gene *intl1* was determined using a qPCR approach. All genes were detected at all sites along the trophic gradient, but their relative abundance varied spatially and seasonally (up to 10,000-fold) (Figure 2; Table S3). Overall, the most frequently detected ARGs were the macrolide resistance gene *ermB* and the tetracycline resistance gene *tetA*, irrespective of season and trophic status. The gene *ermB* was most abundant in the lower layer of the oligotrophic SCB in summer (8.36 copies/*rrn*), while *tetA* was most abundant in the eutrophic VS in winter (6.64 copies/*rrn*). Interestingly, the macrolide resistance-related gene *mphA* was also among the most frequently detected genes along the trophic gradient. The gene *mphA* was more abundant in the coastal area in summer (1.02, 0.7, and 0.3 copies/*rrn* in KBB, SCS, and KS), while it was most frequent in the oligotrophic area and the open sea in winter (0.14, 0.10, 0.11, and 0.09 copies/*rrn* in STB, STS, SCS, and SCB), respectively.

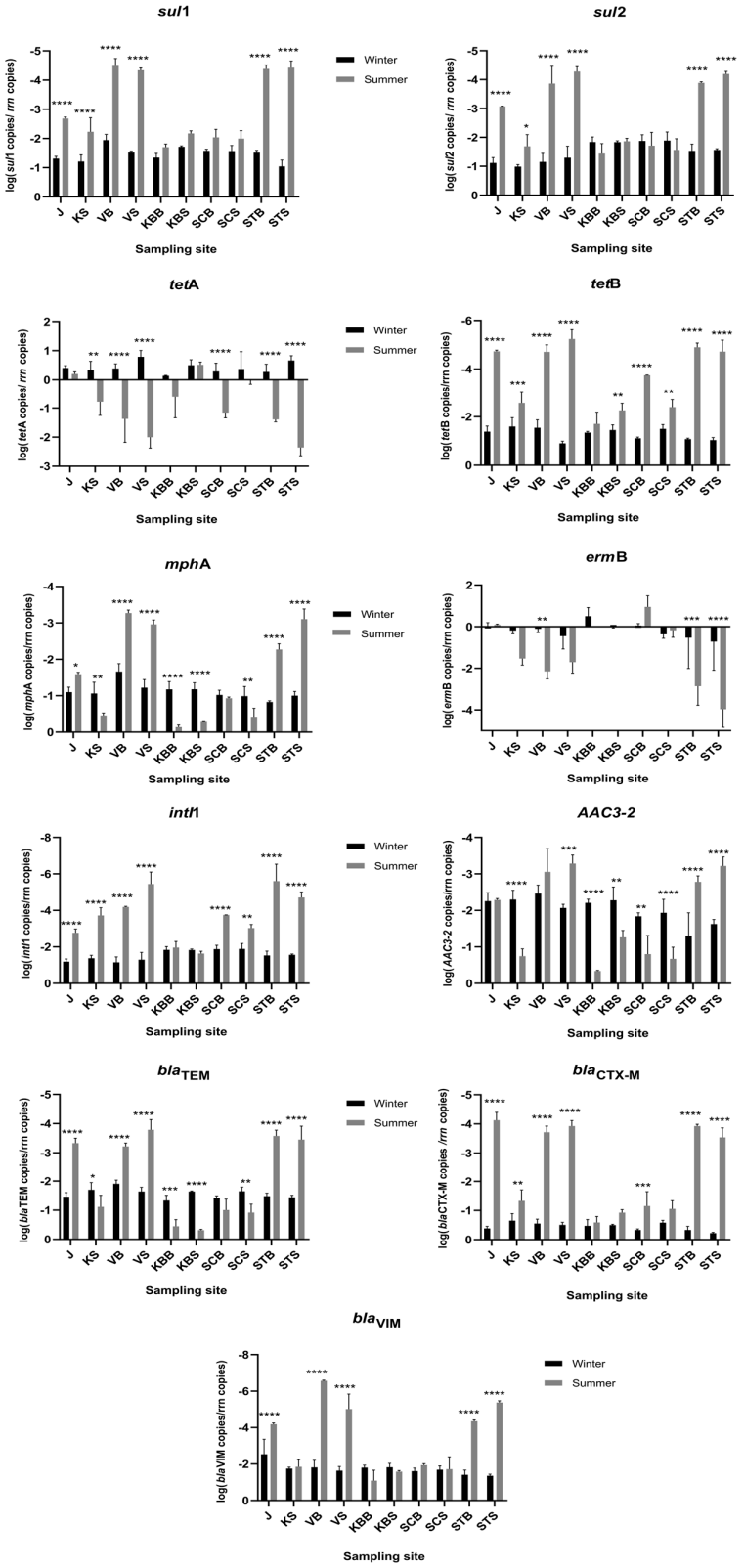

Figure 2. Seasonal and spatial distribution of targeted genes along the trophic gradient represented by their relative abundance (copies of gene/16S rRNA gene). Data were compared using two-way analysis of variance (ANOVA), and seasonal significance level is represented by the *p*-value (Sidak test for multiple comparisons). **** $p < 0.0001$; *** $p < 0.005$; ** $p < 0.001$; * $p < 0.01$.

The abundance of the β-lactam resistance genes (bla_{TEM}, bla_{CTX-M}, bla_{VIM}) showed no clear spatial and temporal character. In particular, bla_{TEM} (detected in a range from 2×10^{-4} in STB to 0.87 copies/*rrn* in KBB) and bla_{VIM} (4×10^{-6} in STS to 0.14 copies/*rrn* in KBB) showed seasonality and were generally found more frequently in summer than in winter, following the trophic gradient. In contrast, bla_{CTX-M} is most abundant in winter (from 0.19 copies/*rrn* at SCS to 0.6 copies/*rrn* at oligotrophic open-sea STS in winter) and was not related to spatial status.

The relative abundance of sulfonamide resistance genes *sul1* and *sul2* appears to be site-specific, although both were uniformly more abundant in winter. Comparing their abundance with each other, *sul2* was generally more abundant along the trophic gradient regardless of season (maximum 0.14 copies/*rrn* in winter and up to 0.04 in summer at KBB).

The relative abundance of the integrase gene *intl1* steadily followed the trophic gradient and occurred more frequently in winter (maximum 0.08 copies/*rrn* at VB) than in summer, when it shifted to the central part of the bay (KBS 0.02 copies/*rrn* at KBS). Moreover, the aminoglycoside resistance gene *aac3-2* was the least abundant ARG across the trophic gradient, with no obvious spatial shifts, while its seasonal variation (up to tenfold) was statistically significant at almost all sites (Figure 2; Table S3).

Taken together, the coastal eutrophic sites (J, KS) showed significant seasonal shifts ($p < 0.0001$) for all genes except *mphA*, *ermB*, *aac3-2*, and *tetA*. The mesotrophic area (KBB, KBS, SCS, and SCB) was seasonally stable, with the exception of bla_{TEM}, *tetB*, *aac3-2*, *mphA*, and *intl1*, which were significantly more abundant in winter ($p <$ [0.0001–0.0017]).

2.3. Seasonal and Spatial Co-Occurrence of Quantified ARGs and intl1

The relationship between the targeted ARGs along the trophic gradient in the summer and winter seasons was represented by the Spearman rank correlation heatmap (Figure 3; Table S4) and the Pearson correlation-based co-occurrence network (Figure 4; Table S5).

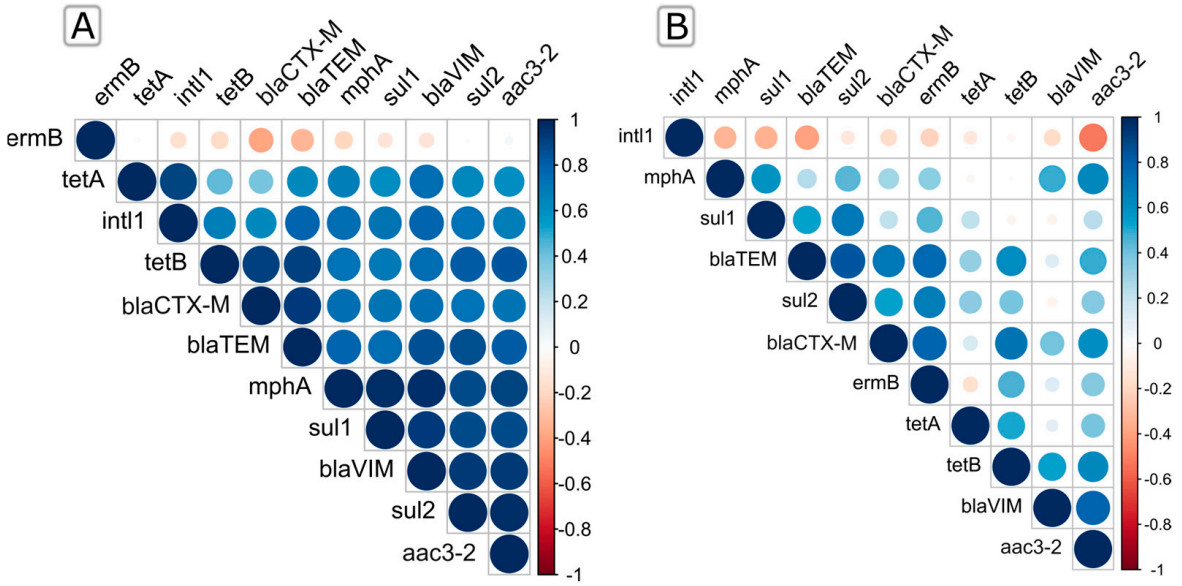

Figure 3. Spearman's rank correlations of the ARGS relative abundances during summer (**A**) and winter (**B**). Positive correlations are displayed in blue, and negative correlations are displayed in red. Color intensity and the size of the circles are proportional to the correlation coefficients.

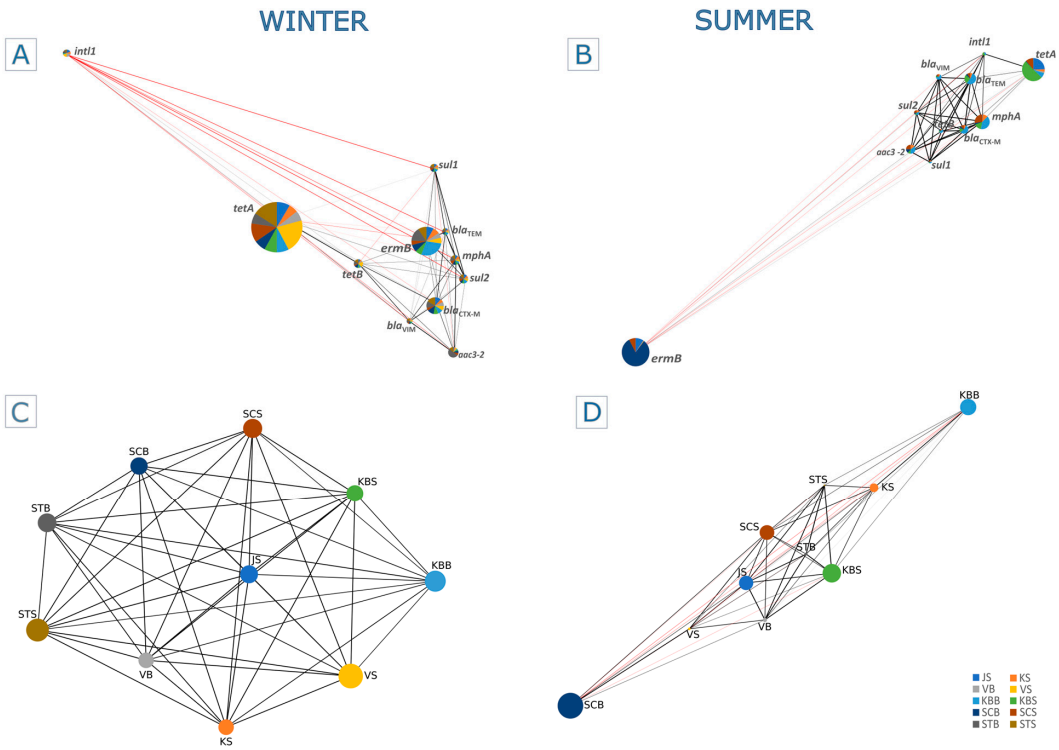

Figure 4. Mentel–Pearson correlation of ARGs distribution along the trophic gradient. The co-occurrence network represents the relations between the ARGs, where the node size is proportional to the abundance of the gene in winter (**A**) and summer (**B**). Sampling sites are represented by different colors, and their distribution within each node is defined by the frequency of a specific gene at that site. The Mentel–Pearson correlation between the sampling sites is shown for winter (**C**) and summer (**D**), with a positive correlation represented by a black line and a negative correlation by a red line.

We found that *ermB* in summer is the only gene negatively correlated with all compared genes except *aac3-2* (Spearman $r < 0.03$), while all other genes were negatively correlated with at least one gene (Figure 3A; Table S4), as also confirmed by the Pearson correlation (Figure 4B). In addition, we found that *sul1*, *sul2*, bla_{TEM}, and bla_{VIM} showed a significant positive correlation (Spearman $r > 0.79$, $p < 0.0001$) with *mphA*. A positive correlation was also observed between $bla_{\text{CTX-M}}$, bla_{TEM}, and *tetB* (Spearman $r > 0.92$, $p < 0.0003$) (Figure 3A; Table S4), which was also confirmed by the Pearson correlation ($r = 0.97$ and 0.99) (Figure 4B; Table S5). The integrase gene *intI1* most significantly correlated with *tetA* (Spearman $r > 0.9$, $p < 0.0003$; Pearson $r = 0.82$), bla_{TEM}, and bla_{VIM} (Spearman $r > 0.8$, $p < 0.006$; Pearson $r = 0.75$ and 0.45), followed by other ARGs ($p < 0.05$), while it only exhibited a negative correlation only with *ermB* (Spearman $r < -0.16$; Pearson $r = -0.2$) (Figure 3A and Table S3; Figure 4B and Table S5).

During winter, the frequencies of ARGs showed very different relations, indicating the considerable influence of seasonality (Figure 3B and Table S4; Figure 4A and Table S5). For instance, the relative abundance of bla_{TEM} showed a significant positive correlation (Spearman $r > 0.83$, $p < 0.002$) with *sul2* and $bla_{\text{CTX-M}}$, while it was negatively correlated with *intI1* (Spearman $r < -0.39$). A positive and statistically significant Spearman correlation was also observed between *ermB* and the β-lactam resistance genes $bla_{\text{CTX-M}}$ and bla_{TEM}, as shown by the Pearson coincidence network. In contrast, the integrase gene *intI1* showed a negative Spearman correlation with all genes examined in winter (Figure 3B; Table S4).

In addition, the Spearman correlation showed a strong linear relationship between trophically similar sites in winter (Figure 4C), with eutrophic/mesotrophic sites clearly grouped against oligotrophic sites. However, this was less clear in summer (Figure 4D),

as the high abundances of *ermB* and *tetA* genes led to a decrease in the linear relationship between these areas.

2.4. Relationship between Environmental Parameters and Gene Abundances

The relationship between environmental factors, ARG, and sampling sites was examined using RDA (Figure 5). Taken together, the environmental variables explained a total of 99.8% of the variation in ARG abundance along the trophic gradient. During winter, the cyanobacteria SYN and PRO acted synergistically and had a strong positive effect on the distribution of most ARGs along the first canonical axis (Figure 5A). In contrast, HNA, HNF, HB, and VLP were negatively correlated with most genes, with the exception of *tetA* at mesotrophic (VS) and oligotrophic (SCS) sites and *ermB* at the eutrophic KBB site. It is noteworthy that the abundance of *intl1* correlated negatively with PE and Chl *a* and positively with FIB along the trophic gradient (STS, VB, and KBS). In terms of physico-chemical parameters, pH was found to have a positive influence on the distribution and abundance of ARGs in the eutrophic coastal zone (KS, J) in winter (Figure 5C). In the oligotrophic zone (STS, SCS), the main driving factors with positive correlation were dissolved inorganic nutrients (NO_3^-, NO_2^-, NH_4^+), while SAL had a negative effect. TEMP was found to have a negative effect on the abundance of ARGs ($R^2 = 0.69$, $p < 0.034$), except in the case of *tetA*, whose abundance was positively affected, especially in the mesotrophic zone (VS). The abundance of *ermB* also showed a very strong positive correlation with SAL and dissolved oxygen in this zone (KBB) (Figure 5C).

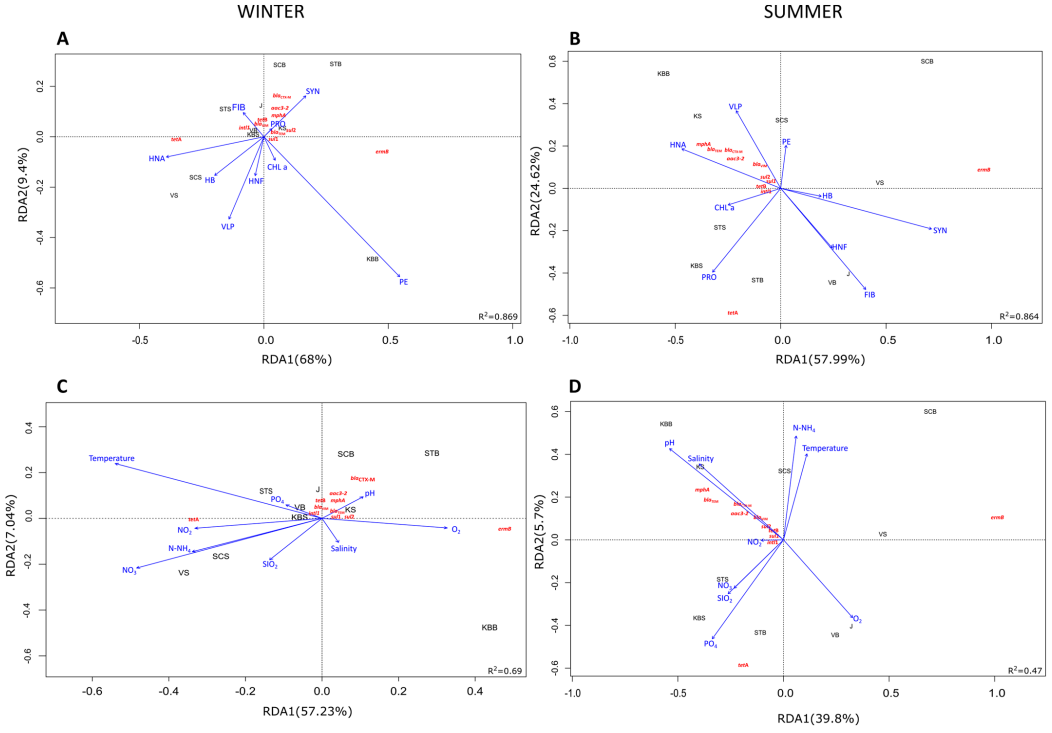

Figure 5. Redundancy analysis (RDA) ordination plot illustrating the correlation between the ARGs and biological properties (**A**,**B**) or physicochemical properties in winter (**C**) and summer (**D**). Abbreviations: temperature (TEMP), salinity (SAL), pH, dissolved inorganic nutrients (NO^{3-}, NO_2^-, NH_4^+, and PO_4^{3-}), dissolved oxygen (O_2), chlorophyll *a* (Chl *a*), *Synechococcus* (SYN), *Prochlorococcus* (PRO), picoeukaryotes (PE), heterotrophic bacteria (HB), heterotrophic nanoflagellates (HNF), virus-like particles (VLP), fecal indicator bacteria (FIB).

Regarding the summer season, the RDA revealed that HNA and VLP were the main biological factors positively influencing the distribution of all ARGs, except *tetA* and *ermB*,

along the trophic gradient. The gene *tetA* was found to be positively correlated only with Chl *a* and PRO in mesotrophic and oligotrophic areas (KBS, STS, STB). However, the substantial influence of HB, HNF, SYN, and FIB at eutrophic (J) and mesotrophic sites (VB) did not affect the abundance of target ARGs in summer (Figure 5B). Moreover, variations in pH and SAL were positively correlated with the abundance of all genes except *tetA* along the trophic gradient, while this ARG was positively affected by some inorganic nutrients (NO_3^- and PO_4^{3-}) (Figure 5D).

2.5. Association of Quantified ARGs with Bacterial Communities along the Trophic Gradient

To investigate the relationships between the ARG profiles obtained in this study and the bacterial communities previously analyzed by Illumina-based metagenomics [8], we performed a Mentel–Pearson correlation by taking the most abundant bacterial classes along the trophic gradient during summer and winter (Figure 6).

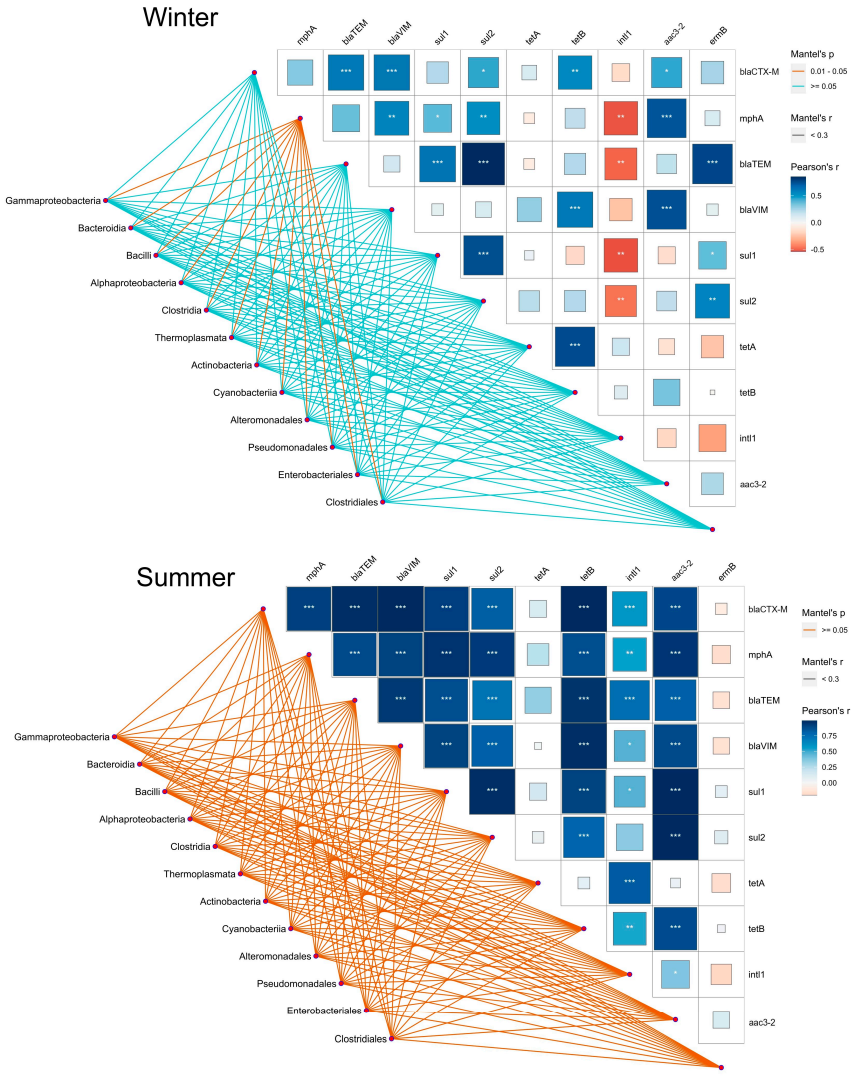

Figure 6. Pairwise comparisons of ARGs and their association with the selected bacterial classes and orders are shown with a color gradient denoting Pearson correlation coefficient. The eight most abundant classes and four orders were selected to provide a better insight seasonally into the differences between eutrophic and oligotrophic sites. The bacterial classes and orders were related to each ARG based on their abundance. The edge width corresponds to the values of Mentel's *r*, and the line color indicates the statistical significance of Mentel's *p* value. *** $p < 0.005$; ** $p < 0.001$; * $p < 0.01$.

In winter, the only statistically significant ($p < 0.01$) relation of classes, including *Gammaproteobacteria*, *Bacilli*, and *Bacteroidia*, and genes were shown for *mphA*, which further positively correlated with *aac3-2*, bla_{VIM}, and *sul2* and negatively correlated with *intl1*. The gene *intl1* displayed a negative correlation with a number of other genes, of which the *sul*-like genes and bla_{TEM} were found to be significant for all studied orders, such as *Pseudomonadales*, *Enterobacteriales*, and *Clostridiales*. On the other hand, a positive but not statistically significant interconnection was observed between bacterial classes and ARGs during the summer. In comparison to winter, *mphA* and bla_{TEM} correlated positively when compared to *tetA* and associated with the aforementioned classes, while this was even more evident in the case of the *intl1* gene. On the contrary, the abundance of *ermB* in bacterial classes and classes during summer shifted to a negative correlation when compared to the *mphA* and *bla* genes (Figure 6).

3. Discussion

By combining culture-dependent and culture-independent techniques, including qPCR in this study and the Illumina-based amplicon sequencing in our previous study [8], we further investigated the distribution of clinically relevant ARGs along the trophic gradient of the Adriatic Sea to obtain a more comprehensive picture of how ARGs disperse, which particular environmental parameters influence them the most, and which bacterial taxa may be their key mediators in the temperate marine environment.

3.1. Seasonality Is a Driving Factor for Antibiotic Resistance and Distribution of ARGs

Heterotrophic bacterial counts revealed that the incidence of resistance to some antibiotics differed significantly when comparing the remote oligotrophic areas with the human-influenced coastal areas, with the incidence generally higher in winter than in the summer.

This could be related to the fact that we have previously observed higher abundances of human gut-associated bacteria in coastal waters in winter as the seawater remains closer to the sewage outlets due to the coastal relief and winds [13]. Less activity of predators (HNF) and weaker insolation and surface temperature in winter could increase the bacterial survival rate compared to summer [14]. In addition, we have already observed that offshore sites are characterized by a shift of ARGs towards the intrinsic resistance mechanisms of autochthonous marine bacteria and are strongly affected by seasonality, which is primarily related to the stratification of the water column in summer [8].

The prevalence of resistance to TET was among the highest across the trophic gradient, although it decreased away from the coast and was lowest in oligotrophic waters. This suggests that it is an anthropogenic feature, as the coastal waters serve as recipients of sewage discharges and effluents from fish farms. Namely, as a broad-spectrum antibiotic, TET serves as a growth promoter in aquaculture [15] and is a widely used antimicrobial in human medicine, which is excreted into the environment in an active form via urine and feces after treatment [16]. Accordingly, *tetA* was found to be the most abundant ARG in winter and the second most abundant in summer along the trophic gradient. An analysis of the global ocean resistome based on data from the TARA Oceans project found *tetA* and *tetB* to be among the most abundant oceanic ARGs [17]. Regarding the surface–bottom layer distribution, TET resistance was more abundant in surface waters in winter, while the bottom and surface layers were somewhat similar in the summer season (Figure 1). Warmer temperatures and higher nutrient loads in the sea, e.g., in estuaries and from sewage in coastal areas, are crucial factors for the growth and dynamics of bacterial communities [4] and affect the survival and distribution of bacteria associated with sewage, leading to a shift in bacterial gene profiles [18]. In our previous study [8], PICRUSt2 functional prediction based on the 16S rRNA amplicon sequencing data showed that the *tetA* and *tetB* genes were highly represented in marine microbial communities of the eutrophic and mesotrophic areas in winter.

The same analysis indicated a higher occurrence of sulfonamide resistance genes *sul1* in the estuary and *sul2* in the bottom layer of the oligotrophic zone [8], which was confirmed by qPCR experiments in this study. *Sul* genes are among the most abundant ARGs in surface marine waters [19], especially in beach waters [20] as well as in rivers [21]. In addition, they generally appear to be more recalcitrant and have a higher potential to increase in abundance than other ARGs in the environment [22,23]. Accordingly, a high relative abundance of these genes was found in eutrophic and mesotrophic zones, while they showed seasonal stability in both the surface and bottom layers of the water column.

As for the prevalence of beta-lactam (CTX) resistance, its seasonal variations reflected the distribution of the $bla_{\text{CTX-M}}$ gene along the trophic gradient, which was more abundant in winter at both mesotrophic and oligotrophic sites. In addition, the high relative abundance of the bla_{TEM} gene (up to 0.87 copies/*rrn* at KBB) corroborates the finding that beta-lactam resistance is highest in coastal waters [20,24], where it correlates strongly with the respective anthropogenic influences [25]. However, the aminoglycoside resistance gene *aac3-2* was the least abundant ARG across the trophic gradient in this study, with no apparent increase, although a relatively higher abundance of this gene had previously been found in the anthropogenically impacted coastal areas [26].

The gene *intl1* had been previously used as an indicator of anthropogenic activities [25], while integrons as mobile genetic elements are mediators of horizontal gene transfer and, thus, important factors for the widespread distribution of ARGs in the environment [27,28]. Consistent with this, the distribution of *intl1* steadily followed the trophic gradient and was more abundant in winter, as were the majority of ARGs analyzed in this study. In summer, relative abundance was particularly high in the mesotrophic area. Nevertheless, previous studies have shown that the scenario in the coastal environment is very complex due to the various external sources of ARGs [3,29].

3.2. Correlation between ARGs, intl1, and Physico-Chemical Parameters Seasonally

In this study, most ARGs showed significant seasonal variation, with relatively higher relative abundance in winter. It is noteworthy that the relative abundance of *intl1* correlated negatively with PE and Chl *a* and positively with FIB along the trophic gradient, indicating *intl1* as a reliable marker of anthropogenic influence in the natural environment. Furthermore, the positive correlation of *intl1* with the majority of clinically important ARGs analyzed in this study, especially the *bla*, *tetA*, and *mphA* genes, underscores the relationships between the anthropogenic impact and the distribution of ARGs. The significant correlation between *tet* genes and *intI1* has also been frequently observed in various aquatic environments [23], where it plays a key role in the integration of exogenous ARGs into the bacterial genome [30,31]. These co-occurring ARGs are often associated with integrons, transposons, or plasmids that enable the transfer of ARGs between different bacterial taxa [32]. In this context, several genetic determinants of antimicrobial resistance, such as bla_{TEM}, *sul1*, and *intI1*, have already been proposed as valuable indicators of contamination to assess the level of antimicrobial resistance in the environment [33]. In addition, a high concentration of organic and inorganic matter introduced by submarine sewage effluents [6] or river inflows stimulates the growth of bacterioplankton [34] and virio-plankton, as the abundance of viruses is closely correlated with the abundance of bacteria [35]. Bourdonnais et al. [36] investigated the abundance of ARGs in the English Channel (North Sea) and found that the *sul1* and *intI1* genes were positively correlated with dissolved oxygen, while the microbial population was also correlated with pH, TEMP, and SAL in addition to dissolved oxygen and turbidity.

In the oligotrophic zone, the main driving factors with positive correlation were dissolved inorganic nutrients, suggesting that nutrient loading may further promote the maintenance of ARGs in the natural aquatic environment [37]. Environmental stresses such as extreme temperatures, pH, and SAL may induce bacteria to cope with these stresses through phenotypic and genotypic adaptations that enable subsequent resistance to similar stresses [38]. Indeed, TEMP was found to have a negative effect on the distribution of all

analyzed ARGs except *tetA* in the mesotrophic zone. Moreover, the abundance of *ermB* showed a strong positive correlation with SAL and dissolved oxygen in the mesotrophic zone in winter. In summer, SAL was positively correlated with all ARGs except *tetA*, whose abundance was positively influenced by inorganic nutrients, as previously shown [39]. Conversely, the distribution of *tetA* at mesotrophic and oligotrophic sites in winter was mainly influenced by HNA, HNF, HB, and VLP. Previously, Ordulj et al. [40] found the lowest virus abundances in summer at coastal sites in the central and southern Adriatic.

The macrolide gene *ermB* correlated positively only with *aac3-2* in summer but with $bla_{\text{CTX-M}}$ and bla_{TEM} in winter. Regarding the biological parameters, *ermB* showed a positive correlation with HNA, HNF, HB, and VLP at eutrophic sites in winter and an opposite correlation in summer. Notably, the distribution of *ermB* did not follow the trophic gradient and also showed no positive correlation with the nutrient/FIB increase, so it cannot be considered a biomarker for tracking antibiotic resistance.

3.3. Association of Quantified ARGs with Bacterial Communities along the Trophic Gradient

The co-occurrence network analysis revealed a significant association of the ARGs quantified in this study with the most abundant bacterial classes along the trophic gradient. A significant relationship between the bacterial populations and the ARGs was observed in both marine [41] and estuarine environments [42]. The *intI1* gene in pollution-impacted surface waters was found to be distributed mainly between *Gammaproteobacteria*, such as clinically relevant families of *Enterobacteriaceae, Pseudomonadaceae*, and *Aeromonadaceae*, and co-occured with genes encoding beta-lactamase, sulfonamide, aminoglycoside, and tetracycline resistance [43]. The *sul1*, *sul2*, and *tetA* are commonly found with *intl1* in broad host range plasmids, contributing to the spread of multiresistance in aquatic environments [44]. *Enterobacteriaceae*, particularly *E. coli*, *Enterobacter* spp., and *Citrobacter* spp., can simultaneously carry these resistance determinants [45,46].

Dželalija et al. [8] reported that *tetA* was associated with *Enterobacterales* (*Proteus* spp.), *Gammaproteobacteria* (*Vibro* spp.), and *Bacilli* (*Bacillus* spp.), increasingly in the winter season, in eutrophic and mesotrophic areas. In this study, a higher positive correlation was found with these taxa and, furthermore, with *Bacteroidia, Pseudomonadales*, and *Clostridiales.* The seasonal shift of *tet* genes toward a higher relative abundance in winter could be related to sewage discharges and runoff from the mainland, as speculated previously [8].

Furthermore, we have already predicted that *sul* genes are associated with a high abundance of *Gammaproteobacteria* in the eutrophic zone, especially in the estuary in winter, and are carried by *Acinetobacter*, *Enterobacter*, and *Aeromonas* further along the trophic gradient [8]. The detection of these genes in high relative abundances by qPCR in this study, thus, provides further evidence of their persistence in the human-impacted aquatic environment, where *Protebacteria* (mainly *Gammaproteobacteria*) are the predominant carriers of ARGs [19,47,48]. Beta-lactam-encoding genes (bla_{TEM} and bla_{VIM}) were detected more frequently in summer, especially in eutrophic areas, which could be associated with a high abundance of *Vibrio*, *Acinetobacter*, and *Citrobacter* as their probable carriers [8]. On the other hand, the abundance of $bla_{\text{CTX-M}}$ did not decrease away from the coast, which could be explained by the shift of the community from *Gammaproteobacteria* in winter to Marine group II in summer at mesotrophic and oligotrophic sites [8].

The macrolide resistance genes *ermB* and *mphA,* which were among the most frequently detected ARGs, may be related to *Acinetobacter*, *Bacteroidetes*, and *Bacillus*, all of which are highly abundant in coastal marine microbiomes [8]. Consistent with the results of our study, Li et al. [19] reported that *Bacteroidetes* are positively correlated with several ARGs in the marine environment, while *Proteobacteria* are correlated with *intl1* [49].

4. Materials and Methods

4.1. Study Area and Determination of Environmental and Biological Parameters

Water samples from the bottom (extension B in sample name) and surface (extension S) layers were collected seasonally in March (winter) and August (summer) 2021 in the

central Adriatic Sea (Croatia), as previously described [8] (Table S1). The six sampling sites were chosen based on geography and anthropogenic impact along the trophic gradient over a 68 km line from the wastewater-influenced estuary to coastal area and the pristine open sea (Figure 7). The eutrophic JS represents the site within the Jadro River estuary affected by unmanaged wastewater from households [5]. The river further flows into the Vranjic Bay, represented by eutrophic coastal VS/VB site. The following KSS site is located near a wastewater-impacted public beach on the coast of the nearby Kaštela Bay [13] and is also eutrophic. KBS/KBB site is mesotrophic and located in the center of Kaštela Bay but is still influenced by the mainland, while the offshore oligotrophic station SCS/SCB further follows the trophic gradient. The last sampling site, an open-sea STS/STB, is located near the island of Vis, one of the most remote islands in the eastern Adriatic, and is considered a pristine marine environment.

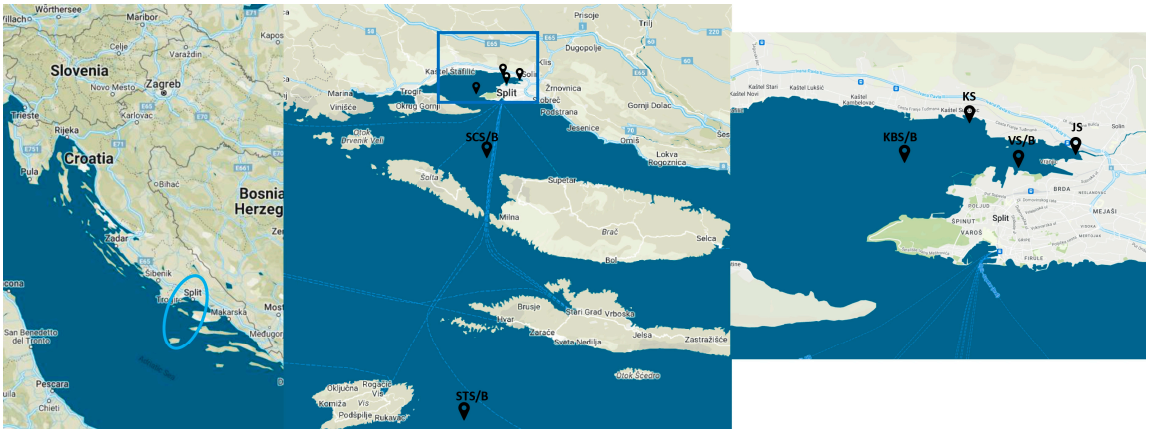

Figure 7. Map of the study area and sampling sites in the central Adriatic Sea. JS, Jadro River; KS, Kaštel Sućurac; VB, Vranjic Bay (bottom layer); VS, Vranjic Bay (surface layer); KBB, Kaštela Bay (bottom); KBS, Kaštela Bay (surface); SCB, Split Channel (bottom); SCS, Split Channel (surface); STB, Stončica (bottom); STS, Stončica (surface).

Physico-chemical and biological parameters were determined previously, and the data and standard protocols were given previously [8] (Table S1). These included the following: temperature (TEMP), salinity (SAL), pH, dissolved inorganic nutrients (NO_3^-, NO_2^-, NH_4^+, and PO_4^{3-}), dissolved oxygen (O_2), chlorophyll *a* (Chl *a*), abundances of *Synechococcus* (SYN), *Prochlorococcus* (PRO), picoeukaryotes (PE), heterotrophic bacteria (HB), heterotrophic nanoflagellates (HNF), and viruses, as well as bacterial production and enumeration of fecal indicator bacteria (FIB) (*E. coli* and intestinal enterococci).

4.2. Antibiotic-Resistant Heterotrophic Bacterial Counts

Aliquots (100 µL and 1 mL) of seawater were plated on Marine agar (MA; BD Difco, Franklin Lakes, NJ, USA) plates without antibiotics, while 10, 25, 50, and 100 mL of seawater were filtered using 0.22 µm pore-size mixed cellulose ester (MCE) membranes (Whatman, Maidstone, UK), which were placed on MA plates supplemented with an appropriate antibiotic from the β-lactam, sulfonamide, macrolide, tetracycline, or aminoglycoside class to enumerate resistant and total heterotrophic bacteria. Based on the minimal inhibitory concentration (MIC) breakpoint interpretive for antibiotic resistance provided by Clinical and Laboratory Standards Institute [50] and The European Committee on Antimicrobial Susceptibility Testing [51], the plates were supplemented with 32 µg/mL of azithromycin (AZM), 4 µg/mL of cefotaxime (CTX), 16 µg/mL of gentamicin (GEN), 512 µg/mL of sulfamethoxazole (SXT), and 16 µg/mL of tetracycline (TET). Experiments were performed in triplicates. Colonies were counted after the incubation at 28 °C for 7 days, and the frequency (%) of antibiotic-resistant bacteria (i.e., number of antibiotic-resistant colony-forming units

(CFU) per ml of water divided by total CFU, multiplied by 100) was determined for each sample. All antibiotics were purchased in the form of powder from Sigma-Aldrich (St. Louis, MO, USA).

4.3. DNA Extraction and Quantification of Target Genes

Two liters of water were filtered in triplicate using fast-flow 0.22 μm pore-size MCE membranes (Whatman), after which the genomic DNA was extracted using PowerWater DNA Isolation Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA concentration was measured using NanoDrop® spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA), and DNA was stored at −20 °C for further analysis.

Quantitative real-time PCR (RT-qPCR) was used to quantify the 16S rRNA gene, class 1 integron-integrase gene (*intl1*), and ten ARGs, including sulfonamide resistance genes (*sul1*, *sul2*), tetracycline resistance genes (*tetA, tetB*), β-lactam resistance genes ($bla_{\text{CTX-M}}$, bla_{TEM}, bla_{VIM}), macrolide resistance genes (*ermB*, *mphA*), and aminoglycoside resistance gene (*aac3-2* encompassing *aac(3′)-IIa/aacC3/aacC2* and *aac(3′)-IIc/aacC2*) using primers and amplification conditions listed in Supplemental Table S6. The specificity of all primers was confirmed by solubility curve analysis. Genes of interest were first amplified from the genomic DNA of antibiotic-resistant bacteria, separated by standard gel electrophoresis, purified using ReliaPrep™ RNA Clean-Up and Concentration System (Promega, Southampton, UK), and then cloned into pGEM®-T Easy Vector or pNORM plasmids (Promega) according to the manufacturer's recommendations. The recombinant plasmids were then transformed into competent *E. coli* JM109 (Promega). After positive clones were selected and verified by PCR, the plasmids were used to prepare qPCR standards. Each qPCR reaction was performed in a total volume of 20 μL using 10 μL Power SYBR® Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA), 1 μM of each primer, and 2 ng of DNA template in 96-well plates. All assays were carried out on the ABI 7500 Fast Real-time PCR System (Applied Biosystems). The efficiency and sensitivity of each assay were determined by generating a standard curve using 10-fold serial dilutions of plasmid DNA. Reliable correlation coefficients ($R^2 > 0.99$) and amplification efficiencies based on slopes between 98% and 105% for standard curves were obtained. Melting curves were generated to confirm amplification specificity. All qPCR assays were performed using three biological replicates and two technical replicates, whereas a non-template sample (containing DNA-free water) was included as a negative control.

4.4. Data Analysis and Statistics

RT-qPCR data were given as the relative abundance of the target gene in each sample, calculated as the ratio of ARG or *intl1*/16S rRNA gene copy number (*rrn*), log10 transformed and subjected to a Shapiro–Wilk test to assess their normal distribution. Data were then compared using a two-way analysis of variance (ANOVA). Sidak's multiple comparisons test was performed to compare the relative abundance of genes at each site between two seasons and visualized using GraphPad Prism V9.01. Statistically significant differences in gene abundances and environmental parameters were evaluated using the program R (v3.4.2) and redundancy analysis (RDA) using the packages vegan, ggplot2, labdsv, MASS, and mvpart. Gene frequencies were normalized using the Hellinger transformation, and environmental parameters were standardized. To investigate the correlation effect of ARGs mutually, a Spearman rank correlation test was performed, and correlation matrices were developed. The statistical analysis was performed with the software Rstudio (v2023.09.1 + 494) using the packages agricolae, stats, and corrplot.

In our previous study [8], we conducted a comprehensive metagenomic analysis. In brief, 16S rRNA amplicon sequencing of the V3-V4 hypervariable region was performed. The 250 bp paired-end raw reads were merged, and high-quality clean tags were obtained after quality filtering with QIIME2 (v2022.2). Sequences with ≥97% similarity were assigned to the same Operational Taxonomic Units (OTUs) by the Closed Reference clustering method. The OTU sequences were classified using the vsearch

method and the SILVA database (v.13.8) to determine relative taxa frequencies and construct a phylogenetic tree. Diversity analysis and functional prediction were performed using QIIME2 (v2022.2) and PICRUSt2 on the Nephele platform [52,53]. Filtering of metagenomic data for visualization was performed using the in-house program MiaViz (https://github.com/apavlinovic/mia-viz; accessed on 22 September 2023) and Python 3.9.0 (library: Pandas, SciPy, Seaborn).

The co-occurrence network was constructed to show the correlations between the genes (Pearson's correlation) using the cooccure package in R. All statistical tests were considered significant at $p < 0.05$. Mantel test and Pearson's correlation were applied to assess the relationships between the bacterial communities at the class level and the abundances of ARGs using the ggcor package in R.

5. Conclusions

In the present study, the distribution of ARGs denoting resistance to five major antibiotic classes was characterized in the central Adriatic by combining culture-dependent and culture-independent techniques, including RT-qPCR and metagenomic sequencing, which provided further insights into the profiles of ARGs and specific bacterial taxa as their carriers in the marine environment. As also revealed by metagenomics, a higher resistance was detected by qPCR in the winter eutrophic zone, which was due to the high relative abundance of *tetA*, $bla_{\text{CTX-M}}$, and bla_{TEM} genes associated with the higher TEMP, higher nutrient loading, and lower SAL, as well as the high abundance of *Gammaproteobacteria* such as *Acinetobacter* and *Citrobacter*. A different trend was observed in the oligotrophic zone, where *tetA* was mainly driven by TEMP and HNF, while dissolved oxygen and SAL were responsible for *ermB*. In addition, *intl1* steadily followed the trophic gradient, positively correlated with FIB, and was more abundant in winter, suggesting that this gene is a favorable gene marker for tracking anthropogenic impacts in the natural environment. A similar pattern was also observed for bla_{TEM} and bla_{VIM}. Water column stratification and geography significantly influence ARGs distribution, favoring eutrophic, especially estuarine, areas, while seasonal changes in oligotrophic sites result from a shift in bacterial communities from *Gammaproteobacteria* in winter to Marine group II in summer.

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3. DISCUSSION

The AR is recognized as a major and a global public health problem, with millions of people dying annually from infections caused by antibiotic-resistant pathogens[1]. The ARGs and ARB have been found in various environments, including hospital settings and diverse natural habitats, whereas human activities tend to play a major role in the spread of AR determinants towards human pathogens. Therefore, it has become imperative to emphasize the significance of studying this phenomenon simultaneously in clinics and the environment, as highlighted by the WHO under the One Health concept.

It is important to note that the ocean and marine ecosystems are the largest natural habitats on Earth, yet are still among the least studied in terms of AR. More research is needed to understand which genetic determinants conferring nonsusceptibility to clinically relevant antimicrobial agents are present in marine bacteria and how they further disseminate to other microorganisms and environments. The aim of this PhD research is therefore to fill this gap by investigating how AR varies seasonally and spatially in the marine environment and what are the key driving factors that shape the marine bacterial community and consequently, the marine resistome. In addition, the aim was to quantify the targeted ARGs by RT-qPCR along the trophic gradient, and molecularly characterize the isolates of WHO 'critical' and 'high priority' pathogens, including Carbapenem-Resistant Enterobacteriaceae (CRE) and vancomycin-resistant *Enterococcus faecium* (VREfm), recovered from the coastal marine environment of the central Adriatic.

3.1 Microbial diversity along the trophic gradient of the central Adriatic Sea

To investigate temporal and spatial variations in the structure and diversity of marine bacterial communities along a trophic gradient, we studied a total of 80 microbiomes, recovered seasonally from the bottom layer and surface waters along a 68-km transect from wastewater-impacted estuary to coastal and pristine open sea in the central Adriatic, using 16S rRNA amplicon sequencing, PICRUSt2 bioinformatics and biostatistics. The PICRUSt2 tool was used to functionally annotate the 16S rRNA amplicon sequences in relation to antibiotic resistance mechanisms according to the KEGG Brite Antimicrobial Resistance Genes Database (KO01504). The indigenous planktonic bacterial community was compared with the antibiotic-

resistant ones, including the TET- and SXT-resistant bacterial communities, to gain insight into their taxonomic and functional structures, which we hypothesize are determined by the specific environmental factors that influence their dynamics as carriers of ARGs and shape the marine resistome.

The central Adriatic has long been anthropogenically influenced, particularly by wastewater discharges, leading to the emergence of clinically relevant pathogens and ARGs in the Jadro River estuary[25] and the coastal waters [140,141]. As expected, counts of faecal indicator bacteria confirmed wastewater pollution at the coastal sites JS and KSS. In contrast, open waters, including Stončica (STS, STB), remained relatively unaffected by long-term human activities and are considered pristine environments. Proteobacteria were found to be the predominant components of the microbial community and the major contributor to the marine resistome across the trophic gradient. This is consistent with previous observations where Proteobacteria were found to be predominant in seawater from both pollution-prone environments [22] and those with minimal human impact[9,34]. Marine Proteobacteria, particularly Alpha- and Gammaproteobacteria, have also been identified as the most abundant phylum in both deep-water[19,142] and shallow coastal Adriatic[29].

Similar to previous reports, Alphaproteobacteria mainly comprised *HIMB11* and *AEGEAN-169* lineages from nearshore to open sea, while *SAR11* (including Ia, II, and III subclades), which dominate the ocean bacterioplankton[143], were highly abundant in oligotrophic waters, particularly during summer. Additionally, open surface waters (STS) in summer were differentially enriched by marine gammaproteobacteria *Litoricola*, *SAR116*, and *SAR86* lineages, all of which were previously identified among the most common proteobacteria genera in similar environments[19]. The predominance of marine *Synechococcus* over other cyanobacteria was also previously observed in the Adriatic[19,29,142]. Furthermore, *Firmicutes*, typically commensals of the human gut, were expected to be among the leading taxa in microbiomes emerging from human-impacted coastlines [144]. Accordingly, they prevailed from eutrophic estuarine sites (JS and KS) to mesotrophic coastal sites (VS, VB, KBS, and KBB), demonstrating their spatial arrangement along the trophic gradient.

3.2 Metagenomics reveals that seasonality is the main driving factor of microbial diversity along the trophic gradient

Microbiomes from sites most impacted by anthropogenic activities, such as river estuaries and nearshore sites, were found to be enriched by human gut-associated bacteria, particularly Clostridiales and Enterobacterales. Open-sea site STB was the most distinct from other sites, primarily due to the high prevalence of classes native to this pristine area, such as marine Alphaproteobacteria, Nitrososphaera, Thermoplasmata, and Cyanobacteria. The abundance of intestinal commensals and soil bacteria decreased away from the coast, resulting in a shift in the resistome towards the intrinsic resistance mechanisms of native marine bacteria. In such habitats, the effect of seasonality is profound and primarily related to the stratification of the water column, driving differences between surface and bottom resistomes in summer compared to the winter period. Notably, the bottom layer microbiome at KBB was the only coastal microbiome highly abundant in intestinal genera (*Blautia*, *Bacteroides*, *Faecalibacterium*) in summer due to stratification of the water column. Far fewer differences were observed between resistomes in summer and winter along the coast, primarily due to the influx of allochthonous bacteria and their genes through sewage discharges and mainland runoff. In winter, higher abundances of intestinal bacteria in coastal waters were observed because seawater is kept closer to the outlets due to coastal relief and winds. Additionally, lower insolation and surface temperature in winter may have facilitated bacterial stress, promoting higher survival rates compared to summer[145]. Communities in summer exhibited higher species richness than in winter and were represented by most identified phyla and classes, with considerable variations in the proportion of the ten most abundant orders and families. Increased human activities in the coastal Adriatic during summer, such as extensive tourist activities and nautical traffic, may contribute to the influx of allochthonous bacteria into the ecosystem, while specific climatic features, such as winds, allow for their further dispersal along the trophic gradient. Additionally, seasonally driven factors contribute to the bloom of native marine bacteria characteristic of this ecosystem, as previously observed by Korlević and colleagues.[29].

Moreover, microbial picoplankton communities in the Adriatic are known to rapidly respond to changes in environmental conditions[146,147]. Moreover, top-down controls such as grazing and viral lysis may have contributed to community richness. The abundance of viruses doubled in summer, indicating their higher activity in this area, as confirmed previously by Ordulj and colleagues [148]. Furthermore, non-selective grazing by heterotrophic nanoflagellates (HNF)

controls the number of bacterial populations, while species-specific viral lysis could determine the size of bacterial groups and thus affect microbial diversity[149]. Additionally, both community richness and diversity decreased with distance from the coast, following the decrease in chlorophyll *a* and a shift from eutrophic to oligotrophic conditions in the studied area[147]. Moreover, most human-influenced sites had elevated richness and diversity indices, while the deepest sampling point in this study, the bottom layer at a depth of 100 meters (STB), had the lowest taxa richness and diversity, supporting the hypothesis that human-derived pollution contributes to microbial diversity[88]. Surface communities differed from bottom layer microbiomes across the trophic gradient, with more significant differences observed with increasing depth.

Notably, after the formation of the thermocline during summer, surface communities at mesotrophic (KBS) and oligotrophic sites (SCS, STS) became more influenced by temperature, depicting biomarkers for different trophic areas. For instance, *Faecalibacterium*, *Blautia*, and *Bacteroides* were characteristic of the KBB microbiome at the bottom layer at 35 meters depth, in contrast to *Cutibacterium* and *Marinobacter* at the surface. *Pseudomonas* was differentially enriched at 50 meters depth in SCB, while alphaproteobacterial subclades II and III of the *SAR11* lineage characterized its surface microbiome. Interestingly, several taxa within *Archaea* were most abundant in the indigenous open-sea microbial community (STB) at 100 meters depth, including *Thermoplasmatota* Marine group II and *Thaumarchaeota "Ca. Nitrosopumilus"* and *"Ca. Nitrosopelagicus."* These bacteria can oxidize ammonia and nitrate as energy sources, allowing them to live heterotrophically in nutrient-deficient environments, mainly in coastal waters[29,150].

3.3 Seasonality is a key driving force for ARGs distribution along the trophic gradient based on qPCR quantification

To gain more comprehensive view on the distribution of clinically relevant ARGs along the trophic gradient, ARGs encoding resistance to five major antibiotic classes, including sulfonamides (*sul1, sul2*), tetracyclines (*tetA, tetB*), β-lactams (*bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{VIM}}$), macrolides (*ermB, mphA*), aminoglycosides (*aac3-2*), and integrase gene (*intl*1) were further quantified in seawater by RT-qPCR. In addition, their distribution was investigated in relation to the effect of environmental factors and the structure of the bacterial community obtained by metagenomics.

Notably, significant seasonal variation was observed for most ARGs, with a relatively higher abundance noted during winter months. The *intl1* gene, which is known as an indicator of anthropogenic activities in aquatic environments [151], is found to mirror the trophic gradient with augmented abundance in winter as were the majority of ARGs analyzed in this study.

It was found that the prevalence of TET resistance was increased across the trophic gradient, diminishing away from the coast and was lowest in oligotrophic waters, indicating an anthropogenic imprint, as coastal waters act as receptors for sewage discharges and effluents from aquaculture facilities. Namely, TET is widely used as a growth promoter in aquaculture[152] and human medicine, and excreted into the environment in an active form[153], thereby contributing to its ubiquitous presence in marine ecosystems. Based on the qPCR results, the *tetA* gene was found to be the most abundant ARG in winter and the second most abundant in summer along the trophic gradient, which is consistent with the results of the TARA Oceans project, where *tetA* and *tetB* were identified as the most prevalent oceanic ARGs[154]. In addition, PICRUSt2 functional prediction based on the 16S rRNA amplicon sequencing showed that the *tetA* and *tetB* genes were highly represented in marine microbial communities of the eutrophic and mesotrophic areas in this study.

Moreover, qPCR analysis confirmed high prevalence of sulfonamide resistance genes *sul1* in the estuary and *sul2* in the bottom layer of the oligotrophic zone, which is consistent also with metagenomic analysis. Moreover, these genes tend to be among the most prevalent ARGs in surface marine waters [155], especially the beach waters [156]. They were found to generally exhibit greater resilience and a higher propensity for increased abundance compared to other ARGs in

the environment [157,158], due to which are highly abundant in both eutrophic and mesotrophic zones and display seasonal stability.

Beta-lactam resistance in this study showed seasonal variation along the trophic gradient and corresponded to the frequency of the *bla*$_{\text{CTX-M}}$ gene, which was more abundant in winter at both mesotrophic and oligotrophic sites. In addition, the *bla*$_{\text{TEM}}$ gene showed an increased relative abundance in coastal waters and showed a strong correlation with anthropogenic influences[151,156,159]. On the other hand, the aminoglycoside resistance gene *aac3-2* exhibited diminished abundance across the trophic gradient, evincing no discernible escalation despite its higher prevalence in anthropogenically impacted coastal areas [160].

The analysis further showed that several ARGs showed strong correlation between each other and regard to specific environmental parameters. Notably, the relative abundance of *intl1* exhibited a negative correlation with particulate organic carbon and chlorophyll *a*, while positively correlating with fecal indicator bacteria along the trophic gradient, underscoring its efficacy as a robust marker of anthropogenic influence in natural environments. Moreover, the positive correlation between *intl1* and the majority of clinically relevant ARGs, particularly *bla, tetA,* and *mphA* genes, highlights the interplay between anthropogenic impact and ARG distribution. The co-occurrence of *tet* genes and *intI1* observed in this study was previously reported in various aquatic environments[161] where it is involved in integrating exogenous ARGs into bacterial genomes[38,162].

Moreover, there is evidence how particular biotic factors influence the distribution of targeted ARGs depending on the trophic status. The abundance of *tetA* at mesotrophic and oligotrophic sites during winter was primarily influenced by specific biological parameters such as high nucleic acid bacteria, heterotrophic nanoflagellates, heterotrophic bacteria, and virus-like particles. Previously, Ordulj and colleagues[148,163] found the lowest viral abundances in summer in coastal waters of central and southern Adriatic.On the other hand, in summer, abundance of *tetA* was positively influenced by inorganic nutrients, as previously shown[164].

This is associated with the fact that organic and inorganic matter from sewage effluents and river inflows stimulates the proliferation of bacterioplankton[19] and virioplankton, as evidenced by the correlation between the abundance of viruses and bacteria[165]. Study conducted in the English Channel (North Sea) revealed positive correlations between *sul1* and *intI1* genes with

dissolved oxygen, pH, temperature, and salinity, in addition to dissolved oxygen and turbidity[166]. On the contrary, *ermB* distribution did not align with the trophic gradient and showed no positive correlation with nutrient or fecal indicator bacteria increase, indicating its unsuitability as a biomarker for AR tracking.

3.4 Quantified ARGs are primarily associated with Gammaproteobacteria along the trophic gradient

The co-occurrence network analysis revealed a significant association between the ARGs targeted by qPCR and the most abundant bacterial classes across the trophic gradient. For instance, the *intI1* gene, prevalent in pollution-impacted surface waters as was the case in this study, was predominantly associated with Gammaproteobacteria, including the Enterobacteriaceae, and frequently co-occurred with genes encoding resistance to multiple classes of antibiotics[167]. Moreover, a high positive correlation of *tet* genes with Enterobacteriales (*Proteus* spp.), Gammaproteobacteria (*Vibro* spp.) and Bacilli (*Bacillus* spp.) was observed particularly in the winter season in eutrophic and mesotrophic areas which could be attributed to sewage discharges and mainland runoff. Furthermore, *sul* genes were also primarily associated with Gammaproteobacteria in the eutrophic zone, and were carried by *Acinetobacter*, *Enterobacter,* and *Aeromonas* further along the trophic gradient, pointing to the relevance of this phylum for ARGs disperse in aquatic environments[155,168,169]. Similarly, the macrolide resistance genes *ermB* and *mphA*, among the most frequently detected ARGs, could be due to *Acinetobacter*, *Bacteroidetes*, and *Bacillus* high abundance in coastal marine microbiomes. These findings align with previous studies reporting positive correlations between Bacteroidetes and several ARGs in marine environments[155] ,

The *bla* genes, on the other hand, did not show unique distribution along the trophic gradient. The bla_{TEM} and bla_{VIM} were more frequently detected in summer, particularly in eutrophic areas, and appear to be associated with high abundance of *Vibrio*, *Acinetobacter*, and *Citrobacter*. However, the relative abundance $bla_{\text{CTX-M}}$ did not decline away from the coast, possibly due to a shift in the community composition from Gammaproteobacteria in winter to Marine group II in summer at mesotrophic and oligotrophic sites.

3.5 Pathogens belonging to Gammaproteobacteria and Firmicutes mostly contributed to the marine resistome, as revealed by metagenomics

The microbiome analysis revealed that the most pathogenic genera belonged to Proteobacteria and Firmicutes, and their taxonomic diversity and contribution to the marine resistome was investigated more in detail. During winter, pathogenic genera typical of sewage-influenced waters persisted in estuaries and nearshore areas (e.g. *Bacteroides, Serratia, Enterococcus, Arcobacter*)[28], while in summer, they were more dispersed along the gradient, affecting the resistome structure. Conversely, *Pseudomonas, Acinetobacter*, and *Vibrio* were present along the gradient throughout the year. Similarly, Zeng and colleagues identified *Escherichia*, *Bacteroides*, and *Clostridium* as the predominant bacterial hosts [170] of ARGs in gut-associated environments, whereas *Alteromonas, Vibrio*, and Proteobacteria were predominant in pristine environments[145]. Moreover, SXT- and TET-resistant microbiomes were found to be highly enriched by species from the Pseudomonadales, supporting their broad spectrum of AR[171]. Notably, we found that multidrug-resistant *Pseudomonas* and *Acinetobacter* isolates recovered from estuarine and coastal waters studied herein owe their resistance phenotype to the overexpression of intrinsic efflux pumps and carriage of various acquired resistance genes, including β-lactamase genes[25,172]. In addition, VREfm were isolated from the wastewater-impacted beach water in this study, which is consistent with the fact that Firmicutes were among the major components of wastewater[173] as well as the effluent-receiving coastal waters[28,174]. It is particularly worrisome, however, that we detected negative shifts in the diversity of beach water microbiome and associated resistome due to the wastewater leakage from the adjacent outfall, even though the beach water was of excellent quality based on legislation currently in force in Croatia.

When referring to pathogens comprising the surface- and seafloor-derived TET- and SXT-resistant communities, it was found that they differed more in summer than in winter, as was also the case for the native communities, demonstrating seasonality. Notably, there were genera that persisted in the resistant communities regardless of the season and contributed significantly to the resistome. Namely, it was found that TET-resistant communities mediated resistance through a combination of mechanisms, including upregulation of multidrug efflux pumps (norB, norC, MexAB-OprM, AcrAB-TolC) and *tet*-like genes (*tet35, tetA, tetB*), predominantly carried by staphylococci, *Pseudomonas*, Enterobacteriaceae, as well as autochthonous marine genera such as *Pseudoalteromonas*, *Vibrio*, and *Halomonas*[175–179]. Similarly, SXT-related

resistomes were predominated by mechanisms driving resistance to SXT, such as multidrug efflux pumps of *Pseudomonas* (MexAB and MexEF) and *Acinetobacter* (AdeABC, AdeIJK)[180], as well as *sul1* and *sul2* genes carried by multiple genera.

In addition, the most common pathogenic genera in the surface beach in summer were *Arcobacter, Acinetobacter, Bacteroides, Prevotella, Pseudomonas* and *Streptococcus*, which is a direct consequence of the wastewater inflow. Consequently, these genera, especially *Arcobacter, Acinetobacter, Bacteroides* and *Pseudomonas*, contributed significantly to the beach resistomes. Notably, *Arcobacter* spp. are found to be resistant to various classes of antibiotics[181]. Similarly, the second most abundant pathogenic genus, *Acinetobacter*, is predominantly associated with the multidrug resistance[182]. This is consistent with results from similar studies, as it is known that a resistance-nodulation-cell division (RND) efflux pump AdeABC expels various drug classes out of the bacterial cell, such as aminoglycosides, tetracyclines, phenicols, fluoroquinolones, and some beta-lactams, leading to the multidrug resistance phenotype[183]. Several other chromosomally encoded RND efflux systems were also found to be more abundant in beach water microbiome, particularly MexAB-OprM, MexJK-OprM, and MexXY-OprM whose overexpression in *Pseudomonas aeruginosa* results by nonsusceptibility to multiple classes of antibiotics[184]. This was also a case for AcrEF-TolC pump[185], which is one of the main instruments of intrinsic resistance in *E. coli* as well as MdtEF-TolC whose increased activity in *E. coli* leads to active export of cationic biocides, including glycopeptide antibiotics[186]. Moreover, it is interesting to note that the beach water microbiome was more enriched in *VraFG* transporter genes, which are involved in the formation of a five-component system that mediates resistance of *Staphylococcus aureus* to cationic antimicrobial peptides[187].

3.6 WHO 'critical' and 'high priority' pathogens are identified in human-impacted estuarine and coastal marine waters of central Adriatic

Anthropogenic activities, including a discharge of wastewater into coastal surface waters, are known to be hotspots for the spread of MDR pathogens into natural water bodies, posing a significant threat to human and environmental health[127]. Human-gut associated bacteria, among which Enterobacteriaceae in particular, contribute greatly to the enrichment of environmental resistome whether by partaking in the exchange of ARGs between environmental microbial populations and their transmission to other human pathogens, or *vice versa*[188,189.]. Considering that aforementioned Illumina-based 16S sRNA amplicon sequencing identified human-gut associated bacteria, particularly enterobacteria and enterococci, in microbial communities recovered from human-impacted coastal waters in this study, we aimed to isolate these pathogens by using culture-dependent methods and perform their molecular characterization in terms of genotyping and identification of their resistance genotype. Herewithin, we report for the first time, the emergence of WHO 'critical' and 'high priority' pathogens including CRE and VREfm in the coastal marine environment of the central Adriatic.

A total of 35 waterborne VREfm was recovered, including 4 isolates from surface waters at public beach Gojača in Kaštel Sućurac and 31 from effluent-receiving coastal waters in Split area, which were compared with 15 surveillance VREfm from University Hospital Centre Split to investigate their resistance background and routes of introduction into the aquatic environment. Notably, VREfm recovered from wastewater, coastal bathing waters and hospital shared similar virulence, multidrug resistance, and sequence type (ST) profiles, posing a major public health threat. Notably, all studied isolates carried the *vanA* gene, while one clinical isolate also possessed the *vanC2/C3* gene. Only a few studies have reported the *van* genotype of marine VREfm isolates, including those from the United States and Puerto Rico, but the isolates have not been further molecularly characterized or compared with hospital strains [190,191]. After its first identification in a hospital strain in 2020[192], the consistent detection of the *vanA* gene in both the environmental isolates and the clinical surveillance isolates in this study evidences its widespread in southern Croatia, as it is already the case in other countries [193,194]. In addition, the hospital strains largely carried the aminoglycoside-resistance genes *aac(6')-Ie-aph(2")-Ia*, but also harbored *aph(2")-Ib* and *aph(2")-Id* which were predominantly detected in the waterborne isolates from this study. Consistent with this, these resistance determinants are commonly associated with high-level aminoglycoside-resistant (HLAR) phenotype in

enterococci[195,196]. The isolates also had a similar virulence profile, with the *hyl* gene, which encodes hyaluronidase, being the most prevalent virulence gene in both environmental and clinical isolates. Moreover, typing revealed that the isolates belonged to 10 different STs, all within the clonal complex CC17, a major epidemic lineage associated with hospital infections and outbreaks, with ST117 and ST889 common to waterborne and hospital isolates. Notably, the fact that the majority of hospital and environmental isolates from this study, including isolates from wastewater and beach water, belonged to these STs indicates a particular importance of these lineages for the spread of VREfm in the community and hospital and emphasizes their sewage-driven dissemination in southern Croatia.

Moreover, a total of 78 WHO 'critical priority' pathogens CRE was isolated from coastal waters of the central Adriatic (Kaštela Bay, public beach Trstenik and estuary of Jadro river) using selective media. Thirty-eight isolates belonging to 9 enterobacterial species were confirmed by PCR to carry at least one carbapenemase encoding gene, among which $bla_{\text{KPC-2}}$, $bla_{\text{OXA-48}}$, or $bla_{\text{VIM-1}}$. A majority of carbapenemase-producing *Klebsiella, Citrobacter* and *Enterobacter* isolates exhibited XDR phenotype, which is frequently associated with infections that are difficult to treat and are of higher mortality[197]. The carbapenemase-producing isolates were further genotyped, screened by PCR for the presence of selected ARGs, including those encoding for ESBLs and AmpC β-lactmases, and their carbapenemase encoding plasmids sequenced and *de novo* assembled.

The $bla_{\text{KPC-2}}$ was the predominant carbapenemase gene identified in 38 isolates, followed by $bla_{\text{OXA-48}}$ in 19, and $bla_{\text{VIM-1}}$ in 7 CRE isolates. Of note, two isolates of *C. freundii* and one each of *K. pneumoniae, K. variicola* and *E. asburiae* simultaneously carried all three identified carbapenemase genes. Importantly, such a combination of carbapenemases had not been previously reported in these species in marine environment. In addition, 16 of 38 carbapenemase-producing *Enterobacteriaceae* co-harbored two carbapenemase genes, mostly $bla_{\text{KPC-2}}$ in combination with $bla_{\text{OXA-48}}$. One potential explanation for this accumulation of carbapenemase genes could be linked to the enhanced usage of carbapenem antibiotics in clinical settings in Croatia [198–200]. Indeed, Croatia is among the top three and five EU countries in terms of carbapenem consumption in hospital and community in 2020 and 2021 (ECDC, 2022), respectively. Moreover, we should also note that a high prevalence of KPC-producing *K. pneumoniae* is reported in University Hospital Split[201]and also consider a leakage of these

strains in the environment via sewage due to the the fact that they were recovered at coastal sites affected by wastewater.

The KPC-producing isolates of *E. coli, K. pneumoniae and C. freundii* were detected in Croatia first in clinical settings at the north of country [199,202] and later in the southern coastal regions[201]. KPC-producing *K. pneumoniae* was reported for the first time outside of hospital settings in the river water downstream from the sewage outlet of the Zabok General Hospital[203], suggesting the leakage of this strain into the environment via hospital wastewater. Furthermore, *bla*$_{\text{KPC-2}}$- and *bla*$_{\text{OXA-48}}$-positive Enterobacteriaceae, mostly *E. coli* and *K. pneumoniae,* were reported in hospital wastewater from Zagreb[204,205] as well as the treated municipal wastewater from the Zagreb wastewater treatment plant[206], emphasizing the role of unadequatly treated wastewater in harboring and spreading of these 'critical-priority' pathogens. Interestingly, the last study identified *bla*$_{\text{KPC-2}}$+*bla*$_{\text{OXA-48}}$+*bla*$_{\text{VIM-1}}$ genotype in *Kluyvera spp.* and *Raoultella* spp., pointing to the fact that this study might be the first to report environmental *C. freundii, K. pneumoniae, K. variicola* and *E. asburiae* with such resistance genotype.

Regarding the reports of KPC-positive CRE in marine environment in Croatia, Kvesić and colleagues reported KPC-2-producing isolates of *E. coli, K. pneumoniae*, and *C. freundii* in the coastal waters receiving submarine effluents from a wastewater treatment plant in city of Split[28]. Notably, one isolate of *K. pneumoniae* concurrently possessed *bla*$_{\text{KPC-2}}$ and *bla*$_{\text{OXA-48}}$ genes, similar to isolates J231 and J232 recovered in this study from the estuarine water of Jadro river. Nevertheless, estuarine isolates differed from this wastewater isolate by other *bla* genes detected, such as *bla*$_{\text{TEM-1}}$, *bla*$_{\text{SHV-1}}$ and *bla*$_{\text{CTX-M-15}}$.

It is notworthy that *bla*$_{\text{OXA-48}}$ was identified in isolates of nine enterobacterial species, including *K. variicola, K. oxytoca, K. pneumoniae, C. freundii, E. asburiae, E. cloacae, E. coli*, *E. bugandensis*, and *R. ornithinolytica*, and mostly together with *bla*$_{\text{KPC-2}}$. In Croatia, *bla*$_{\text{OXA-48}}$ was reported in CRE isolates from hospitals[207] and hospital wastewater[205], but frequently co-occuring with *bla*$_{\text{NDM-1}}$ carbapenemase gene[205,208]. On contrary, *bla*$_{\text{NDM-1}}$ was not detected in isolates from this study, pointing to the different epidemiology of carbapenemase genes in environment of southern Croatia.

The *bla*$_{\text{VIM-1}}$ gene was detected in *E. asburiae*, *K. oxytoca*, and *C. freundii*, often alongside two or three other carbapenemase genes. Notably, the detection of the *bla*$_{\text{VIM-1}}$ gene in *E. asburiae*

was an uncommon finding, as this gene has only recently been reported in clinical isolates in Italy[209], and was not reported previously in the marine environment. This particular XDR *E. asburiae* isolate (T218) co-harboured *bla*$_{\text{VIM-1}}$ and *bla*$_{\text{KPC-2}}$ which were located separately on conjugative plasmids. *De novo* assemblage of *bla*$_{\text{VIM-1}}$-carrying plasmid pEAT218_VIM revealed that this carbapenemase gene was accompanied by ESBL gene *bla*$_{\text{OXA-10,}}$ as well as others conferring resistance to several high-priority antibiotics, including aminoglycosides (*aac(6')-Ib3*), tetracycline (*tetA*), and plasmid-mediated quinolone resistance (*qnrB6*), along with the virulence gene *astA*. Moreover, multiple plasmid replicons were identified, including Col440l, IncN, and IncL/M(pOXA-48), facilitating the transfer of ARGs as documented by previous studies[210,211]. Previously, Vittoria and colleagues identified a 6 Kb cassette comprising the *bla*$_{\text{VIM-1}}$ determinant, surrounded by *intI1* and followed by *aac(6')-Ib-cr, aph(3')-II, aadA, catB, qacE*, and *sul1*. This cassette has been implicated in the successful dissemination of various international *bla*$_{\text{VIM}}$-carrying plasmids among different *Enterobacterales* [209].

Furthermore, sequencing of conjugative *bla*$_{\text{KPC-2}}$-bearing plasmid pEBT200_KPC from MDR *E. bugandensis* T200 isolate revealed a diverse array of co-occuring ARGs, including *bla*$_{\text{OXA-10}}$, *bla*$_{\text{KPC-2}}$, *bla*$_{\text{GES-1}}$, *aac(6')-Ib3*, *sul1*, *sul2*, *armA*, *msr(E)*, and *mph(A)*. Notably, the *msr(E)* gene confers resistance to macrolides, such as erythromycin and azithromycin, commonly utilized in the treatment of respiratory tract infections[212], while the *mph(A)* gene encodes resistance to macrolides and lincosamides, often used in the management of infections caused by Gram-positive bacteria[213]. Furthermore, this plasmid contained multiple plasmid replicons, including Col(IRGK), ColRNAI, IncFIA(HI1) and IncR, suggesting its potential in successful horizontal gene transfer events as previously reported[214]. Notably, IncR replicons have been previously identified as conjugative elements linked to the transmission of *bla*$_{\text{KPC-2}}$ [215]. Similarly, IncR replicon type was also identified in *bla*$_{\text{KPC-2}}$-carrying plasmid pECT224_KPC from XDR *E. coli* T224. However, this particular plasmid also possessed IncP6 replicon type and several ARGs, including the *aac(6')-Ib-cr*, *sul1* and *dfrA27* genes due to which was highly similar to pECT205_KPC from XDR *E. coli*. The IncP6-type plasmids are mobilizable broad-host-range plasmids which emerged as particularly successful vechicles of *bla*$_{\text{KPC-2}}$ among different *Enterobacteriaceae* in clinical settings and the environment[216,217]. The acquisition of *bla*$_{\text{KPC-2}}$ by IncP6 plasmids in *E. coli* from wastewater-receiving coastal waters in Croatia has been reported only recently [141]. Noteworthy, IncP6 replicons were detected in conjugative plasmids from three *E. coli* isolates (T224, T227, and T205) as well PDR *K. pneumoniae* T221

isolate in this study, emphasizing the significance of this plasmid type in dissemination of *bla*$_{KPC-2}$ gene in these 'critical-priority' pathogens.

Regarding the PDR *K. pneumoniae* T221 isolate, *de novo* plasmid analysis revealed that the plasmid pKPT221_KPC contained replicons of plasmid groups A/C2, FIB and FII in addition to IncP6. Environmental dissemination of the *bla*$_{KPC-2}$ gene via IncFIB plasmids has been previously reported in *K. pneumoniae* and appears to be associated with sewage disposal in coastal marine waters[141]. In addition, this isolate had plasmid-encoded resistance to trimethoprim (*oqxA, dfrB1*), fosfomycin (f*osA5*), aminoglycosides (*aadA1*), and *bla*$_{OKP-B}$ variants. The previous investigation of the genetic environment of *fosA5* suggested that *K. pneumoniae* might serve as the origin of plasmid-mediated *fosA5* [218]. In addition, gene cassettes of the class 1 integrons encompassing *aadA1* gene were frequently detected in *K. pneumoniae*; in fact, they are among the most prevalent [219]. Furthermore, this particular PDR *K. pneumoniae* isolate carried various virulence determinants, including the serum resistance-associated factor, *traT*, as well as the *yagX/ecpC* and *yagX/ecpC* associated with pili formation and the development of colistin resistance in clinical settings[220], highlighting its AR and virulence potential and a public health threat.

4. CONCLUSION

1. Seasonality was identified as the main driving factor of microbial diversity and resistome structure along the trophic gradient, but it impacted less with the proximity to the coast. Namely, resistome was more diverse in winter, but multidrug, beta-lactam resistance modules as well as macrolide, phenicol, aminoglycoside, and particularly imipenem resistance genes were much more frequent in winter, suggesting that the diversity of indigenous resistomes is highly dependent on seasonal variations of the water column, driven by thermohaline stratification and nutrients.

2. Although the microbiomes from human-influenced sites significantly differed from those in oligotrophic offshore area, Proteobacteria were the most abundant phylum and the major contributors to the marine resistomes. As the abundance of intestinal commensals (such as *Serratia, Lactobacillus, Bacteroides, Faecalibaculum, Romboutsia, Faecalibacterium, Blautia*) and soil bacteria (*Pseudomonas, Exiguobacterium, Castellaniella*) decreased away from the coast, this resulted in a shift of resistome towards the intrinsic resistance mechanisms of native marine bacteria.

3. Seasonal variations in frequency of targeted ARGs were also confirmed by qPCR, evidencing their higher relative abundances in winter, particularly in eutrophic coastal areas, as a consequence of anthropogenic impact and a fact that the seawater is kept closer to the outlets due to coastal relief and winds. In particular, *intl1*, followed by bla_{TEM} and $bla_{\text{VIM,}}$ were strongly associated with higher FIB counts and nutrient loading, as well as Gammaproteobacteria, such as *Acinetobacter* and *Citrobacter,* as their predominant carriers.

4. Submarine outfalls of a wastewater treatment plant and an offshore discharge of untreated sewage were identified as 'hot spots' for introduction of WHO 'high priority' pathogens VREfm in coastal marine environment. Importantly, VREfm from wastewater, coastal bathing waters and Split University hospital shared similar virulence (*hyl* gene), multidrug resistance (*vanA*, *aac(6')-Ie-aph(2")-Ia, aph(2")-Ib* and *aph(2")-Id*) and ST profiles, with ST117 and ST889 common to waterborne and hospital isolates, evidencing their sewage-driven dissemination and a risk for further dissemination in the environment.

5. WHO 'critical-priority' pathogens carbapenemase-producing *Enterobacteriaceae* belonging to nine enterobacterial species were recovered from coastal waters of central Adriatic, including Kaštela Bay, Jadro river and Trstenik public beach, highlighting the dissemination of these AR traits beyond clinical settings in Croatia and presenting novel challenges for human and environmental health.

6. Beside *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{VIM-1}}$, *bla*$_{\text{KPC-2}}$ was the most frequently detected carbapenemase gene and was mostly located on diverse conjugative plasmids that harboured multiple resistance genes, insertion sequences and plasmid replicons, favouring their horizontal spread as revealed by conjugation experiments and *de novo* plasmid sequencing.

7. The presence of virulence determinants as well as XDR and even PDR phenotypes in some CRE strains isolated in this study, along with their potential of dissemination through horizontal gene transfer, underscores the complexity and adaptability for persistence of these 'critical-priority' pathogens in aquatic environments.

5. LITERATURE

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6. CURRICULUM VITAE

Mia Dželalija was born on August 22, 1993, in Split, where she completed her undergraduate and graduate studies in 2017 with a Master of Education in Biology and Chemistry (mag. educ. biol. et chem.) from the Faculty of Science at the University of Split. Since 2020, she has been employed as an assistant on the HRZZ project "Seasonal and spatial distribution of antibiotic resistance in marine microbial communities along a trophic gradient in the central Adriatic Sea" at the Faculty of Science, University of Split. Her scientific work includes research on the development and spread of bacterial resistance to antibiotics and the composition and structure of bacterial communities in marine environments exposed to intense anthropogenic influences. In the academic year 2020/2021, she enrolled in the postgraduate university doctoral programme in Biology at the Faculty of Science, University of Zagreb. She has been honored with two scholarships awarded by the Federation of European Microbiological Societies (FEMS), enabling her participation in prestigious scientific conferences both domestically and internationally. In addition, she has been distinguished with the Annual Award for Young Scientists "Jasenka Pigac" by the Croatian Society of Microbiology and in the same year by the University of Split with the prestigious Science award in the category of young scientists. She actively participates in the popularisation of science by organising the summer school "Bacterial spoilage in food production plants" in 2023 and has been actively involved in conducting workshops at the Science Festival since 2015. To date, she has authored or co-authored 11 scientific publications, including three as first author, which have been featured in prestigious journals indexed within the Web of Science (WoS) database, as well as 10 abstracts presented at various scientific conferences.

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Prilog u časopisu

Izvorni znanstveni rad

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7. EXTENDED ABSTRACT

Bakterijska rezistencija na antibiotike (AR) je globalni problem današnjice te je u skladu s konceptom „Jednog zdravlja“ (*eng.* One health) važno provoditi simultani nazdor i kontrolu u cjelokupnom prirodnom okolišu. Mora i oceani su najveći ekosustavi u svijetu ali su sa aspekta sastava rezistoma i glavni patogenih nositelja AR najmanje istraženi. Cilj ovog doktorskog rada je bio istražiti sezonske i prostorne značajke AR u morskim mikrobnim zajednicama niz trofički gradijent srednjeg Jadrana duž 68 km od estuarija rijeke Jadro koja je pod utjecajem otpadnih voda, do obalnog i otvorenog mora. Terenskim istraživanjem profilirano je osamdeset mikrobioma korištenjem 16S rRNA sekvenciranja te je određen taksonomski sastav autohtone morske zajednice kao i rezistoma pri dnu i površini morskog stupca vode. Pri tom je otkriveno kako na biogeografske pomake uvelike utječu sezonske promjene, osobito termohalina stratifikacija i zastupljenost organskih tvari. Također, najbogatiji sastav i raznolikost morskog mikrobioma uočena je u područjima visoke antropogene aktivnosti, dok su najudaljenija mjerenja od obale pokazala manju taksonomsku raznolikost. Također, utvrđeno je kako antropogeni utjecaji uvelike oblikuju mikrobnu raznolikost. Tijekom zime, pod utjecajem otpadnih voda s kopna, koljena Proteobacteria i Firmicutes prevladavali su duž trofičkog gradijenta te su bili glavni doprinositelji morskog rezistoma. Najzastupljeniji patogeni rodovi na površini eutrofnog područja tijekom ljeta bili su *Arcobacter*, *Acinetobacter*, *Bacteroides*, *Prevotella*, *Pseudomonas* i *Streptococcus*, što je izravna posljedica dotoka otpadnih voda. Posljedično, ovi rodovi, posebno *Arcobacter*, *Acinetobacter, Bacteroides* i *Pseudomonas*, značajno su pridonijeli AR u priobalnom dijelu. Ciljani geni AR kvantificirani su pomoću RT-qPCR, među kojima je distribucija *tetA*, *bla*$_{\text{CTX-M}}$ i *bla*$_{\text{TEM}}$ u korelaciji s višom temperaturom, opterećenjem hranjivim tvarima i nižim salinitetom. Patogeni 'visokog' i 'kritičnog prioriteta' (*eng*. high priority) , uključujući vankomicin-rezistentni *Enterococcus faecium* i karbapenem-rezistentne Enterobacteriaceae izolirani su iz obalnih područja zahvaćenog otpadnim vodama, što ukazuje na njihovo širenje u morskom okolišu putem kanalizacije. Gen za karbapenemazu *bla*$_{\text{KPC-2}}$ je najčešće identificiran kod svih izolata, a slijede ga *bla*$_{\text{OXA-48}}$ i *bla*$_{\text{VIM-1}}$. Treba napomenuti da su dva izolata *C. freundii* i po jedan izolat *K. pneumoniae*, *K. variicola* i *E. asburiae* istovremeno nositelji sva tri identificirana gena za karbapenemaze što je po prvi put zabilježeno kod ovih vrsta u morskom okolišu. Stjecanje ovih gena za karbapenemaze kod okolišnih izolata moglo bi se povezati s povećanom upotrebom karbapenemskih antibiotika u klinici u Hrvatskoj kao i povećana prevalencija *bla*$_{\text{KPC-2}}$-pozitivnih enterobakterija u KBC-u Split. Enterobacteriaceae izolati nosili su karbapenemaza-kodirajuće gene (*bla*$_{\text{KPC-2}}$, *bla*$_{\text{OXA-48}}$

and *bla*$_{\text{VIM-1}}$) na različitim konjugacijskim plazmidima što pogoduje njihovom horizontalnom širenju, a što je potvrđeno eksperimentima konjugacije i *de novo* sekvenciranjem plazmida. Ovi rezultati pokazuju složeni obrazac AR duž trofičkog gradijenta i važnost antropogenog utjecaja za njezino širenje u morskim ekosustavima, te nužnost sveobuhvatnih strategija usklađenih s paradigmom Jednog zdravlja radi rješavanja problema AR u okolišu.

8. HYPOTHESIS AND SCIENTIFIC CONTRIBUTION

Objective	Hypotheses	Scientific paper
O1 *Investigate spatial and temporal variations of marine bacterial community structure along the trophic gradient of central Adriatic.* ***O2*** *Explore resistome diversity and the distribution of selected antibiotic resistance genes at seasonal and spatial scales in Adriatic.*	**H1** Biological and physicochemical factors diversely influence the bacterial community composition along the trophic gradient, and consequently, lead to shifts in resistome structure at seasonal and spatial scales.	*Dželalija M, Kvesić-Ivanković M, Jozić S, Ordulj M, Kalinić H, Pavlinović A, Šamanić I, Maravić A.* ***Marine resistome of a temperate zone: Distribution, diversity, and driving factors across the trophic gradient.*** *Water research. 2023 Nov 1;246:120688.* *Dželalija M, Fredotović Ž, Udiković-Kolić N, Kalinić H, Jozić S, Šamanić I, Ordulj M, Maravić A****. Large-Scale Biogeographical Shifts of Abundance of Antibiotic Resistance Genes and Marine Bacterial Communities as Their Carriers along a Trophic*** *Gradient. International Journal of Molecular Sciences. 2024 Jan 4;25(1):654.*
O3 *Identify which bacterial genera are the main carriers of antibiotic resistance.*	**H2** In coastal waters under anthropogenic influence resistome will be more diverse and supported predominantly by pathogenic bacteria of human and terrestrial origin.	*Dželalija M, Kvesić M, Novak A, Fredotović Ž, Kalinić H, Šamanić I, Ordulj M, Jozić S, Barišić IG, Tonkić M, Maravić A.* ***Microbiome profiling and characterization of virulent and vancomycin-resistant Enterococcus faecium from treated and untreated wastewater, beach water and clinical sources****. Science of the total environment. 2023 Feb 1;858:159720.*
O4 *Characterize the multidrug-resistant bacterial isolates in terms of antibiotic resistance genes carriage and clonal relatedness.* ***O5*** *Determine the structure and transferability of plasmids carrying carbapenemase-encoding genes.*	**H3** Multidrug-resistant isolates will harbour clinically relevant antibiotic resistance genes, among which carbapenemase-encoding genes which are located on diverse and conjugative plasmids.	**Additional methodology (7.1) and results (7.2)**

9. APPENDIX

9.1 ADDITIONAL METHODOGY

9.1.1. Sampling and bacterial isolation

Seawater samples were collected seasonally in March and August of 2021 from the sites of different anthropogenic impacts and trophic status in southeastern Adriatic, including Jadro (J), Kaštel Sućurac (KS), Vranjic Bay (VB), Kaštela Bay (KB), Split Channel (SC), and Stončica (ST), all of which were described in Chapter 2.2. Additionally, samples were taken from the public beach Trstenik (T; N 43° 30' 8", E 16°27'56") in the city of Split, whose surface waters were of excellent quality (https://vrtlac.izor.hr/ords/kakvoca/kakvoca) according to the criteria established by Croatian law (Regulation on sea bathing water quality; OG 73/08). Aliquots of 300 ml of seawater were vacuum filtered through mixed cellulose ester (MCE) membrane filters with 0.22 um pore size (Whatman, United Kingdom) which were placed on ChromID CARBA SMART (bioMérieux, France) plates and incubated at 37 °C for 48 hours. The criteria for isolate selection was: all colonies of morphotypes represented by less than 5 colonies, half of the colonies of morphotypes represented by 5 to 10 colonies, and about one third of colonies with morphotypes represented by more than 10 colonies [221]. A total of 78 putative Enterobacteriaceae isolates were replated for pure culture on MacConkey agar (Biolife, Italy) at 37°C for 18 h, and identified to species level using matrix-assisted laser desorption/ionization time-of-fight (MALDI-TOF) mass spectrometry and MALDI-Biotyper software version 4.1.80 (Bruker Daltonics, Germany) according to the manufacturer's instructions.

9.1.2. Antibiotic susceptibility testing

The susceptibility of isolates was determined using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Biolife) for the following antibiotics: piperacillin (PIP, 100 µg), piperacillin/tazobactam (PIP/TZP, 100/10 µg), cefoxitin (FOX, 30 µg), cefotaxime (CTX, 30 µg), cefotaxime/clavulanic acid (CTX/CLA, 30/10 µg), ceftazidime (CAZ, 30 µg), ceftazidime/clavulanic acid (CAZ/CLA, 30/10 µg), cefepime (FEP, 30 µg), aztreonam (ATM, 30 µg), imipenem (IPM, 10 µg), meropenem (MEM, 10 µg), ciprofloxacin (CIP, 5 µg), gentamicin (GEN, 10 µg), tetracycline (TET, 30 µg) and trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 µg). After 18-20 hours of incubation at 37 °C zones of inhibition were measured and

interpreted according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [222] .

Thirty-eight CRE isolates (resistant to IPM and/or MEM) were further tested using the Etest (AB Biodisk, Sweden) to determine the minimum inhibitory concentrations (MICs) for carbapenem antibiotics including IPM, MEM and ertapenem (ETP), whereas MIC for colistin (CL) was determined using the broth microdilution method, according to the EUCAST guidelines [222]. Isolates were further tested for the presence of class C AmpC β-lactamases using the disk approximation method using phenylboronic acid (PBA). Inhibition zones for FOX (30 µg) discs alone and in combination with PBA (400 µg) were measured, and the increase of >5 mm in zone diameter in the presence of PBA compared with FOX alone was indicative of an AmpC production (CLSI, 2021) [223]. Production of carbapenemases were tested with MBL Etest (AB Biodisk) and Rapidec Carba NP test (bioMérieux) according to the manufacturers' instructions. The production of ESBLs was tested using clavulanic acid (CLA) combination discs. The phenotype consistent with the production of ESBLs was defined by an increase in zone diameter of ≥5 mm for CAZ and/or CTX in combination with CLA compared to its zone when tested alone (CLSI 2021) [223]

9.1.3 Molecular characterization of CRE isolates

Genomic DNA was isolated using the NucleoSpin™ Microbial DNA kit (Macherey-Nagel™, USA) according to the manufacturer's instructions from a bacterial culture grown overnight on MacConkey agar supplemented with IPM (2 µg/ml). The purity and concentration of the isolated DNA was checked spectrophotometrically using a NanoPhotometer (Implen, Sweden) by measuring the absorbance at 260 and 280 nm.

By PCR, CRE isolates were tested for the presence of genes coding for AmpC beta-lactamases (*bla*$_{FOX}$, *bla*$_{DHA}$, *bla*$_{ACC}$, *bla*$_{ACT}$, *bla*$_{MIR}$), ESBL beta-lactamases (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{GES}$, *bla*$_{PER}$), integrase (*intI*1, *intI*2), sufonamides (*sul1, sul2*), resistance to colistin (*mcr-1, mcr-2*) and carbapenemases (*bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{IMP}$). The primers, amplicon sizes, and PCR conditions are summarized in Table 1. PCR reactions were divided into singleplex and multiplex depending on whether one pair of primers or more were used. For singleplex and multiplex reactions, total volume of the reaction mixture was 20 µL and 50 µL, while the volume of DNA used as a template was 1 µL and 5 µL, respectively. The composition

of the reaction mixture was: 1X PCR buffer (10x $MgCl_2$) (Sigma Aldrich, Massachusetts, United States), 2 mM dNTP Mix (40mM, Promega, Madison, Wisconsin, United States) primers starting with a concentration of 10 µM each (Microsynth, Balgach, Switzerland) and Taq DNA Polymerase from *Thermus aquaticus* (250 U, Sigma Aldrich, Burlington, Massachusetts, United States). PCR products were analysed by gel electrophoresis according to the following parameters: 120V/cm for 30 minutes in 1xTAE buffer. For detection, 20 µL of PCR reaction mixture was mixed with 4 µL of 6x loading dye (0.25% w/v bromphenol-blue, 0.25% w/v xylene-cyanol, 30% v/v glycerol) whereas the 1kb BenchTop Ladder (Promega, Madison, Wisconsin, United States) was used as molecular mass standard. Sybr Safe DNA Gel stain (Invitrogen, Germany) was used to visualize the agarose gels under UV light using the digital photography system OmniDOC (Cleaver Scientific, United Kingdom).

Table 1. Primers, references and conditions for PCR detection of antibiotic resistance genes

Target gene	Primers (5´- 3´)	Product size	References	Conditions
***bla*$_{PER}$**	PERA ATGAATGTCATTATAAAAGC	925 pb	224	Activation for 3 minutes at 94°C 30 cycles of DNA denaturation for 30 seconds at 94°C 35 cycles of primer annealing for 30 seconds at 50°C 35 cycles of chain elongation for 45 seconds at 72°C Final extension for 2 minutes at 72°C Cooling to 4°C
	PERD AATTTGGGCTTAGGGCAGAA			
intI1	intI1L ACATGTGATGGCGACGCACGA	569 pb	225	Activation for 3 minutes at 94°C 35 cycles of DNA denaturation for 30 seconds at 94°C 35 cycles of primer annealing for 30 seconds at 54°C 35 cycles of chain elongation for 1 minute at 72°C Final extension for 7 minutes at 72°C Cooling to 4°C
	intI1R ATTTCTGTCCTGGCTGGCG			
intI2	intI2L CACGGATATGCGACAAAAAGGT	789 pb		
	intI2R GTAGCAAACGAGTGACGAAATG			
***bla*$_{MOX-1}$** ***bla*$_{MOX-2}$** ***bla*$_{CMY-1}$** ***bla*$_{CMY-8-11}$**	MOXM-F GCTGCTCAAGGAGCACAGGAT MOXM-R CACATTGACATAGGTGTGGTGC	520 pb	226	Initial activation for 3 minutes at 94°C 25 cycles of denaturation for 30 seconds at 94°C 25 cycles of primer annealing for 30 seconds at 64°C 25 cycles of chain elongation for 1 minute at 72°C Final extension for 7 minutes at 72°C Cooling to 4°C
***bla*$_{LAT-1-4}$** ***bla*$_{CMY-2-7}$** ***bla*$_{BIL-1}$**	CITM-F TGGCCAGAACTGACAGGCAAA CITM-R TTTCTCCTGAACGTGGCTGGC	462 pb		
***bla*$_{DHA-1}$**	DHAM-F AACTTTCACAGGTGTGCTGGGT	405 pb		
***bla*$_{DHA-2}$**	DHAM-R CCGTACGCATACTGGCTTTGC			
***bla*$_{ACC}$**	ACCM-F AACAGCCTCAGCAGCCGGTTA	346 pb		
	ACCM-R TTCGCCGCAATCATCCCTAGC			
***bla*$_{FOX-1-5b}$**	FOXM-F AACATGGGGTATCAGGGAGATG	190 pb		
	FOXM-R CAAAGCGCGTAACCGGATTGG			
***bla*$_{MIR-1T}$**	EBCM-F TCGGTAAAGCCGATGTTGCGG	302 pb		
***bla*$_{ACT-1}$**	EBCM-R CTTCCACTGCGGCTGCCAGTT			
***bla*$_{CTX-M-15}$**	CTX-M-F1 ATAAAACCGGCAGCGGTG	483 pb	227	Initial activation for 10 minutes at 94°C 35 cycles of denaturation for 1 minute at 94°C 35 cycles of primer annealing for 1 minute at 53°C 35 cycles of chain elongation for 1 minute at 72°C Final extension for 7 minutes at 72°C Cooling to 4°C
	CTX-M-F2 GAATTTTGACGATCGGGG			
***bla*$_{CTX-M-3}$**	CTX-M-3-S CGTCACGCTGTTGTTAGGAA	780 pb	228	
	CTX-M-3-AS ACGGCTTTCTGCCTTAGGTT			
***bla*$_{CTX-M-1-like}$**	M13-upper CGTCACGCTGTTGTTAGGAA	863 pb	229	
	M13-lower TTGGTGACGATTTTAGCCGC			

***bla*$_{KPC}$**	KPC-Fm CGTCTAGTTCTGCTGTCTTG	798 pb	224	Initial activation for 3 minutes at 94°C 40 cycles of denaturation for 30 seconds at 94°C 40 cycles of primer annealing for 1 minute at 58.2°C 40 cycles of chain elongation for 30 seconds at 72°C Final extension for 5 minutes at 72°C Cooling to 4°C
	KPC-Rm CTTGTCATCCTTGTTAGGCG			
sul1	Sul1f GACGGTGTTCGGCATTCT	550 pb	230	Initial activation for 3 minutes at 94°C 30 cycles of denaturation for 30 seconds at 94°C 35 cycles of primer annealing for 45 seconds at 58°C 35 cycles of chain elongation for 1 minute at 72°C Final extension for 7 minutes at 72°C Cooling to 4°C
	Sul1r TTTGAAGGTTCGACAGC			
sul2	Sul2f GCAGGCGCGTAAGCTGA	815 pb		
	Sul2r GGCTCGTGTGTGCGGATG			
***bla*$_{VIM}$**	Vimf GGTGAGTATCCGACAGTC	442 pb	231	Initial activation for 2:30 minutes at 94°C 30 cycles of denaturation for 30 seconds at 94°C 30 cycles of primer annealing for 30 seconds at 55°C 30 cycles of chain elongation for 45 seconds at 72°C Final extension for 2 minutes at 72°C Cooling to 4°C
	Vimr1 CAGCACCAGGATAGAAGAG			
	Vimr2 CAGCACCGGGATAGAAGAG			
***bla*$_{NDM}$**	NDMf ATGGAATTGCCCAATATTATGC	813 pb		
	NDMr TCAGCGCAGCTTGTCGG			
***bla*$_{IMP}$**	IMP-f1 GGCGTTTATGTTCATACTTC	235 pb		
	IMP-f2 GGGTGTTTATGTTCATACATC			
	IMP-r1 GGATTGAGAATTAAGCCACTC			
	IMP-r4 GATCGAGAATTAAGCCACTC			
***bla*$_{TEM}$**	TEM-F ATGAGTATTCAACAT TTCCG	1075 pb	232	Initial activation for 5 minutes at 94°C 35 cycles of denaturation for 30 seconds at 95°C 35 cycles of primer annealing for 30 seconds at 58°C 35 cycles of chain elongation for 1 minute at 72°C Final extension for 7 minutes at 72°C Cooling to 4°C
	TEM-R CTGACAGTTACCATTGCTTA			
***bla*$_{SHV}$**	SHV-F GGGTTATTCTTATTTGTCGC	930 pb		
	SHV-R TTAGCGTTGCCAGTGCTC			
***bla*$_{GES-1}$**	GES-1A ATGCGCTTCATTCACGCAC	860 pb	233	Initial activation for 3 minutes at 94°C 35 cycles of denaturation for 30 seconds at 94°C 35 cycles of primer annealing for 30 seconds at 53°C 35 cycles of chain elongation for 45 seconds at 72°C Final extension for 2 minutes at 72°C Cooling to 4°C
	GES-1B CTATTTGTCCGTGCTCAGG			
mcr-1	CLR5-F CGGTCAGTCCGTTTGTTC	-	234	Initial activation for 10 minutes at 94°C 30 cycles of denaturation for 30 seconds at 95°C 35 cycles of primer annealing for 30 seconds at 56°C 35 cycles of chain elongation for 1 minute at 72°C Final extension for 7 minutes at 72°C Cooling to 4°C
	CLR5-R CTTGGTCGGTCTGTAGGG			
mcr1+2	MCR-1+2-F TATCGCTATGTGCTAAAGCC	-	235	Initial activation for 5 minutes at 94°C 35 cycles of denaturation for 30 seconds at 95°C 35 cycles of primer annealing for 30 seconds at 53.2°C 35 cycles of chain elongation for 45 seconds at 72°C Final extension for 7 minutes at 72°C Cooling to 4°C
	MCR-1+2-R TCTTGGTATTTGGCGGTATC			

Amplicons of interest were purified from the gel using the QIAEX II Gel Extraction kit (Qiagen, Germany) and then subjected to Sanger sequencing of both strands using a 3730XL DNA sequencer (Applied Biosystems, USA) at Macrogen DNA Service (Amsterdam, The Netherlands). The nucleotide alignments were obtained using ClustalW program (www.mbio.ncsu.edu/bioedit/ bioedit.html) and compared with sequences in GenBank using BLAST algorithm (www.ncbi.nlm.nih.gov). Positive and negative controls from our laboratory bacterial collection were included in all PCR experiments.

9.1.4. Genotyping of CRE isolates

Genotypic diversity of *E. coli* (n=6), *Raoultella ornithinolytica* (n=3), *Citrobacter freundii* (n=7), *Klebsiella* spp. (n=16), *Enterobacter cloacae* complex (cplx.) (n=5) was assessed by pulsed-field gel electrophoresis (PFGE), as previously described Jelic and colleagues [231]. DNA of the bacterial isolates was digested in agarose plugs with XbaI (Bio-Rad Laboratories, USA) and separated in a 1% agarose gel using the CHEF-DR III system (Bio-Rad Laboratories, USA) under the following conditions: 6 V/cm; pulse time 6–36 s; final time 19.5 h, at 12 °C. The gels were stained with ethidium bromide and visualized under UV illumination using a Gel Doc XR apparatus (Bio-Rad). The restriction patterns were analysed using BioNumerics software (Applied Maths, Belgium). Dendrograms were generated with a positional tolerance of 1.5% using the UPGMA and DICE similarity coefficient. PFGE clusters were established by identifying similarities that were equal to or greater than 85%.

9.1.5 S1-PFGE and Southern blot hybridization

To determine the size and number of plasmids in CRE isolates genomic DNA was digested using S1 nuclease and separated by PFGE. DNA was embedded in agarose blocks and treated with S1 nuclease as previously described by Jelic and colleagues [199]. In brief, a piece of 3 mm agarose block was cut and incubated in 200 µl of 1X reaction buffer for nuclease S1 (Thermo Fisher Scientific, USA) at 37 °C for 30 minutes. After the buffer was removed, the agarose fragments were incubated for one hour at 37°C in 100 µL restriction buffer (3.5 U S1 nuclease, 1X reaction buffer for nuclease S1; Thermo Fisher Scientific, USA). Degradation was then initiated by removing the restriction buffer and adding 100 µL of 5 X TE buffer (50 mM Tris, 5 mM EDTA, pH 8.0) at 4°C. S1-PFGE gel electrophoresis was performed using the CHEF-DR®III system according to the program from the study Sirichota and colleagues[236] with the following parameters: voltage of 6 V/cm, electric field angle of 80-120°, pulse duration of 6.8 – 38.4 s, at 14°C for 19 hours. Plasmid size was identified using *Salmonella* Braenderup H9812 as the molecular weight marker. The gels were stained with ethidium bromide and visualized under UV illumination using a Gel Doc XR apparatus (Bio-Rad).

Location of carbapenemase encoding genes (*bla*$_{KPC}$ and *bla*$_{VIM}$) was further analysed using Southern blot hybridization performed on genomic DNA as previously described Maravić and colleagues[237] and Jelic and colleagues [199] with minor modifications. DNA were transferred from agarose gel to a nylon membrane (Roche Diagnostics, Germany) and hybridized with digoxigenin-labelled gene fragments using PCR DIG detection system (DIG DNA labelling and detection kit; Roche Diagnostics) according to the manufacturer's instructions. PCR amplicons of amplified carbapenemase genes were used as positive controls in hybridization assays. The size of plasmids carrying *bla*$_{KPC}$ and *bla*$_{VIM}$ genes was determined by S1-PFGE and compared to *Salmonella* Braenderup H9812 ladder.

9.1.6 Conjugation assays

Based on the results of antibiotic susceptibility testing and clonal relatedness, 24 representative CRE isolates were selected to assess the transferability of plasmids containing carbapenemase-encoding genes using the broth-mating conjugation method. The following isolates were used as donors: 9 *Klebsiella* spp. carrying *bla*$_{KPC}$ and 3 carrying *bla*$_{VIM}$, 5 *E. cloacae* cplx. carrying *bla*$_{KPC}$ and 2 carrying *bla*$_{VIM}$, 4 *E. coli* carrying *bla*$_{KPC}$, 5 *C. freundii* carrying *bla*$_{KPC}$ and 2 carrying *bla*$_{VIM}$. The azide-resistant *E. coli* J53 was used as recipient strain. Overnight cultures of donor and recipient cells were grown in Luria–Bertani (LB) broth without antibiotics at 37°C, adjusted to a density of 2 McFarland and mixed at the volumetric rate of 1:2 (100 µL of donor cells and 200 µL of recipient cells). Three hundred millilitres of fresh LB broth was added to the mixture and incubated without shaking for 24 h at 37°C. To select the transconjugants, 150 µL of overnight mixture was plated on LB plates supplemented with azide (100 µg/ml) and MEM (2 µg/ml). Putative transconjugants were checked by PCR (primers and conditions summarized in Table 1) to confirm plasmid acquisition and subjected to antibiotic susceptibility testing using disc-diffusion method against nine antibiotics (ETP, MEM, IPM, FEP, SXT, GM, ATM, CIP, and TET), respectively. The conjugation frequency was determined as the ratio of transconjugants to the initial number of donors.

9.1.7 *De novo* plasmid sequencing and bioinformatics

9.1.7.1 Isolation of plasmid DNA

The CRE isolates were grown on MacConkey agar (Biolife) supplemented with 2 µg of MEM (Sigma-Aldrich, USA) at 37°C overnight, and then inoculated in 250 ml of LB broth supplemented with the same antibiotic. Plasmid DNA was extracted using PureYield Plasmid Midiprep System (Promega, USA) after which the concentration and quality of DNA were assessed using a spectrophotometer (NanoPhotometer N60 ®, Implen). Total of 24 plasmid bands were cut off from the agarose gels after performing S1-PFGE of plasmid DNA as described in Section 4.5. Bands were selected as representatives of resistance plasmids based on positive results of Southern blotting with the *bla*$_{KPC}$- and *bla*$_{VIM}$- labelled probes, antibiograms, species affiliation and the origin of the isolates. After the DNA was extracted from the PFGE gels using the QIAEX II Gel Extraction Kit (Qiagen, Germany) we performed *de novo* sequencing of plasmids.

9.1.7.2 *De novo* assembly, analysis and annotation of bacterial plasmids

On-bead tagmentation of plasmid DNA was performed using the Illumina DNA Prep (M) tagmentation method. In this process, which could accommodate 24 samples per run, bead-linked transposomes (BLTs) were used to simultaneously fragment and tag the DNA with Illumina sequencing primers. Tagged DNA was then amplified by PCR to increase yield and obtain sufficient material for downstream processes. Nextera DNA CD indexes (Illumina) were incorporated to enable multiplexing of plasmid samples within the sequencing library. The individual libraries were pooled to create a composite library, after which a quality control was performed to ensure the integrity and purity of the libraries. Sequencing was done in house on the Illumina iSeq 100 platform, using the iSeq 100 i1 Reagent v2 kit and generating 2 × 151 bp paired-end reads.

Raw reads were quality checked with FastQC (v0.11.9) (Babraham Bioinformatics - FastQC A Quality Control Tool for High Throughput Sequence Data) and subsequently trimmed and quality filtered with Trimmomatic (v0.39)[239]. The trimmed reads were also checked with FastQC, and assembled with SPAdes (v3.14.1)[240] using the plasmid-specific settings. The obtained contigs, based on SPAdes *de novo* assembly and overlapp with reference genomes,

were functionally characterized using available genome databases. Antimicrobial resistance genes were identified using the Comprehensive Antibiotic Resistance Database (CARD)[241] and the CGE-tools (Center for Genomic Epidemiology) including ResFinder 4.1[242,243]. Virulence genes were identified with the aid of the Virulence Factor Database (VFDB)[244]. Based on the VFDB, identity comparison results of ≥90% were statistically analysed. Mobile genetic elements were analysed using ISEScan [245]. Assembled plasmid contigs were quality checked with Quast (v5.2.0)[246] and annotated with Prokka (v1.14.6)[247]. Plasmid typing was carried out with MobSuite (v3.1.0) [248] and Plasmidfinder (v2.1.1) [249]. The various log files were comprised using Multiqc (v1.11)[250]. For visualization, the GFF-3 output files from Prokka and ISEScan were combined using bedtools (v.2.31.0.)[251]. Contig assemblies were visualized with PlasMapper 3.0 [252]. Finally, BRIG (v. 0.95) [253] was used to map all other assembled plasmid contigs against the largest assembly contig pEC-KPCT205, using the annotation of pEC-KPCT205 plasmid as a reference.

9.2 ADDITIONAL RESULTS

9.2.1 Species identification, antibiotic susceptibility and phenotypic detection of β-lactamases in CRE isolates

A total of 78 putative CRE isolates were isolated from ChromID CARBA SMART plates and identified to the species level. As shown in Table 2, these belonged to five genera, out of which *Klebsiella* was the most abundant (29/78, 37.18%). The other four genera were as follows: *E. coli* (22/78, 28.21%), *Citrobacter* (14/78, 17.95%), *Enterobacter* (10/78, 12.82%), and *Raoultella* (3/78, 3.85%). ESBL production was confirmed in 8.97% (7/78) of the isolates, out of which mostly in *E. coli* (n=4), followed by *Klebsiella* spp. and *Enterobacter* spp. On the other hand, more than half of the surveyed CRE isolates (43/78, 55.13%) were found to be AmpC producers, and these predominantly belonged to *Klebsiella* (n=19) and *Citrobacter* (n=12), respectively.

Table 2. Phenotypic detection of β-lactamases and resistance phenotype of 78 CRE isolates obtained in this study.

Species/genus	***Number of isolates***	***ESBL (n/%)***	***AmpC-positive (n/%)***	***MDR (n/%)***	***XDR (n/%)***	***PDR (n/%)***
E. coli	22	4 / 18.2	5 / 22.7	3 / 14	7 / 31.8	0
Klebsiella spp.	29	2 / 6.9	19 / 65.5	3 / 10.3	25 / 86.2	1/ 6.8
Citrobacter spp.	14	0	12 / 85.7	3 / 21.4	7 / 50	0
E. cloacae	10	1 / 10	5 / 50	2/ 20	4 / 40	0
R. ornithinolytica	3	0	2 / 66.67	3 / 100	0	0
Total	78	7 / 8.9	43 / 55.13	14 / 17.9	43 / 55.1	6 / 7.7

Abbreviations: MDR (multi-drug resistant), XDR (extensively drug-resistant), and PDR (pan-drug resistant) phenotype according to Magiorakos and colleagues [254] .

Of the 78 putative CRE isolates, half were designated as XDR, among which most of carbapenem-resistant *Klebsiella* (n=20), followed by *E. coli* and *Citrobacter* (7 isolates each). Noteworthy, one *Klebsiella* isolate was found to exhibit PDR phenotype (Table 2).

Out of 78 putative CRE isolates, 38 isolates belonging to 9 Enterobacteriaceae species were confirmed by PCR to carry at least one carbapenemase gene and their antibiotic resistance phenotypes are given in Figure 7.

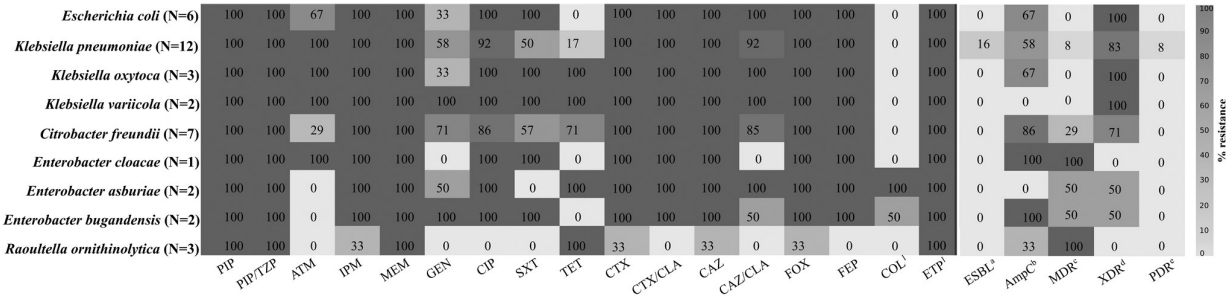


Figure 7. Antibiotic resistance phenotype of 38 CRE isolates. Quantitative values in brackets signify the number of isolates attributed to each species, whereas the numerical data within the cells delineate the observed percentage of resistance. Abbreviations: PIP, piperacilin; PIP/TZP, piperacilin/tazobactam; ATM, aztreonam; IPM, imipenem; MEM, meropenem; GEN, gentamicin; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; CTX, cefotaxime; CTX/CLA, cefotaxime/clavulanic acid; CAZ, ceftazidime; CAZ/CLA, ceftazidime/clavulanic acid; FOX, cefoxitin; FEP, cefepime; CL, colistin; ETP, ertapenem.

Out of 38 carbapenemase-producing isolates, 9 were found to be MDR, 28 were XDR whereas 1 isolate of *K. pneumoniae* was PDR. As expected, these isolates were uniformly resistant to penicillins, cephalosporins and carbapenems, whereas majority remained susceptible to CL. Noteworthy, 2 isolates of *E. asburiae* and 1 isolate of *E. bugandensis* were found to be resistant to CL.

9.2.2 PCR detection of ARGs

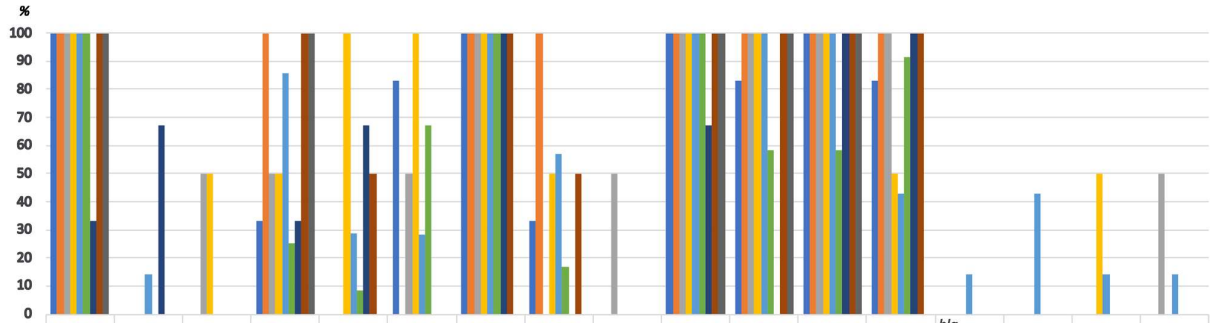
Out of the 78 putative CRE isolates, 38 isolates were positive by PCR for the presence of carbapenemase gene(s). These isolates originated from Kaštela Bay (3 *R. ornithinolytica* isolates), Jadro river (10 isolates of *K. pneumoniae*) and Trstenik public beach (2 *K. variicola,* 3 *K. oxytoca*, 5 *Enterobacter* spp., 6 *E. coli* and 7 *C. freundii* isolates).

Among the carbapenemase genes detected, the $bla_{\text{KPC-2}}$ gene was detected most frequently (36/38, 94.7%), and followed by $bla_{\text{OXA-48}}$ (19/38, 50%) and $bla_{\text{VIM-1}}$ (7/38, 18.4%). It is noteworthy that two isolates of *C. freundii* and one each of *K. pneumoniae*, *K. variicola* and *E. asburiae* simultaneously carried all three identified carbapenemase genes. These isolates were

further subjected to PCR screening for other resistance determinants which revealed from five to up to ten resistance genes simultaneously present in isolates. It was found that integrase genes, *intI1* (37/38, 97.4%) and *intI2* (29/38, 76.3%) were present in the majority of isolates. Similarly, *sul1* (33/38, 86.8%) and *sul2* (28/38, 73.7%) genes responsible for sulfonamide resistance were also found to be commonly present, respectively. Regarding the genes encoding for ESBLs, the bla_{SHV} gene was the most frequently identified (35/38, 92.1%), followed by bla_{TEM} (18/38, 47.4%), and $bla_{CTX\text{-}M\text{-}15}$ (11/38, 28.9%). The $bla_{GES\text{-}1}$ was identified only once, in an isolate of *E. bugandensis*. The AmpC β-lactamase encoding genes were far less detected, among which bla_{ACC} in 3 CRE isolates, $bla_{MIR\text{-}1T}$/$bla_{ACT\text{-}1}$ and $bla_{FOX\text{-}1\text{-}5B}$ genes in 2 isolates and bla_{DHA} in only one isolate.

The distribution of identified β-lactamase genes, integrase and *sul* genes among CRE species can be seen in Figure 8. The resistance genotypes of surveyed CRE isolates are given in Table 3. The bla_{KPC} gene was detected in all 12 carbapenem-resistant isolates of *K. pneumonia*e whereas 4 isolates co-harbored $bla_{OXA\text{-}48}$. Importantly, *K. pneumoniae* T221 and *K. variicola* T254 were found to simultaneously carry $bla_{KPC\text{-}2,}$ $bla_{OXA\text{-}48}$ and $bla_{VIM\text{-}1}$ carbapenemase genes. In addition, *K. oxytoca* T212 co-harbored $bla_{KPC\text{-}2,}$ and $bla_{VIM\text{-}1}$ genes, while *K. oxytoca* T210 carried $bla_{KPC\text{-}2}$ and $bla_{OXA\text{-}48}$ (Table 3). Regarding other β-lactamase genes detected in *Klebsiella* spp., bla_{TEM} gene was detected in 8 isolates, bla_{SHV} gene in all isolates, while the $bla_{CTX\text{-}M\text{-}15}$ was only present in 2 isolates (Table 3, Figure 8). *K. variicola* T254 had both bla_{SHV} and $bla_{CTX\text{-}M\text{-}15}$. The AmpC encoding genes were not observed in *Klebisella* spp. isolates. Moreover, *intI1* was detected in all *K. variicola* and *K. pneumoniae* isolates, while *intI2* was identified in all *K. variicola* and in half (n=7) of carbapenemase-producing *K. pneumoniae* isolates. All *K. oxytoca* and *K. variicola* isolates had *sul1* and *sul2* while *K. pneumoniae* more commonly carried *sul2* then *sul1* (n=11 and n=7) (Table 3, Figure 8).

All six carbapenem-resistant *E. coli* isolates were found to harbor $bla_{KPC\text{-}2}$ gene, while 2 isolates additionally carried $bla_{OXA\text{-}48}$ (Table 3; Figure 8). Moreover, bla_{SHV} was detected in all *E. coli* isolates, followed by bla_{TEM} (n=5) and $bla_{CTX\text{-}M\text{-}15}$ (n=2). Sulfonamide resistance genes *sul1*and *sul2* were identified in almost all *E. coli* isolates. A similar pattern was observed for integrase genes, where only isolate T206 lacked the *intI2*, while the others possessed both. Genes coding for AmpC β-lactamases were not detected in any of the carbapenemase-producing *E. coli* isolates.



	bla_{KPC-2}	bla_{OXA-1}	bla_{OXA-10}	bla_{OXA-48}	bla_{VIM}	bla_{TEM-1}	bla_{SHV-1}	$bla_{CTX-M-15}$	bla_{GES-1}	intI1	intI2	sul1	sul2	bla_{DHA-1} bla_{DHA-2}	bla_{ACC}	bla_{MIR-1T}	$bla_{FOX-1-Sb-}$
	β-lactamase encoding genes									Integrase		Sulfonamide		AmpC β-lactamase			
Escherichia coli	100	0	0	33	0	83	100	33	0	100	83	100	83	0	0	0	0
Enterobacter cloacae	100	0	0	100	0	0	100	100	0	100	100	100	100	0	0	0	0
Enterobacter bugandensis	100	0	50	50	0	50	100	0	50	100	100	100	100	0	0	0	50
Enterobacter asburiae	100	0	50	50	100	100	100	50	0	100	100	100	50	0	0	50	0
Citrobacter freundii	100	14.2	0	85.71	28.6	28.5	100	57.14	0	100	100	100	42.85	14.28	42.85	14.3	14.3
Klebsiella pneumoniae	100	0	0	25	8.3	67	100	16.7	0	100	58.3	58.3	91.6	0	0	0	0
Klebsiella oxytoca	33.3	67	0	33	67	0	100	0	0	67	0	100	100	0	0	0	0
Klebsiella variicola	100	0	0	100	50	0	100	50	0	100	100	100	100	0	0	0	0
Raoultella ornithinolytica	100	0	0	100	0	0	0	0	0	100	100	100	0	0	0	0	0

Figure 8. Distribution of genes encoding for β-lactamases, sulfonamide resistance and integrases in 38 carbapenemase-producing *Enterobacteriaceae* isolates. The percentage of isolates positive for surveyed genes is shown by histogram, and given in table below.

Table 3. Resistance genotype of 38 carbapenemase-producing *Enterobacteriaceae* isolates obtained in this study

Isolate	Species	Resistance genes
T201	*Citrobacter freundii*	bla_{KPC-2}, bla_{SHV-1}, $bla_{CTX-M-15}$, bla_{OXA-48}, intl1, intl2, *sul1*, *sul2*, bla_{DHA}, bla_{VIM-1}
T207	*Citrobacter freundii*	bla_{KPC-2}, bla_{SHV}, *intl1*, *intl2*, *sul1*, bla_{ACC}
T208	*Citrobacter freundii*	bla_{KPC-2}, bla_{SHV}, bla_{OXA-48}, *intl1*, *intl2*, *sul1*, bla_{ACC}
T209	*Citrobacter freundii*	bla_{KPC-2}, bla_{TEM-1}, bla_{SHV}, bla_{OXA-48}, *intl1*, *intl2*, *sul1*
T219	*Citrobacter freundii*	bla_{KPC-2}, bla_{OXA-1}, bla_{SHV}, $bla_{CTX-M-15}$, *intl1*, *intl2*, *sul1*, $bla_{FOX-1-5B}$
T222	*Citrobacter freundii*	bla_{KPC-2}, bla_{TEM-1}, bla_{SHV-1}, $bla_{CTX-M-15}$, bla_{OXA-48}, *intl1*, *intl2*, *sul1*, *sul2*, bla_{ACC}
T251	*Citrobacter freundii*	bla_{KPC-2}, bla_{SHV-1}, $bla_{CTX-M-15}$, bla_{OXA-48}, *intl1*, *intl2*, *sul1*, *sul2*, bla_{VIM-1}, $bla_{MIR-1T/ACT-1}$
T218	*Enterobacter asburiae*	bla_{KPC-2}, bla_{TEM-1}, bla_{SHV}, $bla_{CTX-M-15}$, *intl1*, *intl2*, *sul1*, *sul2*, bla_{VIM-1}, bla_{OXA-10}
T220	*Enterobacter asburiae*	bla_{KPC-2}, bla_{TEM-1}, bla_{SHV}, bla_{OXA-48}, *intl1*, *intl2*, *sul1*, bla_{VIM-1}, $bla_{MIR-1T/ACT-1}$
T200	*Enterobacter bugandensis*	bla_{KPC-2}, bla_{TEM-1}, bla_{OXA-10}, bla_{SHV}, *intl1*, *sul1*, *sul2*, bla_{GES-1}
T214	*Enterobacter bugandensis*	bla_{KPC-2}, bla_{SHV-1}, bla_{OXA-48}, *intl1*, *intl2*, *sul1*, sul2, $bla_{FOX-1-5B}$
T213	*Enterobacter cloacae*	bla_{KPC-2}, bla_{SHV-1}, $bla_{CTX-M-15}$, bla_{OXA-48}, *intl1*, *intl2*, *sul1*, *sul2*
T205	*Escherichia coli*	bla_{KPC-2}, bla_{TEM-1}, bla_{SHV-1}, $bla_{CTX-M-15}$, *intl1*, *intl2*, *sul1*, *sul2*
T206	*Escherichia coli*	bla_{KPC-2}, bla_{TEM-1}, bla_{SHV}, *intl1*, *sul1*, *sul2*
T224	*Escherichia coli*	bla_{KPC-2}, bla_{TEM-1}, bla_{SHV}, $bla_{CTX-M-15}$, *intl1*, *intl2*, *sul1*, *sul2*
T226	*Escherichia coli*	bla_{KPC-2}, bla_{TEM-1}, bla_{SHV}, bla_{OXA-48}, *intl1*, *intl2*, *sul1*, *sul2*
T227	*Escherichia coli*	bla_{KPC-2}, bla_{SHV}, bla_{OXA-48}, *intl1*, *intl2*, *sul1*, *sul2*, bla_{TEM}
T228	*Escherichia coli*	bla_{KPC-2}, bla_{SHV}, *intl1*, *intl2*, *sul2*
T210	*Klebsiella oxytoca*	bla_{OXA-1}, bla_{SHV}, bla_{OXA-48}, *intl1*, *sul1*, *sul2*, bla_{VIM-1}
T212	*Klebsiella oxytoca*	bla_{KPC-2}, bla_{OXA-1}, bla_{SHV}, *sul1*, *sul2*, bla_{VIM-1}
T259	*Klebsiella oxytoca*	bla_{SHV}, *intl1*, *sul1*, *sul2*, bla_{VIM-1}
J14	*Klebsiella pneumoniae*	bla_{KPC-2}, bla_{TEM-1}, bla_{SHV}, *intl1*, *sul1*
J204	*Klebsiella pneumoniae*	bla_{KPC-2}, bla_{TEM-1}, bla_{SHV}, *intl1*, *intl2*, *sul2*
T221	*Klebsiella pneumoniae*	bla_{KPC-2}, bla_{TEM-1}, bla_{SHV}, $bla_{CTX-M-15}$, bla_{OXA-48}, *intl1*, *intl2*, *sul1*, *sul2*, bla_{VIM-1},
T225	*Klebsiella pneumoniae*	bla_{KPC-2}, bla_{SHV-1}, *intl1*, *intl2*, *sul1*, *sul2*
J229	*Klebsiella pneumoniae*	bla_{KPC-2}, bla_{TEM-1}, bla_{SHV}, *intl1*, *intl2*, *sul1*, *sul2*
J231	*Klebsiella pneumoniae*	bla_{KPC-2}, bla_{TEM-1}, bla_{SHV-1}, $bla_{CTX-M-15}$, bla_{OXA-48}, *intl1*, *intl2*, *sul1*, *sul2*
J232	*Klebsiella pneumoniae*	bla_{KPC-2}, bla_{TEM-1}, bla_{SHV}, bla_{OXA-48}, *intl1*, *sul2*
J233	*Klebsiella pneumoniae*	bla_{KPC-2}, bla_{SHV-1}, *intl1*, *sul1*, *sul2*
J237	*Klebsiella pneumoniae*	bla_{KPC-2}, bla_{TEM-1}, bla_{SHV}, *intl1*, *sul2*
J241	*Klebsiella pneumoniae*	bla_{KPC-2}, bla_{SHV}, *intl1*, *intl2*, *sul2*
J244	*Klebsiella pneumoniae*	bla_{KPC-2}, bla_{SHV}, *intl1*, *intl2*, *sul2*
J245	*Klebsiella pneumoniae*	bla_{KPC-2} bla_{TEM-1}, bla_{SHV}, *intl1*, *sul1*, *sul2*
T253	*Klebsiella variicola*	bla_{KPC-2}, bla_{SHV}, bla_{OXA-48}, *intl1*, *intl2*, *sul1*, *sul2*
T254	*Klebsiella variicola*	bla_{KPC-2}, bla_{SHV}, $bla_{CTX-M-15}$, bla_{OXA-48}, *intl1*, *intl2*, *sul1*, *sul2*, bla_{VIM-1}
KZ69	*Raoultella ornithinolytica*	bla_{KPC-2}, bla_{OXA-48}, *intl1*, *intl2*, *sul1*
KZ72	*Raoultella ornithinolytica*	bla_{KPC-2}, bla_{OXA-48}, *intl1*, *intl2*, *sul1*
KZ74	*Raoultella ornithinolytica*	bla_{KPC-2}, bla_{OXA-48}, *intl1*, *intl2*, *sul1*

Regarding the five *Enterobacter* isolates, majority were found to co-harbor two carbapenemase genes. Notably, *bla*$_{KPC-2}$+*bla*$_{OXA-48}$ were identified in *E. cloacae* T213 and E. *bugandensis* T214, whereas *E. asburiae* was found to carry *bla*$_{VIM-1}$ in combination with *bla*$_{KPC-2}$ (isolate T218) or *bla*$_{OXA-48}$ (isolate T220). AmpC β-lactamase genes were also identified, although less frequently; bla$_{FOX-1-5B}$ in *E. bugandensis* T214 and *bla*$_{MIR-1T}$/*bla*$_{ACT-1}$ in *E. asburiae* T220. The *bla*$_{TEM}$ and *bla*$_{SHV}$ in addition to ESBL genes *bla*$_{CTX-M-15}$ or *bla*$_{GES-1}$ were found in both *E. asburiae* isolates. All *Enterobacter* isolates possessed *int1* and *int2* as well as sulfonamide resistance genes *sul1* and *sul2* (Table 3).

Three isolates of *R. ornithinolytica* showed the same antibiotic resistance genotype and contained only *bla*$_{KPC}$ in combination with *int1*, *int2*, *sul1*, and *sul2*. On the other hand, majority of *C. freundii* isolates possessed at least two carbapenemase genes. Isolates T251 and T201 co-harbored
bla$_{KPC-2}$+*bla*$_{OXA-48}$+*bla*$_{VIM-1}$ while isolates T208 and T222 carried *bla*$_{KPC-2}$+*bla*$_{OXA-48}$. AmpC encoding genes *bla*$_{DHA,}$ *bla*$_{FOX-1-5B}$ and *bla*$_{MIR-1T}$/*bla*$_{ACT-1}$ were also detected in these isolates (Table 3).

9.2.3 Molecular epidemiology of CRE isolates

Clonal relatedness was determined by using PFGE genotyping where macrorestriction patterns were utilized to generate a dendrogram, that also depicted similarities in AR profile and ARG (sub)types (Figure 9). Among the 12 *K. pneumoniae* isolates, 2 were not clustered, while 10 belonged to three distinct clusters (A-C) (Figure 9A). The largest cluster A comprised 5 isolates of 90% similarity, although some isolates displayed varying resistance phenotype and genotype. Most of these isolates shared a dominant resistance profile, including resistance to ten antibiotics, and carried $bla_{\text{KPC-2}}$, $bla_{\text{TEM-1}}$, bla_{SHV}, and $bla_{\text{OXA-48}}$ genes. Within cluster B (3 isolates), based on 95% similarity, most isolates shared the same resistance profile, except for one isolate that was sensitive to GEN and lacked the $bla_{\text{TEM-1}}$ gene, unlike the rest of the cluster which possessed both $bla_{\text{TEM-1}}$ and bla_{SHV} genes. In contrast, two isolates of cluster C differed by antibiotic susceptibility and gene profiles. Isolate T221 was the only one resistant to CL and harbored $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-48}}$, $bla_{\text{VIM-1}}$, and $bla_{\text{KPC-2}}$ simultaneously.

Two *K. oxytoca* isolates (Figure 9A) were clonally related (100% similarity) and exhibited identical AR patterns but different phenotypes (cluster D). Isolate T212 lacked the $bla_{\text{KPC-2}}$ or $bla_{\text{OXA-48}}$ genes present in isolate T210. Additionally, two *K. variicola* isolates (cluster E) showed 96% similarity and shared the same AR profile, showing resistance to all antibiotics except CL. They differed in ESBL phenotype, with one isolate containing $bla_{\text{CTX-M-15}}$ and the other not.

For *C. freundii E. coli*, only one cluster (F) were identified, comprising 2 isolates with 95% similarity, while three isolates could not be assigned to any cluster (Figure 9B). Most exhibited varied AR profiles but were all susceptible to CL, and possessed $bla_{\text{KPC-2}}$ and bla_{SHV} genes.

Among the six *E. coli* isolates, one PFGE cluster was identified (cluster G, Figure 9C). Four isolates shared the same AR profile, being resistant to GEN and TET, while two showed resistance to monobactam (ATM). All contained $bla_{\text{KPC-2}}$, in combination to bla_{SHV} and $bla_{\text{TEM-1}}$ genes.

PFGE genotyping was also performed for 5 *Enterobacter* isolates (Figure 9D), which revealed that only two *E. bugandensis* isolates clustered together with 80% of similarity, while others were singletons. As for *R. ornitholytica*, all three isolates clustered together (Figure 9E) while two of them (KZ69 and KZ74) were found to be clones (100% similarity).

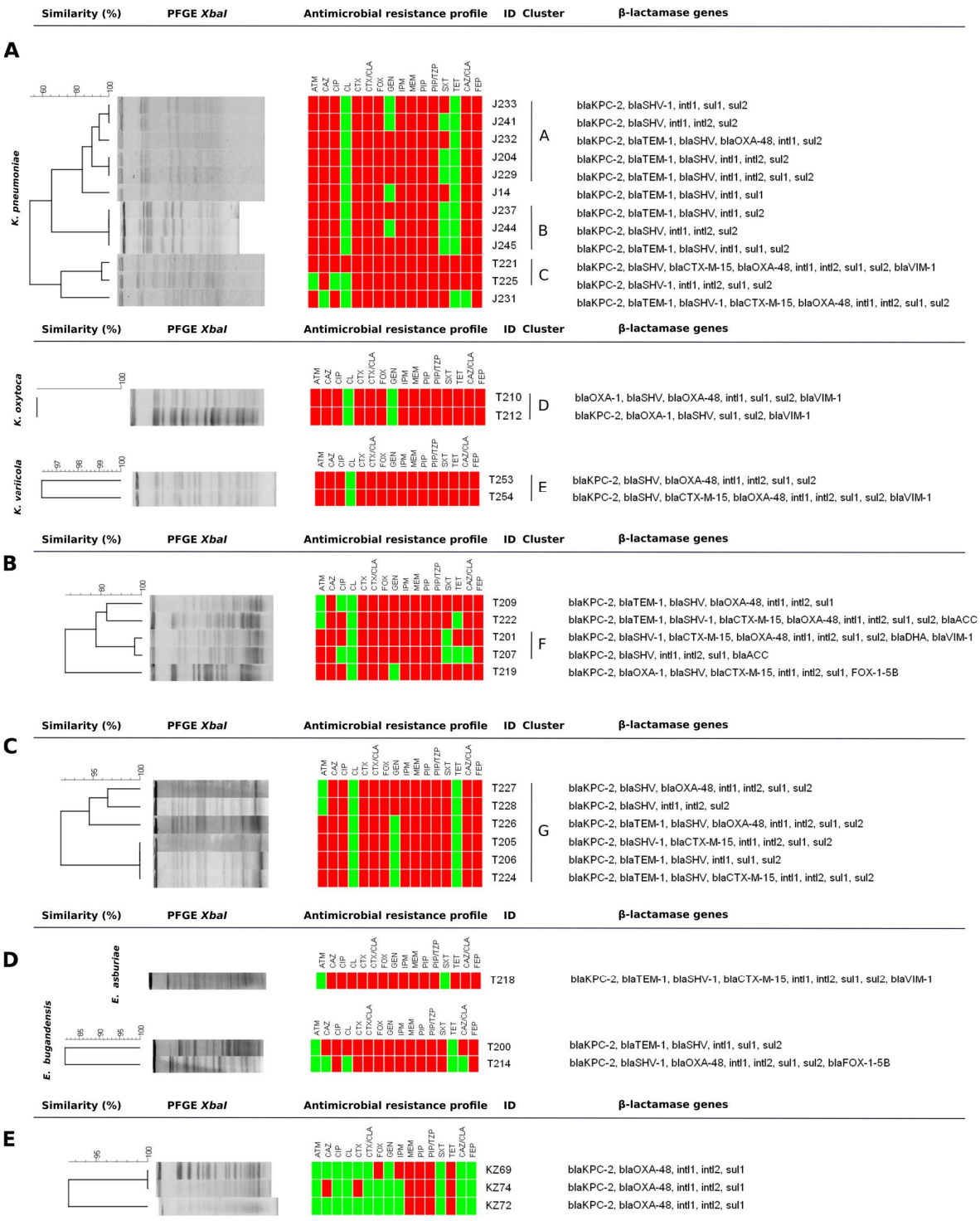


Figure 9. Dendrogram generated with Bionumerics software showing cluster analysis of XbaI-PFGE patterns of (A) *Klebisella* spp., (B) *Citrobacter freundii* , (C) *Escherichia coli*, (D) *Enterobacter* spp. and (E) *Raoulthela orinothlytica* isolates along with their antibiotic resistance phenotypes and ARGs. Red squares represent resistance and green squares represent susceptibility to the indicated antibiotics, ID stands for the name of the isolate.

9.2.4 Molecular identification of *bla*$_{VIM}$ and *bla*$_{KPC}$-bearing plasmids

9.2.4.1 S1-PFGE and Southern blot hybridization

The S1-PFGE was conducted to determine whether the *bla*$_{VIM}$ and *bla*$_{KPC}$ genes were located on plasmids or in bacterial chromosome. Southern blot hybridization of whole-cell DNA from 38 isolates with *bla*$_{KPC}$ and *bla*$_{VIM}$ specific probes revealed the presence of the *bla*$_{KPC}$ gene (Figure 10.) on plasmids in 26 isolates and the *bla*$_{VIM}$ gene in six of them. The plasmid sizes varied regard to bacterial species and ranged from approximately 40 kb to 216.9 kb for the *bla*$_{KPC}$ gene and from 78.2 kb to 310 kb for the *bla*$_{VIM}$ gene (Table 4).

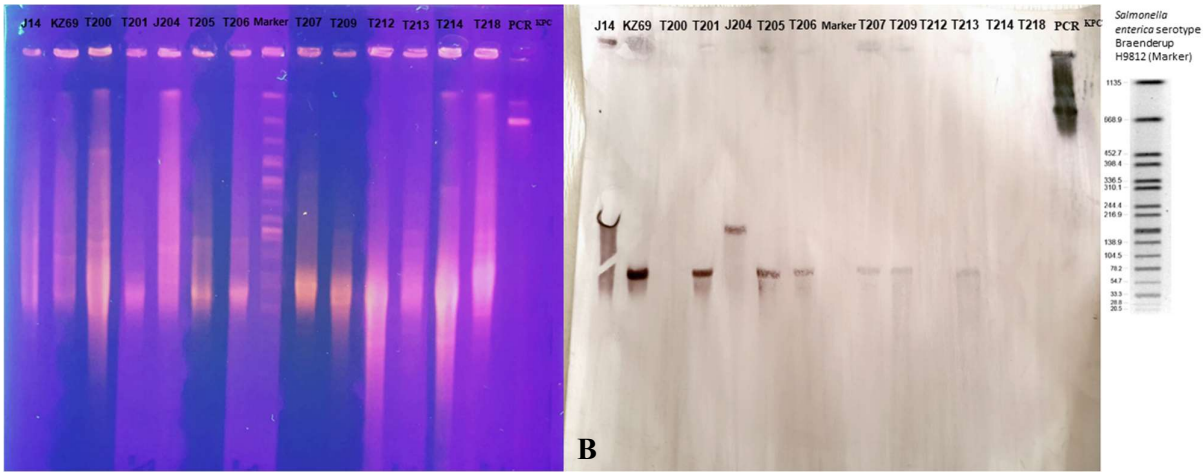


Figure 10. (A) Plasmid profiles of KPC-2-producing Enterobacteriaceae isolates obtained by the S1-PFGE and compared with *Salmonella enterica* serotype Braenderup (H9812) as a reference marker standard. The isolates belong to *K. pneumoniae* (J14, J204), *R. ornithinolytica* (KZ69), *E. bugandensis* (T200, T214), *C. freundii* (T201, T207, T209), *E. coli* (T205, T206), *K. oxytoca* (T202) and *E. cloacae* (T213). **(B)** Corresponding nylon membrane after Southern hybridization with digoxigenin-labelled KPC-2 hybridization probe.

Table 4. Size of plasmids carrying the *bla*KPC and *bla*VIM gene as confirmed by Southern blot hybridization.

Isolate	*Species*	***bla**KPC (~kb)*	***bla**VIM (~kb)*
T201	*C. freundii*	60.5	
T207	*C. freundii*	60.5	
T208	*C. freundii*	78.2	
T209	*C. freundii*	60.5	
T251	*C. freundii*		138.9
T218	*E. asburiae*		244.4
T220	*E. asburiae*		138.9
T213	*E. cloacae*	60.5	
T205	*E. coli*	60.5	
T206	*E. coli*	60.5	
T224	*E. coli*	40	
T226	*E. coli*	40	
T227	*E. coli*	78.2	
T228	*E. coli*	138.9	
T210	*K. oxytoca*		310
J14	*K. pneumoniae*	160	
J204	*K. pneumoniae*	160	
T221	*K. pneumoniae*	40	104.5
J229	*K. pneumoniae*	154	
J231	*K. pneumoniae*	40	
J232	*K. pneumoniae*	114	
J233	*K. pneumoniae*	78.2	
J241	*K. pneumoniae*	78.2	
J244	*K. pneumoniae*	122	
J245	*K. pneumoniae*	216.9	
T253	*K. variicola*	104.5	
T254	*K. variicola*	104.5	78.2
KZ69	*R. ornithinolytica*	60.5	
KZ72	*R. ornithinolytica*	78.2	
KZ74	*R. ornithinolytica*	78.2	

In *K. pneumoniae*, the most common plasmids carrying the *bla*KPC gene were of 160 kb (n=2), 78.2 kb (n=2) and 40 kb (n=2), while other plasmids were 216.9 kb, 154 kb, 122 kb and 114 kb (n=1 each). *K. variicola* carried a 104.5 kb plasmid, while *K. oxytoca* harbored a 78.2 kb size plasmid. Among the isolates of *E. coli*, *C. freundii*, *R. ornithinolytica* and *E. cloacae*, 60.5 kb and 78.2 kb plasmids were the most abundant. Notably, *E. coli* also had a 40 kb plasmid (Table 4). Moreover, *E. asburiae* and *C. freundii* carried ~138.9 kb plasmid bearing *bla*VIM, while *E. asburiae* had an additional 244.4 kb plasmid. The isolates of *K. pneumoniae* and *K. variicola* carried *bla*VIM on plasmids of 104.5 kb and 78.2 kb, respectively.

9.2.5 Horizontal transfer of *bla*$_{KPC-2}$ and *bla*$_{VIM-1}$ carbapenemase genes

Out of 24 carbapenemase-producing Enterobacteriaceae isolates selected for broth-mating conjugation assays, eleven isolates successfully transferred carbapenem resistance to azide-resistant recipient *E. coli* J53 strain with an average efficiency of 1.86×10^{-5}. More in detail, all 11 isolates transferred *bla*$_{KPC-2,}$ among which 4 co-transferred *bla*$_{VIM,}$ as confirmed by PCR (Table 5). As expected, the transconjugants exhibited resistance to β-lactam antibiotics, including carbapenems. Importantly, almost half of the donors co-transmitted resistance to CIP and TET, while one donor strain, *K. pneumoniae* T221, also successfully co-transferred resistance to GEN, enabling its transconjugant resistance to seven antibiotic classes (Table 5).

Table 5. Antibiotic susceptibility of transconjugants and carbapenemase encoding genes transferred to azide-resistant *E. coli* J53. Values in grey depict resistance phenotype.

Species	Donor strain	Transconjugant	Antibiotic susceptibility of transconjugants									Carbapenemase gene transferred
			TET	MEM	IPM	SXT	GEN	ATM	CIP	FEP	ETP	
E. bugandensis	T200	T5	27	11	15	6	21	28	30	21	11	***bla*$_{KPC-2}$**
E. coli	T205	T8	23	8	14	6	30	36	22	14	14	***bla*$_{KPC-2}$**
K. oxytoca	T212	T14	10	6	15	6	25	12	6	15	8	***bla*$_{KPC-2,}$ *bla*$_{VIM-1}$**
E. asburiae	T218	T17	7	6	6	31	20	30	20	13	6	***bla*$_{KPC-2}$ *bla*$_{VIM-1}$**
E. asburiae	T220	T19	6	6	12	25	20	32	19	11	12	***bla*$_{KPC-2}$ *bla*$_{VIM-1}$**
K. pneumoniae	T221	T20	6	6	12	6	6	7	18	12	6	***bla*$_{KPC-2}$ *bla*$_{VIM-1}$**
C. freundii	T222	T21	25	14	16	6	24	35	35	20	15	***bla*$_{KPC-2}$**
E. coli	T224	T22	26	6	14	6	35	8	32	9	8	***bla*$_{KPC-2}$**
E. coli	T227	T25	24	10	14	6	26	30	30	18	18	***bla*$_{KPC-2}$**
K. variicola	T254	T36	6	9	8	24	18	36	30	10	16	***bla*$_{KPC-2}$**
E. coli	T228	T38	23	10	15	6	30	30	26	20	16	***bla*$_{KPC-2}$**

9.2.6. Whole plasmid sequencing and *de novo* assembly

Following PFGE genotyping of carbapenemase-producing isolates, S1-PFGE and Southern blot hybridization of their genomic DNA with *bla*$_{KPC-2}$ and *bla*$_{VIM-1}$-labelled probes, and the conjugation experiments conducted, eight plasmid bends were selected for whole plasmid sequencing. The obtained contigs, based on SPAdes *de novo* assembly and overlap with reference genomes, were functionally characterized using available genome databases. As can be seen in Table 6, the lowest GC content was found in pEAT218_VIM (52.84%) and highest in pKPT221_KPC (56.55%). Moreover, the largest contig was assembled in pECT205_KPC with 39,412 bp, while in pECT224.2_KPC was the smallest 6,801 bp.

Table 6. QuastQC and Prokka annotation of de novo assembled resistance plasmids.

Plasmid	No. of contigs	Largest contig (bp)	GC content (%)	N_{50} (bp)	L_{50}
pECT218_KPC	5,936	7,137	54.14	269	2225
pEAT218_VIM	660	9,371	52.84	703	122
pKPT221_KPC	16,608	7,733	56.55	444	4175
pECT224_KPC	8,022	8,078	53.71	261	3423
pECT224.2_KPC	1,883	6,801	54.79	262	785
pECT227_KPC	7,925	8,138	53.97	261	3404
pEBT200_KPC	2,837	26,040	54.36	271	940
pECT205_KPC	4,663	39,412	52.31	262	1959

Notably, *bla*$_{KPC-2}$ was detected on 6 plasmids while *bla*$_{VIM-1}$ on one (pEAT218_VIM plasmid of *E. asburiae*) (Table 7). Plasmids were found to carry up to five *bla* genes, which besides the carbapenemases genes, included *bla*$_{TEM-1,}$ *bla*$_{OXA-10,}$ *bla*$_{OKP-B,}$ and *bla*$_{GES-1}$. Apart from *bla* genes, sequencing revealed determinants encoding for resistance to other classes of antibiotics including quinolones (*qnrB6*), fluoroquinolones (*aac(6')-Ib-cr),* aminoglycosides *(aac(6')-Ib3),* sulfonamides (*sul1, dfrA27, dfrB1*), macrolides (*mphE*)*,* and tetracyclines (*tetA*), respectively. Eight Inc replicons were identified, among which IncF-types in five plasmids and IncP6 in four of them. In total, eight sequenced plasmids possessed 21 ARGs, 12 plasmid replicons and 5 genes associated with bacterial virulence (Table 7).

Table 7. Molecular characteristics of eight *de novo* assembled resistance plasmids.

Plasmid	Insertion sequences	Incompatibility group	Resistance genes	Virulence genes
pECT218_KPC	IS1, IS3, IS4, IS5, IS6, IS21, IS30, IS66, IS91, IS110, IS1182, IS256, IS481, IS630, ISL3, ISNCY	Col440I, FII(pECLA)	*aac(6')-Ib3, qnrB6, sul1, tet(A)*	*astA*
pEAT218_VIM	IS1, IS3, IS5, IS6, IS30, IS66, IS91, IS110, IS256, ISNCY	Col440I, N L/M(pOXA-48)	*aac(6')-Ib3,* bla_{OXA-10} bla_{VIM-1}, *sul1, tet(A)*	*astA*
pKPT221_KPC	IS3, IS4, IS5, IS6, IS21, IS66, IS91, IS110, IS1182, IS200/IS605, IS256, ISNCY, ISAS1, ISL3	A/C2, FIB(K), FIB(pKPHS1), FII, P6	*aadA1,* bla_{KPC-2}, bla_{TEM-1}, $bla_{OKP-B-16}$, $bla_{OKP-B-5}$, $bla_{OKP-B-3}$, *fosA5, OqxA, sul1, tet(A), dfrB1*	*clpK2, traT, yagZ/ecpA, yagX/ecpC*
pECT224_KPC	IS3, IS5, IS6, IS21, IS30, IS66, IS91, IS110, IS1182, IS200/IS605, IS256, IS481, IS630, ISL3	Col440I, ColRNAI, A/C, FII, P6, R	*aac(6')-Ib-cr* bla_{KPC-2}, *sul1, dfrA27*	
pECT224.2_KPC	IS1, IS3, IS4, IS5, IS6, IS21, IS91, IS110, IS1182, IS481, ISAS1, IS630	ColRNAI	bla_{KPC-2}	
pECT227_KPC	IS1, IS4, IS5, IS6, IS66, IS91, IS1182, IS256, IS481, ISAS1, IS630	Col(IRGK), P6, Q1	*aac(6')-Ib3,* bla_{KPC-2} bla_{TEM-1}	
pEBT200_KPC	IS1, IS3, IS5, IS6, IS110, IS1182, IS200/IS605, IS630, ISNCY, IS91, IS1380	Col(IRGK) ColRNAI FIA(HI1), R	aac(6')-Ib3, armA, bla_{GES-1}, bla_{OXA-10}, bla_{KPC-2}, bla_{TEM-1}, *msr(E), mph(E)*	*traT*
pECT205_KPC	IS1, IS3, IS4, IS5, IS6, IS21, IS91, IS110, IS481, ISL3	Col440I, P6	*aac(6')-Ib-cr,* bla_{KPC-2}, bla_{TEM-1}, *sul1, dfrA27*	

De novo assembled pECT205_KPC from *E. coli* T205 strain was found to be a multidrug resistance plasmid that included the fluoroquinolone resistance gene *aac(6')-Ib-cr*; *bla* genes coding for broad-spectrum β-lactamase TEM-1 and carbapenemase KPC-2, sulfonamide resistance gene *sul1* and trimethoprim resistance gene *dfrA27*. A variety of insertion sequences detected in this strain, such as IS1, IS3, IS5, IS6, IS21, and IS110, likely contribute to genetic diversity and adaptation of plasmids, facilitating its horizontal transfer and modulating gene expression. To get a closer look of its backbone, the pECT205_KPC plasmid was reconstructed to a size of ~40 kb and compared to other *de novo* assembled plasmids from this study (Figure 11). On contrary, another plasmid from *E. coli*, the plasmid pECT227_KPC which was isolated from *E. coli* T227 strain also possessed plasmid replicons of IncP6 and Col-type, but carried only three ARGs, conferring resistance to β-lactams (*bla*$_{KPC-2}$, and *bla*$_{TEM-1}$ genes) and aminoglycosides (*aac(6')-Ib3* gene).

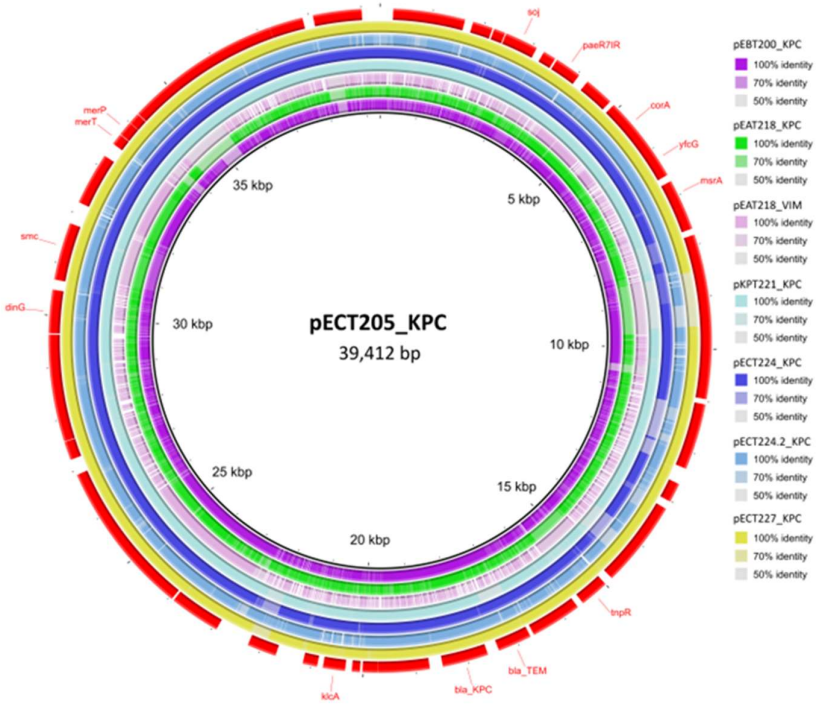


Figure 11. Mapping of *bla*$_{KPC-2}$-harbouring plasmid pECT205_KPC with other *de novo* assembled plasmids from this study. The red ring depicts the coding genes identified by Prokka. The plasmids were visualized using BRIG software.

Interestingly, *E. coli* T224 was found to carry two bla_{KPC}-bearing plasmids (pECT224_KPC and pECT224.2_KPC), both of which contained ColRNAI replicons. Nevertheless, plasmids differed by number of contings and GC content (Table 6), whereas the pECT224_KPC possessed genes conferring multiple resistance to antibiotics due to *aac(6')-Ib-cr*, *sul1,* and *dfrA27* genes (Table 7).

Moreover, the backbone of plasmid pEBT200_KPC from *E. bugandensis* T200 strain was found to be strikingly similar (100% of query cover and 99% identity) with other previously sequenced plasmids of IncR and IncFIA(HI1) groups (Figure 11). It coded for resistance to multiple antibiotic classes including aminoglycosides (*aac(6')-Ib3*, *armA*), β-lactams (bla_{GES-1}, bla_{OXA-10}, bla_{KPC-2}, bla_{TEM-1A}), and macrolides (*msrE*, *mphE*). Additionally, it harbored the virulence gene *traT* known for its role in promoting biofilm formation and improved serum resistance. Furthermore, the plasmid contained multiple transfer elements, namely IS3, IS5, IS6, IS1, IS1182, IS91, IS110, IS4, IS1380, IS21 and IS630, indicating potential horizontal transfer capability and genetic mobility.

Regarding the plasmids originating from *E. asburiae,* T218 strain was found to harbor two annotated plasmids: pEAT218_KPC (7,137 bp) carrying the bla_{KPC} and pEAT218_VIM (9,371 bp) carrying the bla_{VIM} gene. Apart from the different carbapenemases gene in their arrangement, two plasmids differed also by Inc replicons as well as the set of additional ARGs. Interestingly, both carried a virulence gene *astA* encoding for enteroaggregative *E. coli* heat-stable enterotoxin 1 EAST-1 and shared ten insertion sequences (Table 7).

Plasmid pKPT221_KPC (7733 bp) isolated from *K. pneumoniae* T221 strain carried four plasmid replicons, including IncA/C2, IncFIB, IncFII and IncP6. This plasmid carried eleven ARGs which is the most when compared to other analyzed plasmids. These included five *bla* genes, such as bla_{KPC-2}, bla_{TEM-1}, $bla_{OKP-B-5}$, $bla_{OKP-B-16}$, and $bla_{OKP-B-3}$, in addition to resistance determinants associated with aminoglycoside, quinolone, sulfonamide, tetracycline and trimethoprim resistance. This plasmid was also unique because it contained the most virulence genes; four of them, including *clpK2, traT /ecpA, yagX/ecpC.* ClpK2 is involved in protein degradation and regulation of stress responses, while TraT/ecpA and yagX/ecpC are associated with bacterial membrane integrity and resistance to host immune defenses. Additionally, yagZ is linked to the Type VI secretion system (T6SS), contributing to bacterial survival and dissemination in host environments.

9.3. SUPPLEMENTAL MATERIAL TO 1st SCIENTIFIC PAPER

Microbiome profiling and characterization of virulent and vancomycin-resistant *Enterococcus faecium* from treated and untreated wastewater, beach water and clinical sources

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Supplemental material – figures S1 to S8

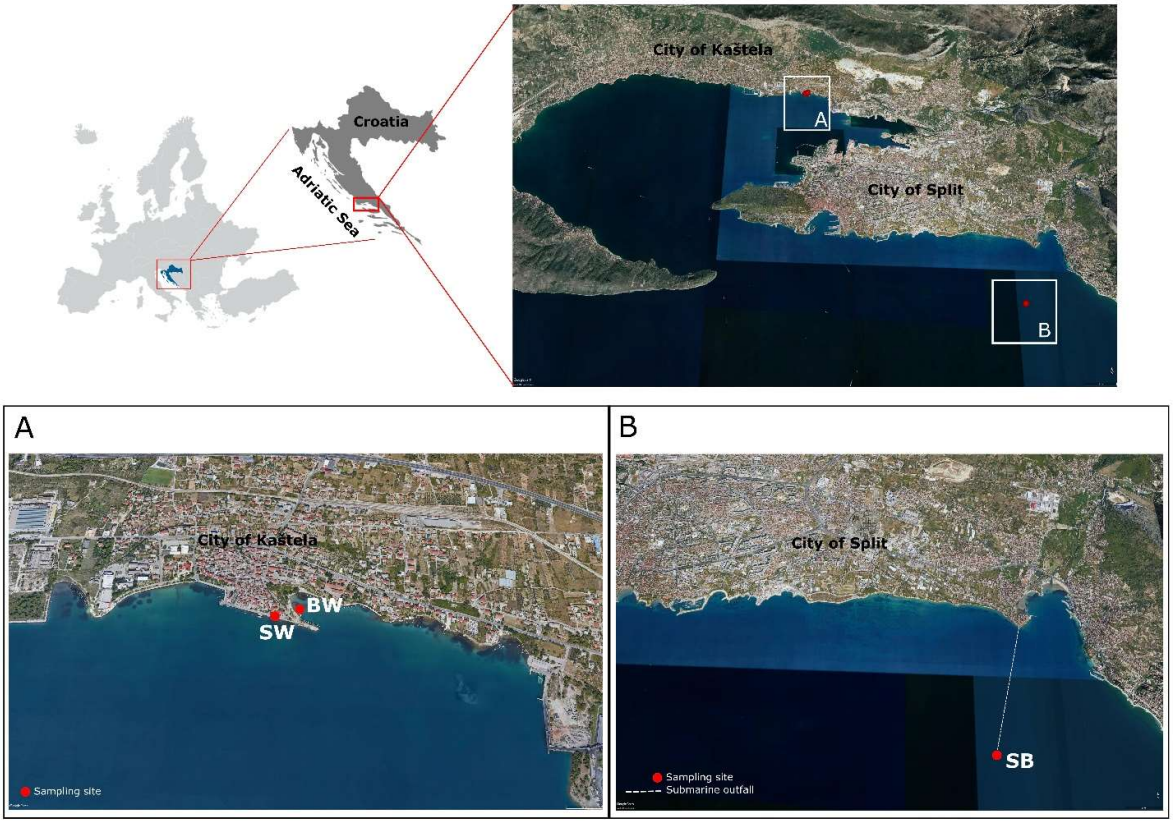

Fig S1. The locations of sampling sites in the coastal area of Split, central Adriatic Sea, Croatia. The water samples were collected near the submarine WWTP outfall (SB), the untreated sewage discharge (SW), and the adjacent public beach (BW).

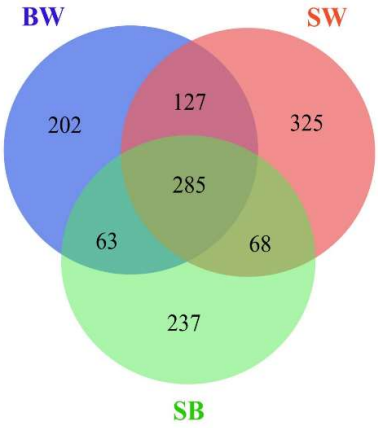

Fig. S2. Venn diagram based on OTUs diversity with values in overlapping parts representing number of OTUs common to more than one microbiome.

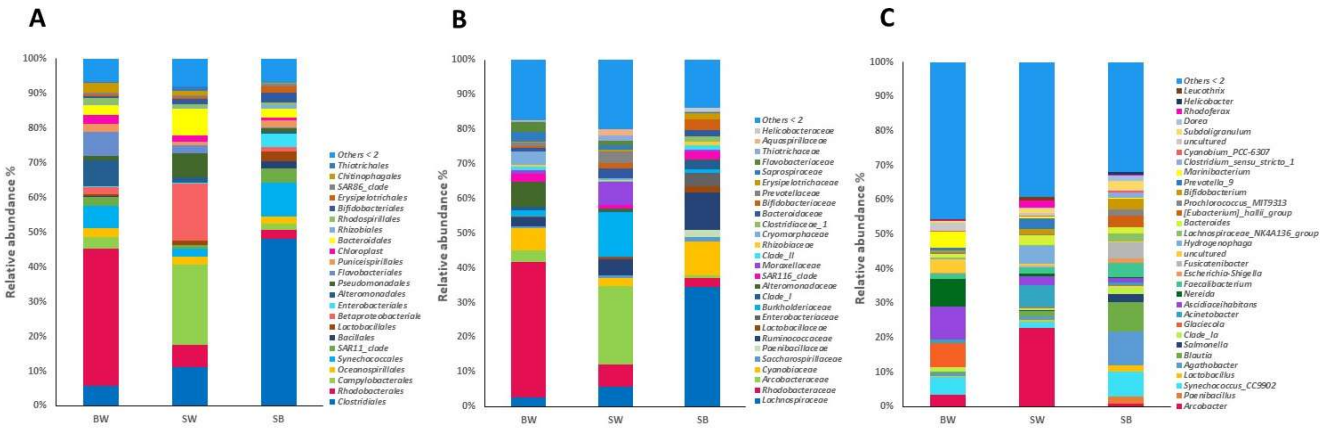

Fig. S3. Relative abundance (%) of bacterial orders (A), families (B) and genera (C) in the studied samples. The abundance is expressed as the percentage of the individual taxa in the total number of reads.

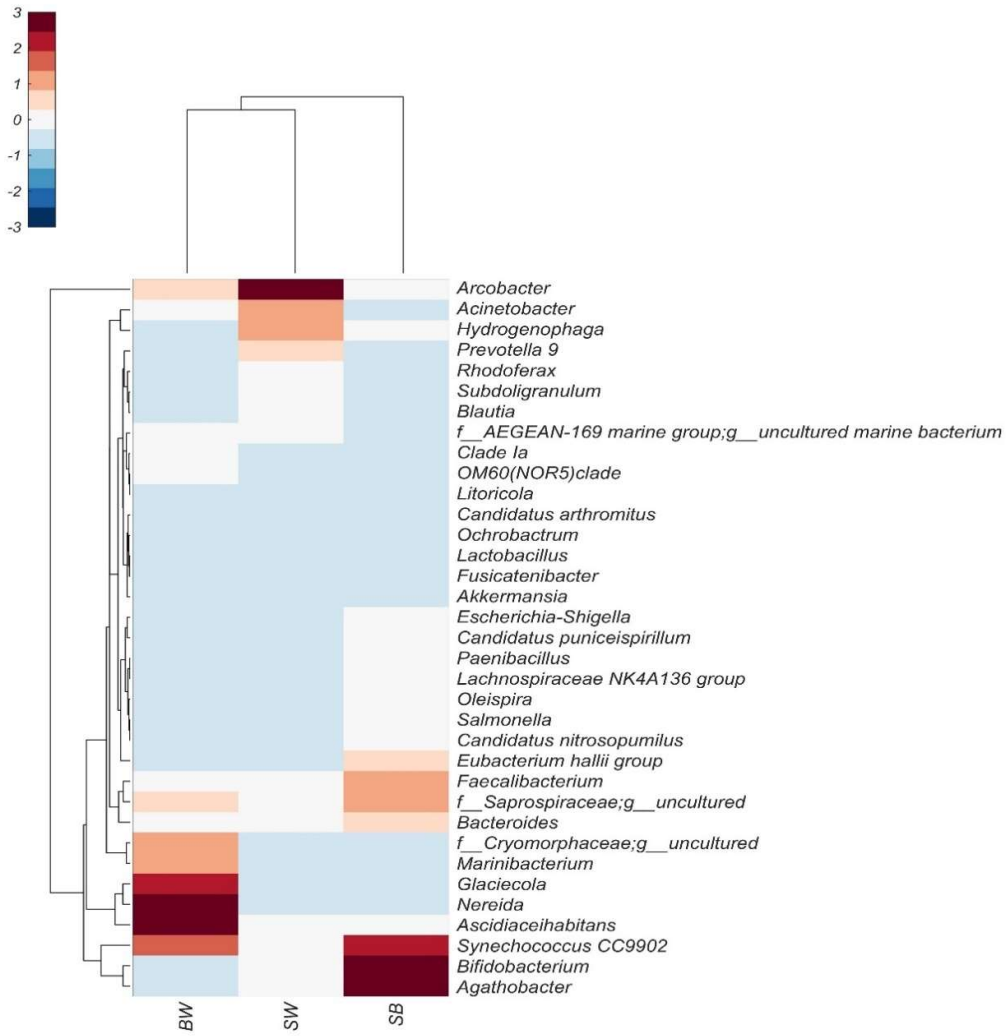

Fig. S4. Cluster heatmap of the relative abundance plotted by sample name and the 35 most abundant genera in microbiomes originated from submarine WWTP effluent (SB), untreated sewage water (SW) and receiving beach water (BW). The absolute 'z' value represents the distance between the raw score and the mean of the standard deviation. 'Z' is negative when the raw score is below the mean, and *vice versa.*

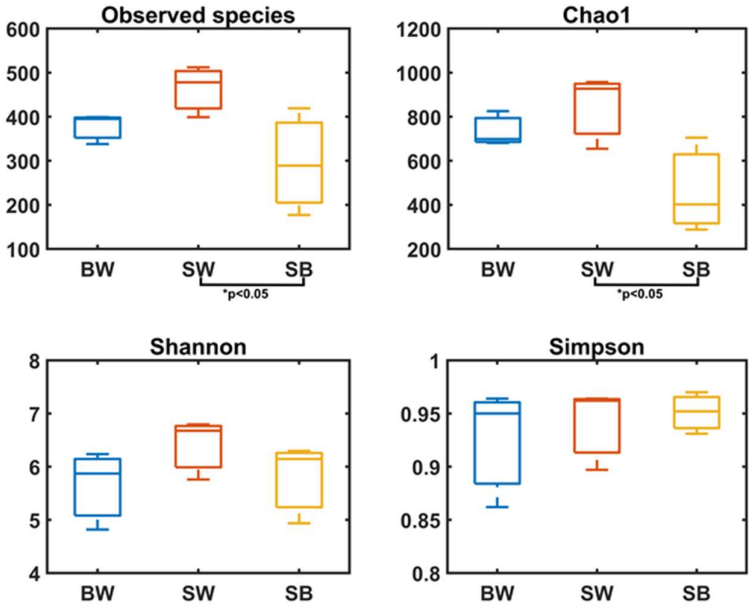

Fig. S5. Boxplots representing alpha diversity by community richness (observed species and Chao1 indices) and diversity (Shannon and Simpson indices) between bacterial communities at three sites. BW (blue), SW (orange), SB (yellow). Alpha indices that were significantly different between groups ($p < 0.05$) are marked with an asterisk.

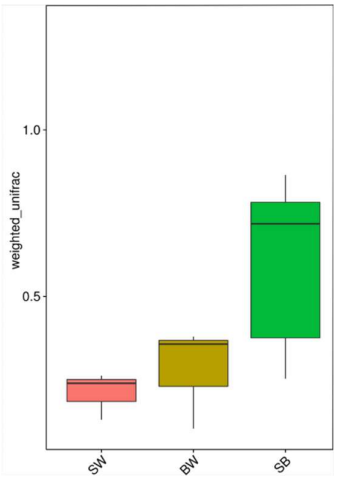

Fig. S6. Weighted UniFrac beta-diversity indices were statistically significant ($p<0.05$, Wilcox test) in case of SW and SB.

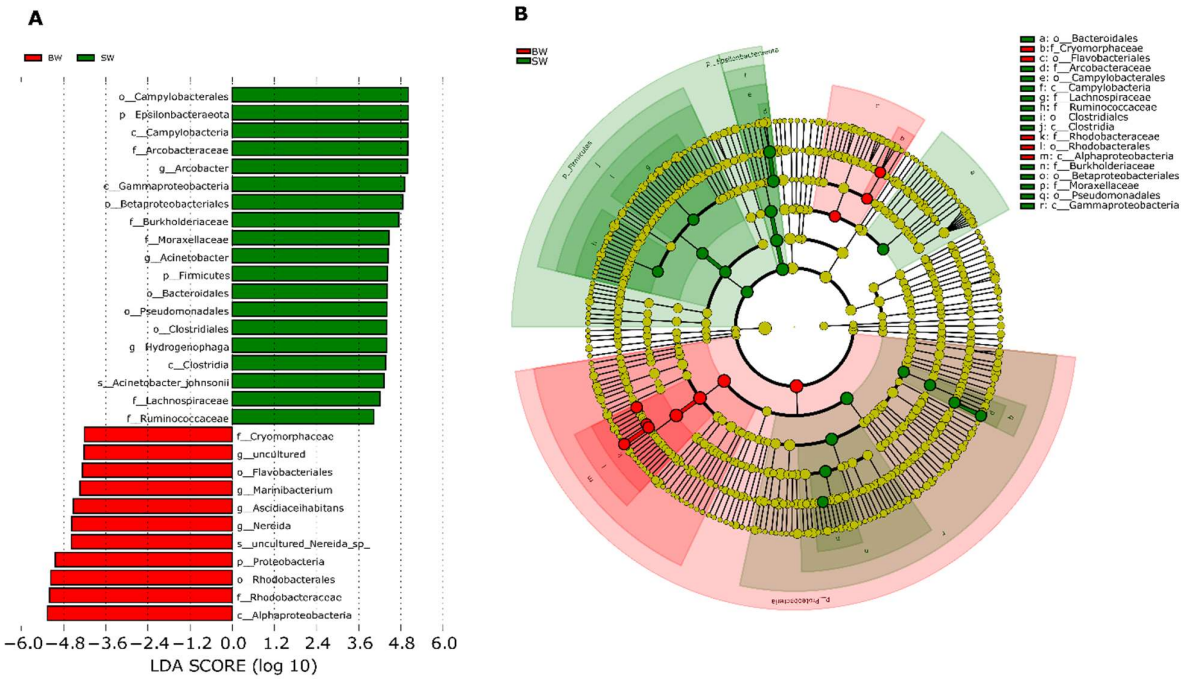

Fig. S7. Histogram of the LDA scores (A) and cladogram (B) showing the phylogenetic distribution of the microbial communities associated with BW and SW with LDA values of 2.0 or higher as determined by LEfSe analysis. Red indicates BW and green indicates SW; yellow represents insignificant difference. The diameter of each circle is proportional to a taxon's abundance. Circles from inner region to outer region represent the phylogenetic levels from class to genus.

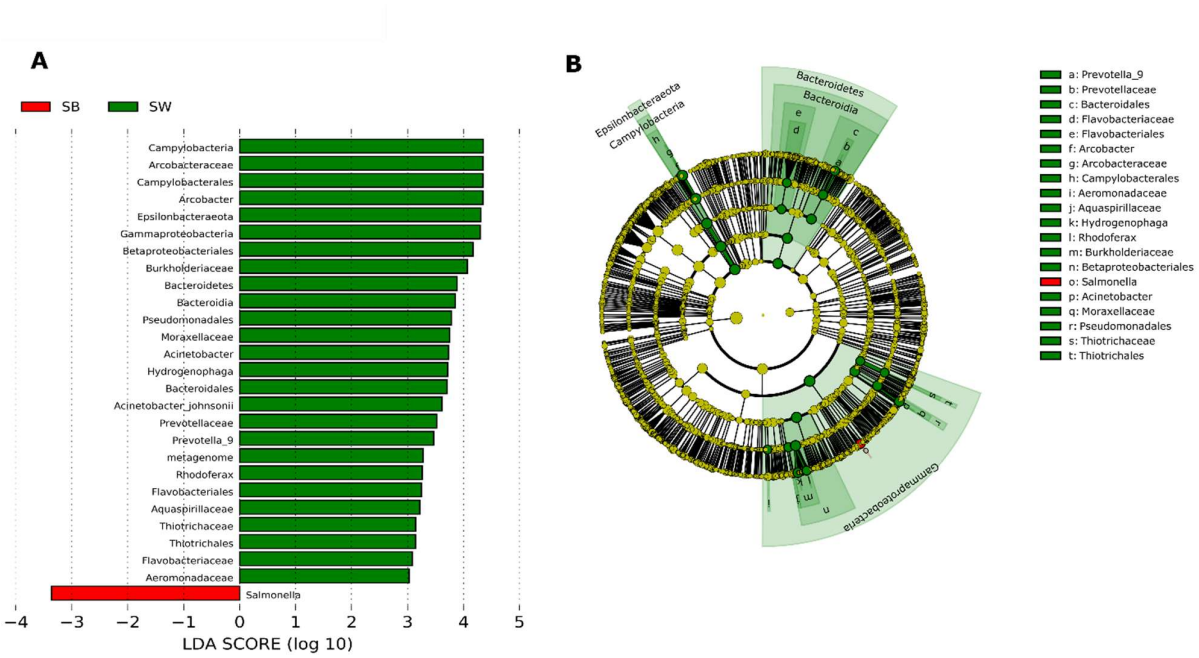

Fig. S8. Histogram of the LDA scores (A) and cladogram (B) showing the phylogenetic distribution of the microbial communities associated with SB and SW with LDA values of 2.0 or higher as determined by LEfSe analysis. Red indicates SB and green indicates SW; yellow represents insignificant difference. The diameter of each circle is proportional to a taxon's abundance. Circles from inner region to outer region represent the phylogenetic levels from class to genus.

Table S1. Physico-chemical and microbiological parameters of the beach water (BW), untreated sewage effluent (SW) and effluent-receiving seawater adjacent to submarine sewer outfall of Stobreč-Stupe (SB) WWTP.

Parameter	Unit	BW	SW	SB
Microbiological				
E. coli	CFU/100 mL	21.67	$4.1x10^5$	123.9
Intestinal enterococci	CFU/100 mL	53.33	$1x10^5$	33.33
Physical				
Temperature	°C	22.9	12.2	16.25
Salinity	‰	33.3	20.2	38.73
pH		8.17	7.99	8.1
Chemical				
NO_3	µmol/L	5.81	3.03	0.07
NO_2	µmol/L	0.021	3.32	0.03
$N\text{-}NH_4$	µmol/L	0.37	135.6	0.04
PO_4	µmol/L	0.085	6.2	0.05
PTOT	µmol/L	0.242	9.27	0.15
NTOT	µmol/L	11.92	174	6.29

Abreviations: TDS, Total dissolved solids; SO42-, Sulfates; NO3, Nitrates; NO2, Nitrites; N-NH4, Ammonia; NTOT, Total Nitrogen; PO4, Phosphates; PTOT, Total phosphorus.

Table S2. Relative abundances (%) of the bacteria at genus, family, order and class level. Values of ≥ 2% are marked in red.

Seasonal and spatial distribution of antibiotic resistance in marine microbial communities along a trophic gradient in central Adriatic Sea

Table S3. Alpha diversity indices showing the richness (observed species, Chao1 indices), diversity (Shannon and Simpson indices) and evenness of bacterial communities at analyzed sites.

Indices	**BW**	**SW**	**SB**
Observed species	377	463[a]	295[a]
Chao1	734,96	846,20[b]	464,92[b]
Shannon	5,641	6,41	5,792
Simpson	0,925	0,941	0,951
Evenness	0,95	1,07	1,01
Pathogenic genera[c]	9,28	42,15	17,78

[a, b] Significant different (p-value<0.05) by Wilcox-test.

c Abundance (%) was defined as the ratio of the read count of the genus pertotal number of read counts in the sample.

Table S4. Results of multi-response permutation procedure (MRPP) analysis evaluating the variation among and within bacterial communities from submarine WWTP effluent (SB), untreated sewage water (SW) and receiving beach water (BW).

	MRPP			
Group	A	observed-delta	expected-delta	Significance
BW-SW	0.3128[a]	0.3869[b]	0.563	0.1
BW-SB	0.1641[a]	0.6156	0.7364	0.034[b]
SW-SB	0.1891[a]	0.5956	0.7345	0.041[b]

[a] Variations among groups are larger than variation within groups.

[b] Statistically significant (p-value < 0.05).

Table S5. PICRUSt2-predicted ARGs content associated with the microbiomes originating from the submarine WWTP outfall (SB), the untreated sewage discharge (SW), and the adjacent public beach (BW), represented as the average values of the absolute abundances of sets and variants of ARGs from KEGG Brite Antimicrobial Resistance Genes database.

h6	Average of each station		
	BW	SW	SB
23S rRNA methyltransferases	2.8566667	5.33333333	2.236667
ADP-ribosyl transferases	1.14	1.19	0.14
Acetyltransferases	73.776667	216.103333	60.31
VraFG transporter	38	8	4
dltABCD operon	432.77333	2488.39333	343.3667
lysyl-phosphatidylglycerol (L-PG) synthase MprF	31.76	35.8833333	73.17667
protease PgtE	40.36	110.303333	91.29333
Class A	0.1433333	1.5	1.133333
Class B	1.75	10.5	1
Class C	6	18.6666667	14
Class D	6	18.6666667	14
Dihydropteroate synthase	0	0	2.165
Imipenem resistance, repression of porin OprD	89.9	366.093333	55.36
AbcA	17.38	20.99	84.64
AcrEF-TolC	180.43667	782.613333	170.4267
AdeABC	162.33333	823	103.6633
BpeEF-OprC	124	632.5	73.44333
MdtEF-TolC	191.15667	804.573333	157.5667
MepA	0	0	189
MexAB-OprM	526.45333	2639.37667	440.71
MexCD-OprJ	13	16.5	15
MexEF-OprN	23.166667	60.5	37.5
MexJK-OprM	262.47333	1321.97	192.6867
MexPQ-OpmE	14.333333	16.3333333	11.5
MexXY-OprM	170.14	841.903333	135.5767
NorB	13.066667	4.8	1.776667
QacA	32.76	153.023333	30.29667
repression of porin OmpF	6.78	6.78	55.10667
N-Acetyltransferases	4.4266667	8.31	149.14
O-Nucleotidyltransferases	32.12	160.95	122.0967
O-Phosphotransferases	9.9266667	6.85666667	10.68333
Other	8.85	24.5	6.8
Others	40.85	78.4666667	128.9567
Phosphotransferase	0	2	101
Tetracycline resistance, efflux pump Tet38	10.716667	15.5833333	94.99
Transporter	32.76	153.023333	30.29667
Transporters	72.216667	259.586667	384.3267
Vancomycin resistance, D-Ala-D-Lac type	59.833333	103.076667	167.19
Vancomycin resistance, D-Ala-D-Ser type	9.32	25.0633333	15.76
beta-Lactam resistance, Bla system	58.353333	85.3966667	57.71333

Table S5. (Extended table data) PICRUSt2-predicted ARGs content associated with the microbiomes originating from the submarine WWTP outfall (SB), the untreated sewage discharge (SW), and the adjacent public beach (BW), represented as the average values of the absolute abundances of sets and variants of ARGs from KEGG Brite Antimicrobial Resistance Genes database.

h6	BW.1	BW.2	BW.3	SW.1	SW.2	SW.3	SB.6	SB.7	SB.9	stat_BWSW	p_BWSW	stat_SWSB	p_SWSB	stat_BWSB	p_BWSB
23S rRNA methyltransferases	3.71	2.43	2.43	3.29	3.71	9	4.14	1.43	1.14	-1.313026233	0.259433524	1.495348139	0.209146437	0.592570084	0.585345026
ADP-ribosyl transferases	1.14	1.14		0.14	2.14	1.29	0.14								
Acetyltransferases	62.28	81.28	77.77	174.17	205.65	268.49	31.88	63.84	85.21	-5.023402131	0.007367661	4.905092707	0.008014621	0.81326524	0.461701536
Cationic antimicrobial peptide (CAMP) resistance, VraFG transporter [MD:M00730]		8	68			8			4						
Cationic antimicrobial peptide (CAMP) resistance, dltABCD operon [MD:M00725]	73.36	532.96	692	3628.16	1822.04	2014.98	623.06	97.4	309.64	-3.415275729	0.026896634	3.61965986	0.022363947	0.372162242	0.728650066
Cationic antimicrobial peptide (CAMP) resistance, lysyl-phosphatidylglycerol (L-PG) synthase MprF [MD:M0072	8.54	17.24	69.5	26.94	26.84	53.87	143.02	48.1	28.41	-0.195846849	0.8542769	-1.021558166	0.364744978	-1.030847507	0.360860758
Cationic antimicrobial peptide (CAMP) resistance, protease PgtE [MD:M00744]	24.24	52.42	44.42	155.29	88.33	87.29	137.76	67.79	68.33	-2.913453113	0.04352748	0.587823505	0.588234275	-2.062046145	0.10820118
Class A [MT]	0.14	0.07	0.22	1	1.21	2.29	2	1.33	0.07	-3.375066537	0.027912518	0.529360298	0.624571799	-1.744766174	0.15596691
Class B [MT]	3	0.5		25.5	5.5	0.5			1						
Class C [MT]	4	8	6	22	17	17			14	-6.247161518	0.003346954				
Class D [MT]	4	8	6	22	17	17			14	-6.247161518	0.003346954				
Dihydropteroate synthase							3	1.33							
Imipenem resistance, repression of porin OprD [MD:M00745]	54.28	101.14	114.28	246.5	356.2	495.58	6	2.66	157.42	-3.715376768	0.020557284	3.518468047	0.024483885	0.637384928	0.55852999
Multidrug resistance, efflux pump AbcA [MD:M00700]	1.67	10.14	40.33	15.54	21.47	25.96	214.81	27.67	11.44	-0.297991478	0.780546837	-0.974386568	0.385038751	-1.014482831	0.367728062
Multidrug resistance, efflux pump AcrEF-TolC [MD:M00696]	108.8	204.36	228.15	1100.21	618.6	629.03	208.37	165.67	137.24	-3.695233061	0.020922569	3.822193082	0.018743021	0.238780707	0.823010357
Multidrug resistance, efflux pump AdeABC [PATH:map01501] [MD:M00649]	86	237	164	573	788	1108	6	3.99	301	-4.092631067	0.014942889	3.907239932	0.017434432	0.543880997	0.615418679
Multidrug resistance, efflux pump BpeEF-OprC [MD:M00698]	65	177.5	129.5	424	607	866.5	2	1.33	217	-3.839294298	0.018470678	3.801091564	0.019085731	0.641325121	0.556212948
Multidrug resistance, efflux pump MdtEF-TolC [MD:M00697]	114.8	218.44	240.23	1124.92	636.56	652.24	189.62	126.88	156.2	-3.721222853	0.020452702	4.012219524	0.015967145	0.786143578	0.47574075
Multidrug resistance, efflux pump MepA [MD:M00705]							189								
Multidrug resistance, efflux pump MexAB-OprM [MD:M00718]	281.79	618.65	678.92	3446.41	2111.59	2360.13	460.43	337.65	524.05	-4.93596725	0.007839331	5.317463323	0.006015841	0.634520282	0.560218705
Multidrug resistance, efflux pump MexCD-OprJ [MD:M00639]	9	7.5	22.5	12	15	22.5			15	-0.613940614	0.572452697				
Multidrug resistance, efflux pump MexEF-OprN [MD:M00641]	24	11.5	34	83	50.5	48			37.5	-2.86802611	0.045559154				
Multidrug resistance, efflux pump MexJK-OprM [MD:M00642]	136.99	323.57	326.86	1421.07	1132.79	1412.05	147.41	92.88	337.77	-9.331434804	0.000734206	9.38980744	0.00071677	0.71798253	0.512468209
Multidrug resistance, efflux pump MexPQ-OpmE [MD:M00769]	11	7	25	11	14	24	2		21	-0.297408958	0.780960434				
Multidrug resistance, efflux pump MexXY-OprM [MD:M00643]	83.99	195.57	230.86	1104.87	663.79	757.05	144.41	91.55	170.77	-4.753425703	0.008948328	5.185294694	0.006582301	0.691027804	0.527558513
Multidrug resistance, efflux pump NorB [MD:M00702]	0.2	4	35	2.4	5	7	2	1.33	2	0.744640667	0.497858579	2.23906939	0.088713459	1.024161143	0.36365288
Multidrug resistance, efflux pump QacA [MD:M00714]	17	45.64	35.64	98.43	147.71	212.93	21.14	15.04	54.71	-3.515921015	0.024540259	3.468900883	0.02560923	0.165130636	0.876850587
Multidrug resistance, repression of porin OmpF [MD:M00746]		11.28	2.28	6.86	4.62	8.86	107.62	51.08	6.62			-1.65212221	0.173853674		
N-Acetyltransferases	2	2.64	8.64	2.43	12.57	9.93	415.14	20.71	11.57	-1.049297496	0.353254397	-1.058388061	0.349559296	-1.087718693	0.337871871
O-Nucleotidyltransferases	16.34	45.88	34.14	104.97	157.58	220.3	286.28	24.64	55.37	-3.742474621	0.020077845	0.436334219	0.685112207	-1.083861632	0.339388452
O-Phosphotransferases	4.64	11.14	14	6.14	4.14	10.29	19.14	2.91	10	0.927787941	0.40603271	-0.760060254	0.489551732	-0.138758163	0.89634672
Other	7.26	9.07	10.22	23	25.21	25.29	2	1.33	17.07	-13.69823834	0.00016452	3.408344844	0.027068575	0.393446791	0.714060117
Others	57.88	26.54	38.13	97.51	70.78	67.11	263.21	80.52	43.14	-2.839630427	0.046885178	-0.735361329	0.502908353	-1.284330911	0.268364753
Phosphotransferase					2		195	7							
Tetracycline resistance, efflux pump Tet38 [MD:M00704]	2.16	8.33	21.66	15.67	13	18.08	239.05	40.59	5.33	-0.819532325	0.458504137	-1.091344236	0.336451907	-1.154858602	0.312442256
Transporter	17	45.64	35.64	98.43	147.71	212.93	21.14	15.04	54.71	-3.515921015	0.024540259	3.468900883	0.02560923	0.165130636	0.876850587
Transporters	49.56	89.49	77.6	202.85	252.04	323.87	916.4	141.8	94.78	-5.053292865	0.007214394	-0.46425198	0.66660916	-1.1705051	0.306774959
Vancomycin resistance, D-Ala-D-Lac type [MD:M00651]	64	45.61	69.89	98.86	102.92	107.45	376.67	78.57	46.33	-5.60036142	0.004990977	-0.609546964	0.575087707	-1.018500367	0.366031582
Vancomycin resistance, D-Ala-D-Ser type [MD:M00652]	1.68	9.28	17	25.08	21.28	28.83	29.28	4	14	-3.193090725	0.033118359	1.213442822	0.291711765	-0.750718052	0.494571969
beta-Lactam resistance, Bla system [MD:M00627]	44.53	36.85	93.68	89.91	80.96	85.32	81.61	59.39	32.14	-1.503370006	0.20717346	1.90435775	0.129585976	0.028024083	0.978985376

Table S6. Percentage of pathogenic genera that are associated with the ARGs and modules in each microbiome based on KEGG BRITE ARGs. (can be found on link below, QR code)

Seasonal and spatial distribution of antibiotic resistance in marine microbial communities along a trophic gradient in central Adriatic Sea

Here are cited all references included in the paper that were not cited before in this doctoral dissertation [255–294] .

9.4. SUPPLEMENTAL MATERIAL TO 2nd SCIENTIFIC PAPER

Marine resistome of a temperate zone: distribution, diversity, and driving factors across the trophic gradient

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Supplemental material – Figures S1 to S4

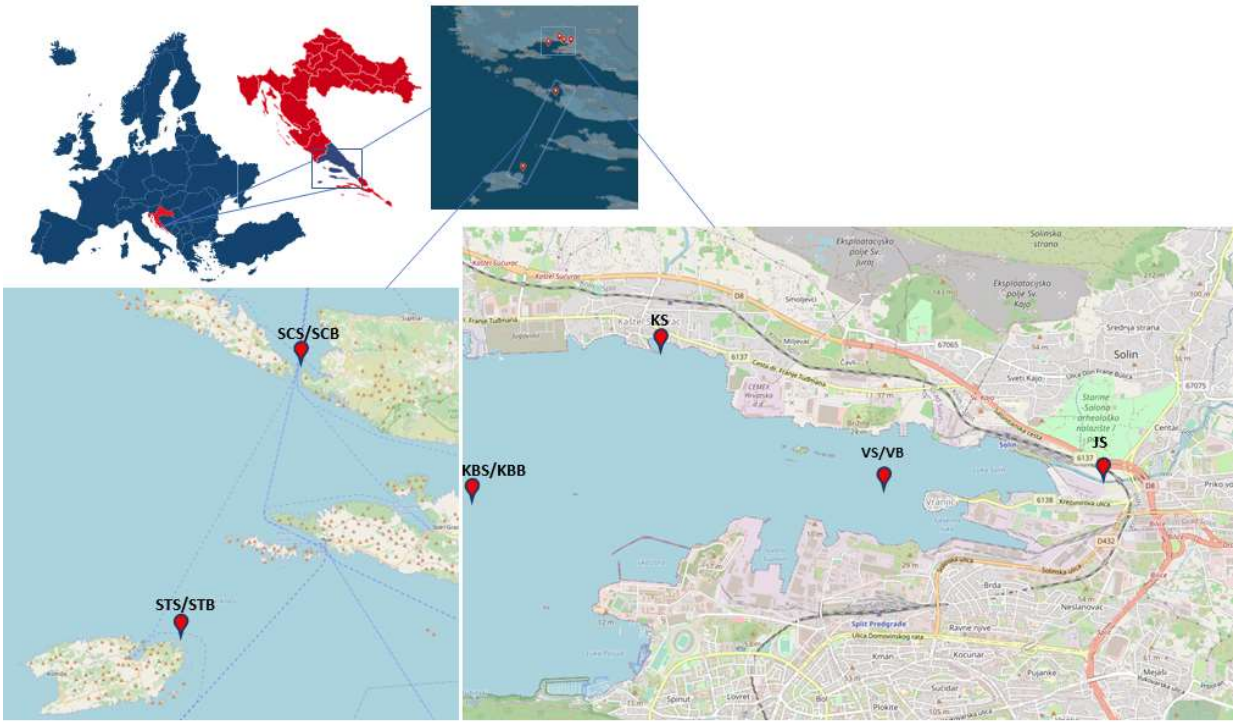

Fig S1. The sampling sites in the central Adriatic Sea (Mediterranean Sea), Croatia. KSS, Kaštel Sućurac (surface); JS, Jadro river (surface); VBS, Vranjic Bay (surface)/VBB, Vranjic Bay (bottom); KBS, Kaštela Bay (surface)/KBB, Kaštela Bay (bottom); SCS, Split Channel (surface)/SCB, Split Channel (bottom); STS, Stončica (surface)/STB, Stončica (bottom).

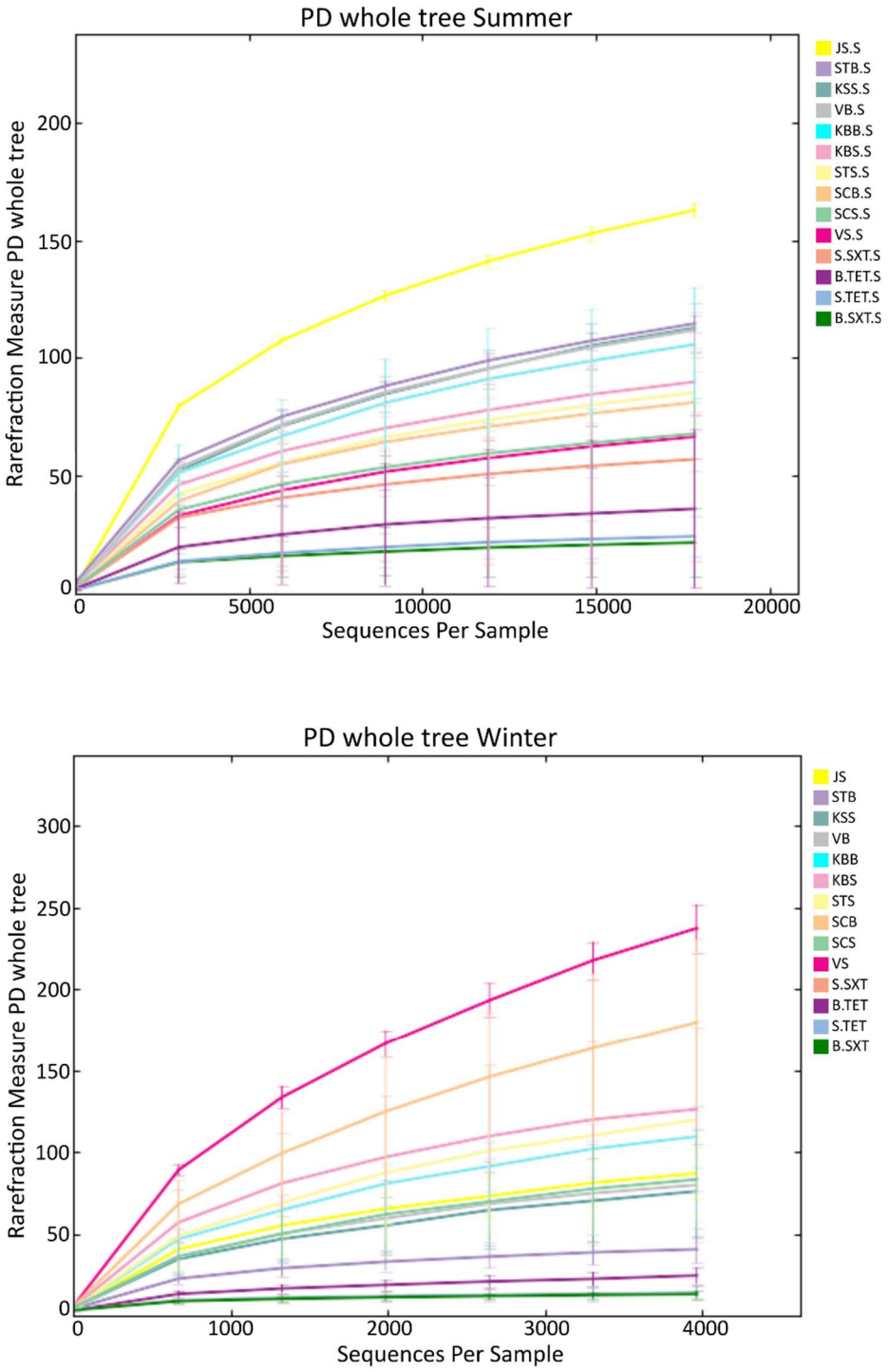

Fig S2. Rarefaction curve is created by selecting randomly certain amount of sequencing data from the samples, then counting the number of the species they represent (i.e. the number of OTUs). The Rarefaction curves can directly reflect the rationality of the sequencing data volume and indirectly reflect the richness of microbial community in the samples

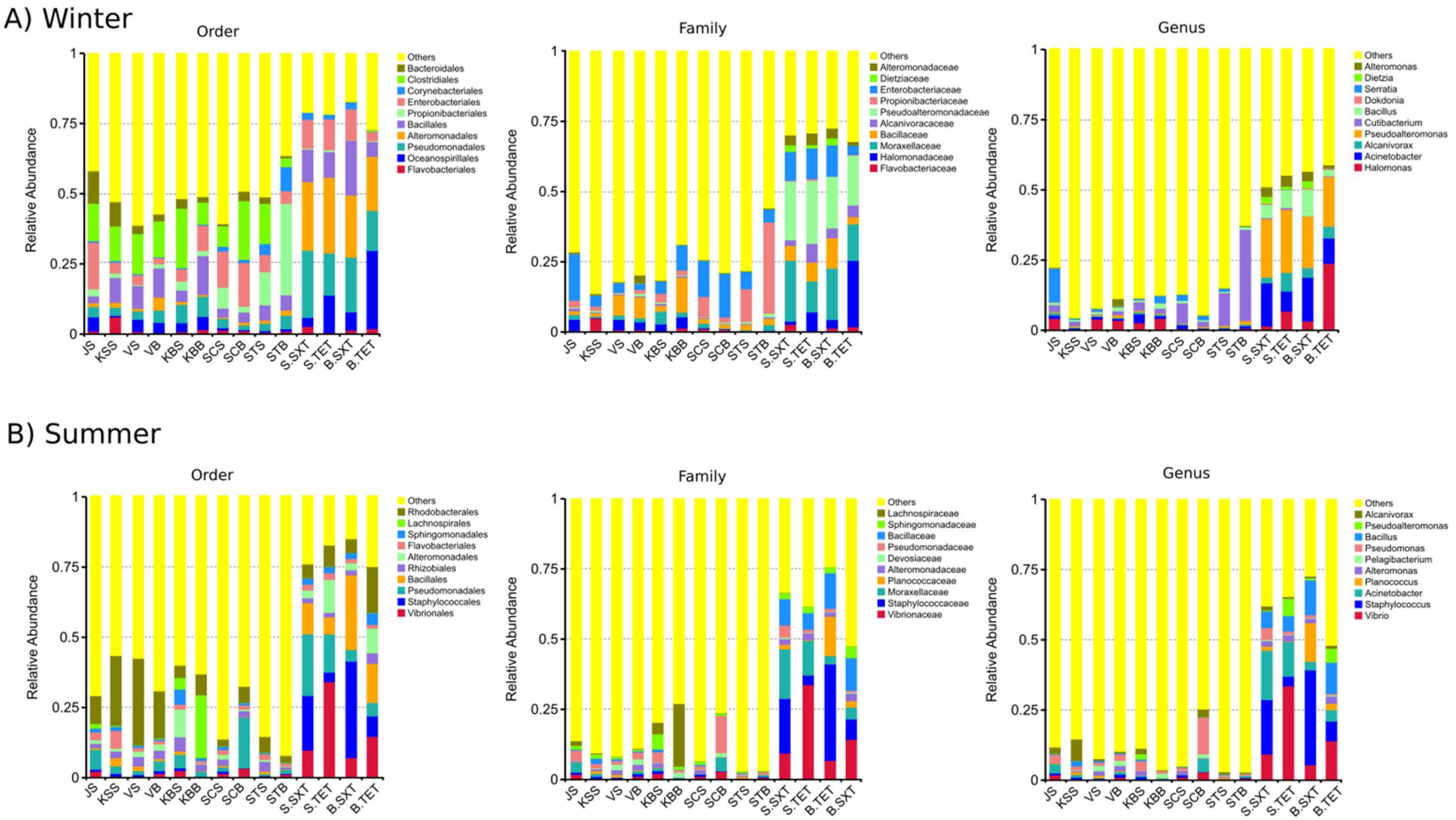

Fig S3. Relative abundance (%) of bacterial taxa at order, family, and genus levels during winter (A) and (B) summer periods, depicting the 10 most prevalent taxa. The abundance is expressed as the percentage of the individual taxa in the total number of reads. The extensions .SXT and .TET refer to SXT- and TET-resistant marine communities, while without extensions is referred to indigenous community.

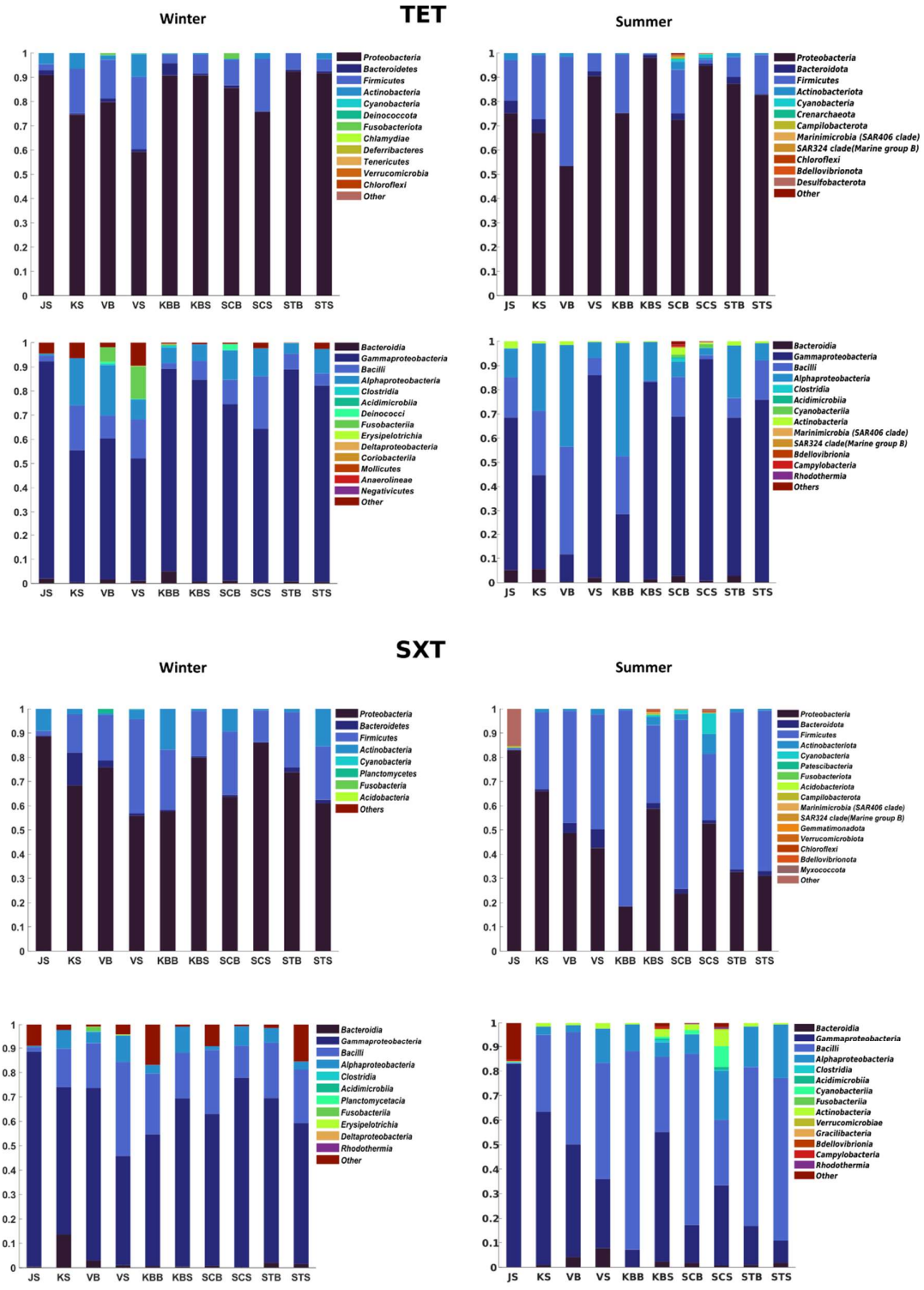

Fig S4. Relative abundance up to 16 most prevalent taxa at phylum and class levels in TET- and SXT-resistant bacterial marine communities during winter and summer seasons.

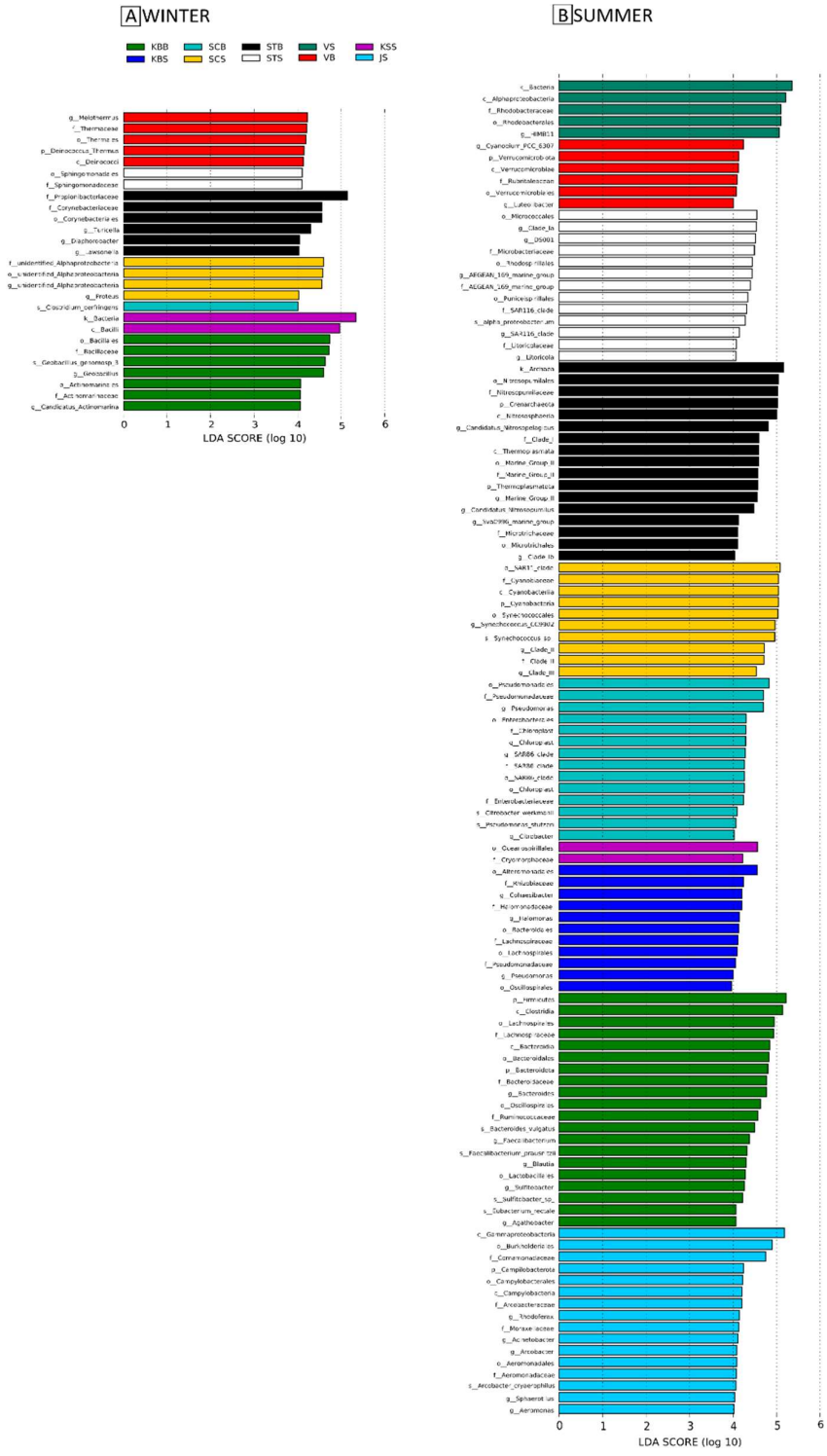

Fig S5. Histogram of the LDA scores (2.0 or higher) based on LEfSe analysis showing the differentially enriched taxa in marine microbiomes along the trophic gradient during winter and summer seasons.

Supplement text S1.

1. Alpha diversity calculations

To analyze the complexity of the biodiversity of the microbiome, we determined the alpha diversity (within sample) for a sample by calculating several indices: Chao1 is an indicator of species richness (total number of species in a sample) that is sensitive to rare OTUs (singletons and doubletons); Shannon species diversity considers the number of species living in a habitat (richness) and their relative abundance (evenness). Gini-Simpson indices to measure the probability of interspecific encounters between stations along the trophic gradient. All measures were calculated and displayed using the microbiome and phyloseq package, which uses OTU tables, sample information tables, and taxon tables for calculations, with R software (version 4.2.4) using the following packages: phyloseq, microbiome, and vegan (Oksanen et al., 2020; McMurdie and Holmes, 2013; Lahti and Shetty, 2017).

Significant differences in bacterial richness and diversity between coastal and open sea sites were determined using the nonparametric Wilcoxon test. In this test, difference denotes the mean difference between groups, p-value is calculated to what extent two groups are statistically different, and its value is labeled as follows (significance or not. p-value < 0.05, signed *, p < 0.01, signed **; p < 0.0001, signed ***).

2. Beta diversity calculations

To assess differences in species complexity among samples, we applied beta-diversity analysis. Beta diversity metrics (between samples) summarize which samples differ from each other by considering sequence abundances or presence/absence of sequences only. Beta diversity based on the Bray-Curtis dissimilarity matrix was calculated using R software (version 4.2.4). The Bray–Curtis dissimilarity index (Bray et al. 1957) measures the compositional dissimilarity between the microbial communities of two samples i and j based on counts in each sample. It is defined as

$$\mathrm{BC} = 1 - \frac{2C_{ij}}{\mathrm{S}_i + \mathrm{S}_j},$$

where C_{ij} is the sum of the smallest values for only the taxa that samples i and j have in common, and s_i and s_j are the total number of taxa counted in samples i and j, respectively. This index

ranges from 0 (the two samples have all taxa in common) to 1 (the two samples have no taxa in common). It gives more weight to shared taxa (Borcard et al., 2018). BC dissimilarity is calculated pairwise between all samples.

Differences in bacterial community structure between sites from coastal to open sea were analyzed using a phylogeny-based weighted UniFrac distance metric. Weighted UniFrac distances between two samples account for the phylogenetic tree and thus phylogenetic distances between community members (Lozupone et al. (2007). In UniFrac, the distance is calculated as a fraction of the branch length, and in weighted UniFrac, branch lengths are weighted according to the relative abundance of the sequences. The sum of unshared branch lengths is divided by the sum of all tree branch lengths, resulting in the fraction of total unshared branch lengths, defined as follows:

$$\sum\sum_{i}^{n} b_i \times \left[\frac{A_i}{A_T} - \frac{B_i}{B_T}\right]$$

Lozupone et al. (2007) defined n as the total number of branches in the tree, bi as the length of branch i, A_i and B_i as the number of sequences descended from branch i in communities A and B, respectively, and A_T and B_T as the total number of sequences in communities A and B, respectively. To account for unequal sampling effort, A_i and B_i are divided byA_T and B_T (Lozupone et al., 2007).

PCoA was performed to obtain principal coordinates and visualize complex, multidimensional data. A distance matrix of the Bray-Curtris distance. PCoA results were performed in R version 4.2.4 (R Core Team, 2022) using the following packages: phyloseq, microbiome, and vegan (Oksanen et al., 2020; McMurdie and Holmes, 2013; Lahti and Shetty, 2018). UPGMA clustering was performed as a type of hierarchical clustering method to interpret the weighted UniFrac distance matrix using average linkage and was performed using QIIME software (version 1.7.0).

3. Relative abundance cluster heatmap

Heatmap was generated using the relative abundance of bacteria at genus level and represents the hierarchical clustering of microbiomes based on the relative abundance of the top 35 genera in indigenous and SXT- and TET-resistant bacterial communities. The x-axis represents the distribution of bacterial communities and y-axis represents the genus-level taxonomy. The distance was calculated using the weighted UniFrac metric and in R software version 4.2.4 (R: The R Project for Statistical Computing,2022) using package: pheatmap (Kolde, 2012). Scaling was done by z-score (Z score = [relative abundance of bacterial taxa in a specific community - mean of relative abundance of the same taxa in all analyzed communities]/standard deviation). The positive Z scores indicate values above the mean, while negative Z scores values are below the mean in units of standard deviation.

4. Differential abundance analysis

Differences between microbial communities at different sites were analyzed using linear discriminant analysis (LDA) effect size (LEfSe). The LEfSe method was used to identify the most abundant taxa between all sites to identify potential microbial biomarkers. LEfSe is a high-dimensional biomarker discovery and explanation algorithm that identifies genomic features (genes, pathways, or taxa) that characterize differences between two or more biological conditions (or classes). The LEfSe algorithm was performed on a matrix with n rows and m columns, where each of the n features is represented by a positive-scoring vector containing its frequencies in the m samples, and each sample is associated with values describing its class and optionally its subclass and/or its subject of origin. Then, three steps were performed by the computational tool: the Kruskal-Wallis rank sum test for classes, the pairwise Wilcoxon test[296,297] between subclasses of different classes, and the LDA (Fisher, 1936) on the relevant features.

The Multiple Response Permutation Procedure (MRPP) is used to determine whether the difference in microbial community structure between groups is significant. The observed delta is the weighted mean of the within-group distance from the actual data, while the expected delta is the weighted mean of the distance from the permutations. The P value represents the significance of the delta, and A represents the chance-adjusted within-group agreement. A small value in the "Observed Delta" column means that the within-group variation is small, while a

large value in the "Expected Delta" column means that the within-group variation is large. A positive A value indicates that the between-group variation is larger than the within-group variation, while a negative value indicates the opposite relationship. The difference between groups is significant if the value in the significance column is less than 0.05. The upper confidence interval (UCL) and the lower confidence interval (LCL) form a corridor within which a quality characteristic reaches the desired value or a normal deviation. Outside the limits of UCL and LCL, the measured quality is considered abnormal and requires intervention in the corresponding process.

Table S1. Environmental analyses

Sampling date (dd/mm/y)	Station	GPS coordinates	Depth (m)	BP (10^4 cells mL^{-1} h^{-1})	HB (10^6 mL^{-1})	HNA (%)	LNA (%)	VLP (10^6 mL^{-1})	HNF (10^3 mL^{-1})	SYN (10^3 mL^{-1})	PRO (10^3 mL^{-1})	PE (10^3 mL^{-1})	Chl *a* mg m^{-3}	TEMP (°C)	SAL (‰)	pH	O_2(ML/L)	NO_3 [µmol L^{-1}]	NO_2 [µmol L^{-1}]	N-NH_4 [µmol L^{-1}]	PO_4 [µmol L^{-1}]	SIO_2 [µmol L^{-1}]
31/03/2021	JS	43° 32' 1"; 16° 29' 9"	0	0.11	0.326	53.4	46.6	5.234	3.534	14.83	0.63	4.8	0.37	13.8	0.2	8.5	5.781	0.43	0.005	0.01	0.003	1.841
9.7.2021	JS	43° 32' 1"; 16° 29' 9"	0	0.13	0.56	55.7	44.3	7.152	2.483	6.37	2.15	2.33	0.13	18	0.8	7.4	6.99	1.12	0.03	0.08	0.021	1.062
24/03/2021	VS	43° 31' 57"; 16° 27' 14"	0	0.27	0.806	32.9	67.1	6.275	1.49	81.43	2.053.1	15.15	1.18	12.68	37.13	8.19	5.755	3.409	0.216	0.735	0.202	5.006
24/03/2021	VB	43° 31' 57"; 16° 27' 14"	18	0.2	0.817	27.3	69.7	6.394	1.21	36.13	0.27	6.88	1.11	12.94	37.95	8.19	5.705	0.929	0.244	0.194	0.151	2.379
17/08/2021	VS	43° 31' 57"; 16° 27' 14"	0	0.31	1.31	34	66	16.682	1.74	3.8	0.8	2.23	0.64	27.004	37.42	8.23	5.215	0.743	0.037	0.268	0.05	2.035
17/08/2021	VB	43° 31' 57"; 16° 27' 14"	18	0.42	1.24	31.5	68.5	15.08	1.54	18.77	0.88	2.3	0.88	24.789	38.031	8.18	5.16	0.386	0.143	0.454	0.09	2.808
31/03/2021	KS	43° 54' 5"; 16° 42' 83"	0	0.12	0.786	58.7	41.3	8.758	2.237	0.47	2.07	0.8	0.37	15	33.5	8.16	5.781	5.678	0.187	1.018	0	3.493
9.7.2021	KS	43° 54' 5"; 16° 42' 83"	0	0.11	0.87	43	57	4.481	0.589	38.65	0.28	2.25	0.13	22.6	32.1	7.91	4.894	0.483	0.043	0.268	0.021	1.062
24/03/2021	KBS	43° 31' 10"; 16° 22' 52"	0	0.11	0.436	24.3	75.7	5.9	1.56	7.53	29.5	77.85	0.47	12.22	37.62	8.21	5.806	0.319	0.094	0.117	0.013	1.841
24/03/2021	KBB	43° 31' 10"; 16° 22' 52"	35	0.19	0.673	26	74	4.917	0.96	46.24	2.094	6.72	0.96	13.34	38.16	8.18	5.493	0.913	0.212	0.01	0.053	2.487
17/08/2021	KBS	43° 31' 10"; 16° 22' 52"	0	0.09	0.4	84.9	15.1	11.681	0.83	1.52	0.58	1.53	0.2	27.01	37.63	8.25	4.827	0.378	0.043	0.335	0.011	0.581
17/08/2021	KBB	43° 31' 10"; 16° 22' 52"	35	0.2	0.64	81.3	18.7	10.227	0.55	5.65	0.9	1.68	0.45	16.53	38.56	8.17	4.879	0.238	0.063	0.257	0.035	2.443
29/03/2021	SCS	43° 26' 13"; 16° 23' 44"	0	0.08	0.48	28.1	71.9	5.696	0.83	8.28	0.13	1.83	0.27	13.88	37.97	8.17	5.87	0.429	0.004	0.52	0.027	1.336
29/03/2021	SCB	43° 26' 13"; 16° 23' 44"	50	0.19	0.469	28	72	3.848	0.72	9.05	0.85	1.83	0.33	14.3	38.73	8.16	5.281	1.374	0.193	0.224	0.109	1.498
18/08/2021	SCS	43° 26' 13"; 16° 23' 44"	0	0.29	0.56	45.1	54.9	12.896	0.65	3.67	0.18	0.75	0.06	26.873	37.678	8.12	4.894	0.292	0.063	0.268	0	0.858
18/08/2021	SCB	43° 26' 13"; 16° 23' 44"	50	0.17	0.36	31.6	68.4	9.201	0.4	5.75	0.88	5.13	0.66	15.077	38.741	8.07	4.964	0.646	0.339	0.628	0.075	3.777
30/03/2021	STS	43° 2' 39"; 16° 17' 6"	0	0.08	0.423	25.7	74.3	3.331	0.7	27.59	0.15	3.2	0.2	14.2	38.6	8.18	5.627	0.516	0.071	0	0.027	1.245
30/03/2021	STB	43° 2' 39"; 16° 17' 6"	100	0.09	0.271	28.3	71.7	2.471	0.23	0.62	0.008	0.32	0.06	14.61	38.93	8.14	5.136	2.042	0.573	0.128	0.067	1.268
19/08/2021	STS	43° 2' 39"; 16° 17' 6"	0	0.11	0.28	33.3	66.7	10.769	0.55	0.37	0.1	0.53	0.05	26.435	38.843	8.11	4.779	0.483	0.089	0.022	0.057	1.265
19/08/2021	STB	43° 2' 39"; 16° 17' 6"	100	0.07	0.24	26.4	73.6	4.166	0.06	0.67	5.13	1.37	0.07	15.235	39.017	8.06	4.796	3.239	0.257	0.071	0.137	2.894

BP (10^4 cells mL^{-1} h^{-1})- bacterial production
HB (mL^{-1}) – Heterotrophic bacteria
HNA (%) – High Nucleic Acid bacterial group
LNA (%) – Low Nucleic Acid bacterial group
VLP (mL^{-1}) - Virus like particles
HNF (mL^{-1}) – Heterotrophic nanoflagellates
SYN (mL^{-1}) – *Synechococcus* sp.
PRO (mL^{-1}) – *Prochlorococcus* sp.
PE (mL^{-1}) - Picoeukaryotes
Chl a (mg m^{-3}) – Chlorophyl a

TEMP (°C) - Temperature
SAL (‰) – Salinity
pH
TDS – Total dissolved solids
NO3 [µmol L-1] - Nitrates
NO2 [µmol L-1] - Nitrites
N-NH4 [µmol L-1] - Ammonia
PO4 [µmol L-1] - Phosphates
SIO2 [µmol L-1] - Silicates

Table S2. Total counts of FIB (CFU±SD/100mL), total heterotrophic bacteria (CFU± SD/1mL) and estimated percentage of the viable heterotrophic bacteria resistant (%R) to SXT and TET

Bacteria	Site	Winter	Summer
E. coli	JS	1966 ± 48.1	3168 ± 186.7
	KS	175.5 ± 36.1	46.5 ± 19.1
	VB	3.5 ± 4.9	1 ± 1.4
	VS	9.5 ± 0.7	0
	KBB	7 ± 9.9	2.5 ± 3.5
	KBS	0.5 ± 0.7	0
	SKB	0	0
	SKS	1 ± 1.4	0
	STB	0	0
	STS	0	0
Intestinal enterococci	JS	1611 ± 253.1	1028 ± 39.6
	KS	597.5 ± 137.9	37 ± 4.2
	VB	2 ± 2.8	2 ± 2.8
	VS	16.5 ± 9.2	3 ± 4.2
	KBB	9 ± 12.7	2.5 ± 3.5
	KBS	0	3 ± 4.2
	SCB	0	6 ± 8.5
	SS	0.5 ± 0.7	0
	STB	0	2 ± 2.8
	STS	0	2.5 ± 3.5
Total viable heterotrophic bacteria	JS	2545 ± 5	2740 ± 100
	KS	13070 ± 120	1580 ± 140
	VB	415 ± 35	585 ± 85
	VS	1030 ± 10	1310 ± 70
	KBB	236.7 ± 35.1	1850 ± 86.6
	KBS	686.7 ± 41.6	1030 ± 36.1
	SCB	153.3 ± 68.1	66.7 ± 25.2
	SCS	526.7 ± 71	310 ± 55.7
	STB	223.3 ± 37.9	295 ± 45
	STS	110 ± 10	293.3 ± 55.1
% R-SXT	JS	1.83	20.00
	KS	0.01	4.75
	VB	0.04	0.09
	VS	0.24	0.10
	KBB	0.21	0.45
	KBS	0.01	0.11
	SCB	0.46	2.21
	SCS	0.32	0.03
	STB	0.24	0.41
	STS	0.64	0.69
% R-TET	JS	39.55	39.27
	KS	35.84	5.96
	VB	12.44	7.14
	VS	12.01	5.34
	KBB	4.56	5.90
	KBS	27.96	13.28
	SCB	11.69	22.50
	SCS	6.32	22.23
	STB	2.12	14.89
	STS	3.10	4.03

Table S3. Alpha diversity (can be found on link below, QR code)

Table S4. Beta diversity (can be found on link below, QR code)

Table S5. MRPP analysis (can be found on link below, QR code)

Table S6. Autochthonous microbial community (can be found on link below, QR code)

Table S7. TET resistant microbial community (can be found on link below, QR code)

Table S8. SXT resistant microbial community (can be found on link below, QR code)

Marine resistome of a temperate zone distribution diversity and driving factors across the trophic gradient

Here are cited all references included in the paper that were not cited before in this doctoral dissertation [299–320].

9.5. SUPPLEMENTAL MATERIAL TO 3rd SCIENTIFIC PAPER

Large-scale biogeographical shifts of abundance of antibiotic resistance genes and marine bacterial communities as their carriers along a trophic gradient

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Table S1. *Taken from Dželalija, M.; Kvesić-Ivanković, M.; Jozić, S.; Ordulj, M.; Kalinić, H.; Pavlinović, A.; Šamanić, I.; Maravić, A. Marine Resis-tome of a Temperate Zone: Distribution, Diversity, and Driving Factors across the Trophic Gradient. Water Res. 2023, 246, 120688, doi:10.1016/j.watres.2023.120688.

Table S2. Frequency of resistance to five major classes of antibiotics in summer and winter along the trophic gradient encompassing eutrophic (JS, KS), mesotrophic (VBS/VBB, KBS/KBB), and olig- otrophic (SCS/SCB, STS/STB) marine areas. The percentage was calculated as the ratio between the number of bacteria grown on agar infused with antibiotics per mL and the total heterotrophic bacteria.

WINTER

	AZM		CTX		GEN		SXT		TET	
	Bottom	Surface	Bottom	Surface	Bottom	Surface	Bottom	Surface	Bottom	Surface
J	/	0.85285285	/	4.168168168	/	1.674474	/	1.674474	/	36.27628
KS	/	0.06536211	/	16.81258826	/	0.018277	/	0.018277	/	56.70163
VB/VS	0.12903226	0.02241715	0.36258065	6.736842105	2.458065	0.145517	0.035484	0.145517	9.996774	7.236842
KBB/KBS	0.58767773	0.00264484	0.66113744	0.937027708	9.582938	0.007557	0.138863	0.007557	3.07109	14.50882
SCB/SCS	1.80652174	0.00411392	3.33913043	0.005212025	1.571739	0.318987	0.46125	0.318987	11.68696	6.318987
STB/STS	0.13970149	0.04484848	0.68171642	1.998484848	0.961567	0.636364	0.24291	0.636364	2.121269	3.1

SUMMER

	AZM		CTX		GEN		SXT		TET	
	Bottom	Surface	Bottom	Surface	Bottom	Surface	Bottom	Surface	Bottom	Surface
J	/	1.473412	/	1.446824	/	2.042836	/	12.1418	/	23.84047
KS	/	0.30563	/	2.831099	/	3.163539	/	6.032172	/	7.576408
VB/VS	0.028269	0.02297	0.778077	0.549188	2.55	1.240835	0.058462	0.035267	4.823077	4.87239
KBB/KBS	0.427273	0.183003	0.260227	0.528676	0.268371	1.282586	0.235133	0.074557	3.102273	8.558916
SCB/SCS	0.445	0.025806	3.6	3.172043	4.775	2.7	2.21	0.030108	22.5	22.22581
STB/STS	0.317344	1.295455	2.535	4.032955	1.90125	4.609091	0.2275	0.686932	8.23875	4.032955

Station seasonal differences	
STATION	p value(t.test)
J	0.835795649
KS	0.322233909
VBB/VBS	0.155947597
KBB/KBS	0.205754497
SCB/SCS	0.069408777
STB/STS	0.020357541

Seasonal antibiotic variations	
ANTIBIOTIC	p value t.test
AZM BOTTOM	0.341072
AZM SURFACE	0.105641
CTX BOTTOM	0.318636
CTX SURFACE	0.296386
GEN BOTTOM	0.651976
GEN SURFACE	0.014681
SXT BOTTOM	0.333359
SXT SURFACE	0.203187
TET BOTTOM	0.430988
TET SURFACE	0.367859

Table S3. The abundance of ten ARGs (*sul1, sul2, tetA, tetB, mphA, ermB, aac3-2,* bla_{TEM}, bla_{CTX-M}, bla_{VIM}) and the integrase gene intl1 was determined using a qPCR approach (can be found on link below, QR code)

Large-scale biogeographical shifts of abundance of antibiotic resistance genes and marine bacterial communities

Table S4. Spearman's rank correlations between genes seasonal.

SUMMER

SUMMER_Spearman's rank correlations between genes

	bla_{CTX-M}	*mphA*	bla_{TEM}	bla_{VIM}	*sul1*	*sul2*	*tetA*	*tetB*	*intl1*	*aac3-2*	*ermB*
bla_{CTX-M}	1	0.75	0.93	0.75	0.72	0.73	0.38	0.92	0.62	0.72	-0.39
mphA	0.75	1	0.79	0.96	0.98	0.88	0.67	0.73	0.76	0.9	-0.2
bla_{TEM}	0.93	0.79	1	0.85	0.76	0.87	0.62	0.92	0.79	0.82	-0.33
bla_{VIM}	0.75	0.96	0.85	1	0.93	0.94	0.76	0.76	0.79	0.94	-0.14
sul1	0.72	0.98	0.76	0.93	1	0.88	0.61	0.7	0.72	0.88	-0.13
sul2	0.73	0.88	0.87	0.94	0.88	1	0.65	0.82	0.73	0.98	0.01
tetA	0.38	0.67	0.62	0.76	0.61	0.65	1	0.43	0.9	0.6	0
tetB	0.92	0.73	0.92	0.76	0.7	0.82	0.43	1	0.68	0.83	-0.19
intl1	0.62	0.76	0.79	0.79	0.72	0.73	0.9	0.68	1	0.68	-0.16
aac3-2	0.72	0.9	0.82	0.94	0.88	0.98	0.6	0.83	0.68	1	0.03
ermB	-0.39	-0.2	-0.33	-0.14	-0.13	0.01	0	-0.19	-0.16	0.03	1

WINTER

WINTER_Spearman's rank correlations between genes

	bla_{CTX-M}	*mphA*	bla_{TEM}	bla_{VIM}	*sul1*	*sul2*	*tetA*	*tetB*	*intl1*	*aac3-2*	*ermB*
bla_{CTX-M}	1	0.28	0.71	0.39	0.21	0.54	0.14	0.72	-0.18	0.6	0.79
mphA	0.28	1	0.25	0.49	0.59	0.44	-0.03	0.01	-0.33	0.65	0.33
bla_{TEM}	0.71	0.25	1	0.12	0.54	0.83	0.32	0.61	-0.39	0.49	0.77
bla_{VIM}	0.39	0.49	0.12	1	-0.05	-0.05	0.09	0.54	-0.18	0.79	0.13
sul1	0.21	0.59	0.54	-0.05	1	0.71	0.21	-0.04	-0.35	0.24	0.45
sul2	0.54	0.44	0.83	-0.05	0.71	1	0.35	0.38	-0.12	0.36	0.68
tetA	0.14	-0.03	0.32	0.09	0.21	0.35	1	0.52	-0.12	0.38	-0.15
tetB	0.72	0.01	0.61	0.54	-0.04	0.38	0.52	1	-0.03	0.64	0.48
intl1	-0.18	-0.33	-0.39	-0.18	-0.35	-0.12	-0.12	-0.03	1	-0.53	-0.21
aac3-2	0.6	0.65	0.49	0.79	0.24	0.36	0.38	0.64	-0.53	1	0.37
ermB	0.79	0.33	0.77	0.13	0.45	0.68	-0.15	0.48	-0.21	0.37	1

Table S4. (Extension) Spearman's p values for gene correlations seasonal.

SUMMER

SUMMER_Spearman's p values for gene correlations

	bla$_{CTX-M}$	*mphA*	*bla*$_{TEM}$	*bla*$_{VIM}$	*sul1*	*sul2*	*tetA*	*tetB*	*intl1*	*aac3-2*	*ermB*
***bla*$_{CTX-M}$**		0.0133	0.0001	0.0133	0.0186	0.0158	0.2763	0.0002	0.0537	0.0186	0.2665
mphA	0.0133		0.0061	0	0	0.0008	0.033	0.0158	0.0111	0.0003	0.5784
***bla*$_{TEM}$**	0.0001	0.0061		0.0016	0.0111	0.0012	0.0537	0.0002	0.0061	0.0038	0.3544
***bla*$_{VIM}$**	0.0133	0	0.0016		0.0001	0	0.0111	0.0111	0.0061	0	0.7001
sul1	0.0186	0	0.0111	0.0001		0.0008	0.06	0.0251	0.0186	0.0008	0.7126
sul2	0.0158	0.0008	0.0012	0	0.0008		0.0425	0.0038	0.0158	0	0.9867
tetA	0.2763	0.033	0.0537	0.0111	0.06	0.0425		0.2145	0.0003	0.0667	1
tetB	0.0002	0.0158	0.0002	0.0111	0.0251	0.0038	0.2145		0.0289	0.0029	0.6021
intl1	0.0537	0.0111	0.0061	0.0061	0.0186	0.0158	0.0003	0.0289		0.0289	0.6628
aac3-2	0.0186	0.0003	0.0038	0	0.0008	0	0.0667	0.0029	0.0289		0.9336
ermB	0.2665	0.5784	0.3544	0.7001	0.7126	0.9867	1	0.6021	0.6628	0.9336	

WINTER

WINTER_Spearman's p values for gene correlations

	bla$_{CTX-M}$	*mphA*	*bla*$_{TEM}$	*bla*$_{VIM}$	*sul1*	*sul2*	*tetA*	*tetB*	*intl1*	*aac3-2*	*ermB*
***bla*$_{CTX-M}$**		0.425	0.0217	0.26	0.5563	0.1076	0.7009	0.0186	0.6272	0.0667	0.0061
mphA	0.425		0.4888	0.1497	0.0739	0.2004	0.9338	0.9867	0.3466	0.0425	0.3466
***bla*$_{TEM}$**	0.0217	0.4888		0.7514	0.1076	0.0029	0.3655	0.06	0.26	0.1497	0.0092
***bla*$_{VIM}$**	0.26	0.1497	0.7514		0.881	0.881	0.8028	0.1076	0.6272	0.0061	0.7261
sul1	0.5563	0.0739	0.1076	0.881		0.0217	0.5563	0.9074	0.3282	0.5109	0.1869
sul2	0.1076	0.2004	0.0029	0.881	0.0217		0.3282	0.2763	0.7514	0.3104	0.0289
tetA	0.7009	0.9338	0.3655	0.8028	0.5563	0.3282		0.1276	0.7514	0.2763	0.6761
tetB	0.0186	0.9867	0.06	0.1076	0.9074	0.2763	0.1276		0.9338	0.0479	0.1615
intl1	0.6272	0.3466	0.26	0.6272	0.3282	0.7514	0.7514	0.9338		0.1173	0.5563
aac3-2	0.0667	0.0425	0.1497	0.0061	0.5109	0.3104	0.2763	0.0479	0.1173		0.2931
ermB	0.0061	0.3466	0.0092	0.7261	0.1869	0.0289	0.6761	0.1615	0.5563	0.2931	

p ***[0.0001-0.001]

p **[0.001-0.01]

p*[0.01-0.05]

Here are cited all references included in the paper that were not cited before in this doctoral dissertation [321–340].

Table S5. Pearson correlation-based co-occurrence network.

WINTER											
ARG-ARG	***bla*** $_{CTX-M}$	***mphA***	***bla*** $_{TEM}$	***bla*** $_{VIM}$	***sul1***	***sul2***	***tetA***	***tetB***	***intl1***	***aac3-2***	***ermB***
bla $_{CTX-M}$	1	0.306015	0.600764	0.605806	0.22079	0.440407	0.10313	0.53686	-0.14931	0.438511	0.244427
mphA	0.306015	1	0.34961	0.5516	0.374779	0.513247	-0.05497	0.189909	-0.53604	0.721786	0.10767
bla $_{TEM}$	0.600764	0.34961	1	0.125829	0.614565	0.852201	-0.05637	0.215831	-0.48947	0.182602	0.756334
bla $_{VIM}$	0.605806	0.5516	0.125829	1	0.064783	0.117309	0.27888	0.600554	-0.24024	0.733887	0.061623
sul1	0.22079	0.374779	0.614565	0.064783	1	0.741307	0.042301	-0.15779	-0.53877	-0.14043	0.362497
sul2	0.440407	0.513247	0.852201	0.117309	0.741307	1	0.20186	0.215116	-0.46418	0.18661	0.559139
tetA	0.10313	-0.05497	-0.05637	0.27888	0.042301	0.20186	1	0.744468	0.142307	-0.12171	-0.23732
tetB	0.53686	0.189909	0.215831	0.600554	-0.15779	0.215116	0.744468	1	0.082932	0.335776	-0.01074
intl1	-0.14931	-0.53604	-0.48947	-0.24024	-0.53877	-0.46418	0.142307	0.082932	1	-0.16961	-0.34816
aac3-2	0.438511	0.721786	0.182602	0.733887	-0.14043	0.18661	-0.12171	0.335776	-0.16961	1	0.225923
ermB	0.244427	0.10767	0.756334	0.061623	0.362497	0.559139	-0.23732	-0.01074	-0.34816	0.225923	1

WINTER										
SITE-SITE	**JS**	**KS**	**VB**	**VS**	**KBB**	**KBS**	**SCB**	**SCS**	**STB**	**STS**
JS	1	0.995096	0.998959	0.97747	0.80399	0.999247	0.997386	0.982606	0.938729	0.993624
KS	0.995096	1	0.993771	0.959577	0.854035	0.993846	0.995888	0.967785	0.963733	0.980574
VB	0.998959	0.993771	1	0.979521	0.799992	0.998739	0.99561	0.983501	0.93555	0.993998
VS	0.97747	0.959577	0.979521	1	0.679989	0.983109	0.962484	0.999115	0.852625	0.994265
KBB	0.80399	0.854035	0.799992	0.679989	1	0.793741	0.830199	0.701547	0.954772	0.738004
KBS	0.999247	0.993846	0.998739	0.983109	0.793741	1	0.995388	0.987721	0.931911	0.996154
SCB	0.997386	0.995888	0.99561	0.962484	0.830199	0.995388	1	0.968977	0.955663	0.985441
SCS	0.982606	0.967785	0.983501	0.999115	0.701547	0.987721	0.968977	1	0.867385	0.996091
STB	0.938729	0.963733	0.93555	0.852625	0.954772	0.931911	0.955663	0.867385	1	0.897175
STS	0.993624	0.980574	0.993998	0.994265	0.738004	0.996154	0.985441	0.996091	0.897175	1

Table S5. (Extension) Pearson correlation-based co-occurrence network.

SUMMER											
ARG-ARG	***bla*** $_{CTX-M}$	***mphA***	***bla*** $_{TEM}$	***bla*** $_{VIM}$	***sul1***	***sul2***	***tetA***	***tetB***	***intl1***	***aac3-2***	***ermB***
bla $_{CTX-M}$	1	0.906185	0.972453	0.98629	0.916421	0.811628	0.13633	0.992312	0.573659	0.880129	-0.06266
mphA	0.906185	1	0.874569	0.902187	0.952889	0.928763	0.224579	0.860999	0.536065	0.944057	-0.15901
bla $_{TEM}$	0.972453	0.874569	1	0.930934	0.859759	0.736563	0.326692	0.951166	0.745161	0.801515	-0.13199
bla $_{VIM}$	0.98629	0.902187	0.930934	1	0.902768	0.807185	0.023302	0.978816	0.458167	0.873764	-0.12811
sul1	0.916421	0.952889	0.859759	0.902768	1	0.971254	0.140909	0.901342	0.460394	0.985336	0.066537
sul2	0.811628	0.928763	0.736563	0.807185	0.971254	1	0.064453	0.789619	0.33912	0.983097	0.083186
tetA	0.13633	0.224579	0.326692	0.023302	0.140909	0.064453	1	0.066564	0.816977	0.045731	-0.16706
tetB	0.992312	0.860999	0.951166	0.978816	0.901342	0.789619	0.066564	1	0.518568	0.868534	0.028766
intl1	0.573659	0.536065	0.745161	0.458167	0.460394	0.33912	0.816977	0.518568	1	0.368998	-0.19922
aac3-2	0.880129	0.944057	0.801515	0.873764	0.985336	0.983097	0.045731	0.868534	0.368998	1	0.131047
ermB	-0.06266	-0.15901	-0.13199	-0.12811	0.066537	0.083186	-0.16706	0.028766	-0.19922	0.131047	1

SUMMER										
SITE-SITE	**JS**	**KS**	**VB**	**VS**	**KBB**	**KBS**	**SCB**	**SCS**	**STB**	**STS**
JS	1	0.369489	0.987065	0.775374	-0.01743	0.81499	0.449103	0.73476	0.870514	0.821222
KS	0.369489	1	0.424675	0.058746	0.803327	0.616582	-0.18436	0.669885	0.609676	0.674329
VB	0.987065	0.424675	1	0.664697	0.028282	0.893401	0.301087	0.676207	0.934283	0.895904
VS	0.775374	0.058746	0.664697	1	-0.18457	0.280992	0.912005	0.764476	0.377644	0.305148
KBB	-0.01743	0.803327	0.028282	-0.18457	1	0.29169	-0.2732	0.379188	0.21347	0.318407
KBS	0.81499	0.616582	0.893401	0.280992	0.29169	1	-0.13387	0.50356	0.987202	0.992148
SCB	0.449103	-0.18436	0.301087	0.912005	-0.2732	-0.13387	1	0.584311	-0.03446	-0.10612
SCS	0.73476	0.669885	0.676207	0.764476	0.379188	0.50356	0.584311	1	0.580088	0.560845
STB	0.870514	0.609676	0.934283	0.377644	0.21347	0.987202	-0.03446	0.580088	1	0.989104
STS	0.821222	0.674329	0.895904	0.305148	0.318407	0.992148	-0.10612	0.560845	0.989104	1

Table S6. Quantitative real-time PCR (RT-qPCR) amplification conditions and using primers.

					WINTER		SUMMER	
Target gene	**Primer name**	**Primers (5´- 3´) for qPCR amplificatior**	**Amplicon s**	**Reference**	**Accuracy(R^2)**	**Efficiency(%)**	**Accuracy(R^2)**	**Efficiency(%)**
ermB	erm(B)-91f	GATACCGTTTACGAAATTGG	364 bp	Milaković et al. 2019	0,912	97,188	0,674	101,358
	erm(B)-454r	GAATCGAGACTTGAGTGTGC						
16S rRNA	534R	CCTACGGGAGGCAGCAG	174 bp	Lopez et al.,2004	0,991	100,716	0,997	87,458
	341F	ATTACCGCGGCTGCTGGCA						
*intI*1	intl1_LC1	GCCTTGATGTTACCCGAGAG	196 bp	Barraud et al., 2010	0,887	91,594	0,912	96,983
	intl1_LC5	GATCGGTCGAATGCGTGT						
bla_{TEM}	blaTEM-F	TTCCTGTTTTTGCTCACCCAG	113 bp	Cacace et al. 2019	0,923	95,481	0,988	90,18
	blaTEM-R	CTCAAGGATCTTACCGCTGTTG						
Sul1	qSUL_1-653f	CCGTTGGCCTTCCTGTAAAG	67 pb	Heuer and Smalla, 2007	0,965	84,788	1	99,268
	qSUL_1-719r	TTGCCGATCGCGTGAAGT						
Sul2	qSUL2_595f	CGGCTGCGCTTCGATT	59 bp	Heuer and Smalla, 2007	0,896	82,522	0,972	90,406
	qSUL2_654r	CGCGCGCAGAA GGATT						
bla_{VIM}	VIM-F	AGTGGTGAGTATCCGACAG	212 bp	Milaković et al. 2019	0,993	99,851	0,974	96,646
	VIM-UR	TCAATCTCCGCGAGAAG						
bla_{CTX-M}	CTXM-F	CTATGGCACCACCAACGATA		Cacace et al. 2019	0,989	92,086	0,953	121,167
	CTXM-R	ACGGCTTTCTGCCTTAGGTT						
tetA	Tet(A)-F	CAGGCAGGTGGATGAGGAA	174 bp	Huang et al. 2015	0,905	103,76	0,91	100,231
	Tet(A)-R	GGCAGGCAGAGCAAGTAGAG						
tetB	Tet(B)-F	GTCATTGCCGATACCACCTC	131 bp		0,978	104,301	0,997	93,186
	Tet(B)-R	GGACTATGCGGTGAAATCTCTC						
aac3-2	aac(3')-IIa/aacC3	/TCGGTCGCCATCGAGAAG	154 bp	Lam et al. 2017	0,971	93,241	0,96	96,783
	aac(3')-IIc/ aacC2	ATGTCCTGCGCGTCGAA						
mphA	mphA-01	CTGACGCGCTCCGTGTT	154 bp	Helsens et al. 2020	0,838	91,739	0,846	91,908
	mphA-01	GGTGGTGCATGGCGATCT						

9.6 ABBREVIATIONS

ABC - ATP-binding cassette

AR - Antibiotic resistance

ARB - Antibiotic-resistant bacteria

ARGs - Antibiotic resistance genes

CAZ - Ceftazidime

CIP - Ciprofloxacin

CLA - Clavulanic acid

CLSI - Clinical and Laboratory Standards Institute

CRE - Carbapenem-resistant Enterobacteriaceae

CTX - Cefotaxime

DNA - Deoxyribonucleic Acid

ESBL - Extended-Spectrum Beta-Lactamase

ETP - Ertapenem

EUCAST - European Committee on Antimicrobial Susceptibility

Testing FEP - Cefepime

FOX - Cefoxitin

GEN - Gentamicin

Inc – Incompatibility

IR – inverted repeat

IPM - Imipenem

MEM - Meropenem

MIC - Minimum inhibitory concentration

MATE - Multidrug and toxic compound extrusion

MDR - Multidrug resistance

MIs - Mobile integrons

MFS - Major facilitator superfamily

NGS - Next-generation sequencing

ONT – Oxford Nanopore Sequencing

ORF - Open reading frame

PAP - Periplasmic adapter protein

PBPs - Penicillin-binding proteins

PBRT - PCR-based replicon typing

PDR - Pandrug resistant

PIP - Piperacillin

pMLST - Plasmid multilocus sequence typing

RND - Resistance nodulation division

RT-qPCR - Real Time Quantitative Polymerase Chain Reaction

SMR - Small multidrug resistance

SXT - Sulfamethoxazole

TET - Tetracycline

TZP – Tazobactam

VREfm - vancomycin-resistant Enterococcus faecium

WHO - World Health Organization

XDR - Extensively Drug-Resistant