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CIRCULATION OF PATHOGENS AND RESISTANCE GENES BETWEEN HUMANS, ANIMALS AND THE COASTAL ENVIRONMENT IN NHA TRANG (VIETNAM) AS REVEALED BY A COMBINATION OF PHENOTYPIC AND OMICS APPROACHES

Présentée par Son-Hoang TRAN
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Sous la direction de Yvan BETTAREL
et Quang-Huy NGUYEN et Jean-Christophe AUGUET

Devant le jury composé de

Delphine DESTOUMIEUX, Directrice de recherche CNRS - IHPE à Montpellier, France	Président du jury et Examinatrice
Didier HOCQUET, Professeur des Université de Franche-Comté, France	Examinateur
Carles BORREGO, Professeur à l'université de Girone, Espagne	Rapporteur
Gwenaelle le BLAY, Professeure des Université - LEMAR à Brest, France	Rapportrice

UNIVERSITÉ DE MONTPELLIER

Abstract

Infectious diseases (IDs) and antimicrobial resistance (AMR) pose significant threats to global health and biodiversity, yet their environmental and socio-economic drivers remain partly understood. While numerous research has focused on inland systems, marine environments, an important reservoirs for pathogenic bacteria (PPB) and AMR genes, have received less attention. Aquaculture, one of the fastest-growing sources of animal protein, contributes about 5.7% of global antibiotic use, contributing significantly to AMR emergence and spread in coastal ecosystems. Despite this, critical knowledge gaps hinder effective risk assessment and management, including a lack of inventory of marine pathogens, inconsistent data on antibiotic use in aquaculture, and unclear impacts of farming practices on microbial communities.

To address the research gaps, this thesis aims to firstly develop a full-length 16*S rRNA* gene dataset (NEMESISdb) to enable the identification and tracking of potentially pathogenic bacteria (PPB) in humans, fish, and crustaceans. The second objective is to investigate the dynamics of both potentially pathogenic bacteria (by utilizing the NEMESISdb) and antibiotic resistance genes (ARGs) in various environmental compartments. The final objective focuses on the potential circulation of PPB and ARGs between marine environments and human or aquaculture hosts.

Results from metabarcoding and bacterial isolation analyses reveal a significant increase in potential human pathogenic bacteria, such as *Prevotella copri*, *Faecalibacterium prausnitzii*, *Escherichia coli*, and *Vibrio parahaemolyticus* within human and marine animals (both wildlife and livestock) and in areas influenced by intensive anthropogenic activities. In contrast, these bacteria are less prevalent in natural environments, and areas with lower levels of anthropogenic impact. Antibiotic resistances of the bacteria also reflect the intensive use of diverse antibiotics in aquaculture practices, mostly from tetracycline, beta-lactam, MLS and multidrug. The resistome is primarily characterized by a high relative abundance of specific ARGs, including *tet*Q (21.5%), *Cfx*A6 (17.7%), *tet*W (5.0%), *tet*O (4.2%) and *Erm*F (4.1%). Phenotypically, beta-lactam resistance is also predominant, as indicated by the presence of the *bla*KPC and *bla*VIM genes. Anthro-

pogenic input, such as urban sewage runoff and/or aquaculture practice, is recognized as the most decisive driver beside seasonality and substrate type for the structure of both pathobiome and resistome of Nha Trang Bay. These factors contribute to higher abundances of both PHPB and ARGs, increase MAR indices in farmers, animals and aquaculture related areas compared to the nature environments, which are further supported by the widespread presence of *intI*1. Moreover, floating cage ecosystems also promote exposure rates to pathogen-rich environment and antibiotic residues, increasing selection pressures and circulation of pathobiome and resistome between humans, animals, and the surrounding environment.

Overall, results from this thesis offers insights to help us better understand the health risks from marine pathogens and antimicrobial resistance (AMR) between human, animals and envrionment in contrasted areas of the Vietnamese coast.

Résumé

Les maladies infectieuses (MI) et la résistance aux antimicrobiens (RAM) représentent des menaces importantes pour la santé et la biodiversité mondiales. Pourtant, leurs facteurs environnementaux et socio-économiques restent partiellement incompris. Si de nombreuses recherches se sont concentrées sur les systèmes continentaux, les environnements marins, importants réservoirs de bactéries pathogènes (PPB) et de gènes RAM, ont reçu moins d'attention. L'aquaculture, l'une des sources de protéines animales connaissant la croissance la plus rapide, contribue à environ 5.7% de l'utilisation mondiale d'antibiotiques, contribuant ainsi significativement à l'émergence et à la propagation de la RAM dans les écosystèmes côtiers. Malgré cela, d'importantes lacunes dans les connaissances entravent l'évaluation et la gestion efficaces des risques, notamment l'absence d'inventaire des agents pathogènes marins, des données incohérentes sur l'utilisation des antibiotiques en aquaculture et l'impact flou des pratiques agricoles sur les communautés microbiennes.

Pour combler ces lacunes, cette thèse vise, dans un premier temps, à développer un ensemble de données génétiques complètes de l'ARNr 16S (NEMESISdb) afin de permettre l'identification et le suivi des bactéries potentiellement pathogènes (PPB) chez l'homme, les poissons et les crustacés. Le deuxième objectif est d'étudier la dynamique des bactéries potentiellement pathogènes (en utilisant la base de données NEMESISdb) et des gènes de résistance aux antibiotiques (GRA) dans divers compartiments environnementaux. Le dernier objectif porte sur la circulation potentielle des PPB et des GRA entre les environnements marins et les hôtes humains ou aquacoles.

Les résultats des analyses de métabarcoding et des isolements bactériens révèlent une augmentation significative des bactéries potentiellement pathogènes pour l'homme, telles que *Prevotella copri*, *Faecalibacterium prausnitzii*, *Escherichia coli* et *Vibrio parahaemolyticus*, chez l'homme et les animaux marins (faune et bétail) et dans les zones soumises à des activités anthropiques intensives. En revanche, ces bactéries sont moins répandues dans les milieux naturels et les zones à faible impact anthropique. La résistance des bactéries aux antibiotiques reflète également l'utilisation intensive de divers an-

tibiotiques dans les pratiques aquacoles, principalement des tétracyclines, des bêta-lactamines, des MLS et des multidrogues. Le résistome est principalement caractérisé par une abondance relative élevée d'ARG spécifiques, y compris *tet*Q (21.5%), *Cfx*A6 (17.7%), *tet*W (5.0%), *tet*O (4.2%) et *Erm*F (4.1%). Phénotypiquement, la résistance aux bêta-lactamines est également prédominante, comme l'indique la présence des gènes *bla*KPC et *bla*VIM. Les apports anthropiques, tels que le ruissellement des eaux usées urbaines et/ou les pratiques aquacoles, sont reconnus comme le facteur le plus déterminant, après la saisonnalité et le type de substrat, pour la structure du pathobiome et du résistome de la baie de Nha Trang. Ces facteurs contribuent à des abondances plus élevées de PHPB et d'ARG, augmentent les indices MAR chez les agriculteurs, les animaux et les zones liées à l'aquaculture par rapport aux environnements naturels, ce qui est en outre soutenu par la présence généralisée d'intI1. De plus, les écosystèmes de cages flottantes favorisent également les taux d'exposition à un environnement riche en agents pathogènes et aux résidus d'antibiotiques, augmentant les pressions de sélection et la circulation du pathobiome et du résistome entre les humains, les animaux et le milieu environnant.

Dans l'ensemble, les résultats de cette thèse offrent des perspectives pour mieux comprendre les risques sanitaires liés aux agents pathogènes marins et à la résistance aux antimicrobiens (RAM) entre l'homme, les animaux et l'environnement dans des zones contrastées de la côte vietnamienne.

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List of Abbreviations

AMR Antimicrobial Resistance.

ARB Antibiotic-Resistant Bacteria.

ARGs Antibiotic Resistance Genes.

ASP Antimicrobial Stewardship Programs.

AST Antibiotic Susceptibility Testing.

ASVs Amplicon Sequence Variants.

CLSI Clinical & Laboratory Standards Institute.

DDDs Defined Daily Doses.

DNA Deoxyribonucleic Acid.

DOM Dissolved Organic Matter.

EUCAST European Committee on Antimicrobial Susceptibility Testing.

HGT Horizontal Gene Transfer.

HICs High-Income Countries.

HIV/AIDS Human Immunodeficiency Viruses/Acquired immunodeficiency syndrome.

IDs Infectious Diseases.

IPC Infection Prevention and Control.

LICs Low-Income Countries.

LMICs Low and Middle-Income Countries.

MAGs Metagenome-Assembled Genomes.

MALDI-ToF MS Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry.

MAR Multiple Antibiotic Resistance.

MDR Multidrug Resistant.

MGEs Mobile Genetic Elements.

MSW Municipal solid Waste.

NGS Next-Generation Sequencing.

OTUs Operational Taxonomic Units.

PCR Polymerase Chain Reaction.

PHPB Potential Human Pathogenic Bacteria.

PPB Potentially Pathogenic Bacteria.

qPCR Quantitative Polymerase Chain Reaction.

QRDRs Quinolone Resistance-determining Regions.

RNA Ribonucleic Acid.

SNPs Single Nucleotide Polymorphisms.

VBNC Viable-but-non-culturable.

VFDB Virulence Factor Database.

WGS Whole-genome sequencing.

WHO World Health Organization.

XDR-TB Extensively Drug-Resistant Tuberculosis.

Introduction

1 A Global Health Threat: Antimicrobial Resistance & Emerging Pathogens

Antimicrobial Resistance (AMR) occurs when microorganisms, including bacteria, viruses, fungi, or parasites, develop mechanisms that make existing treatments ineffective. This poses a significant threat to modern medicine. In 2019, AMR was directly responsible for an estimated 1.27 million deaths and contributed to nearly 5 million additional deaths, making it one of the leading causes of global mortality, even surpassing Human Immunodeficiency Viruses/Acquired immunodeficiency syndrome (HIV/AIDS) and malaria (C. J. Murray et al., 2022). In addition to AMR, emerging and re-emerging pathogens present ongoing risks of outbreaks, epidemics, and pandemics. Pathogenic transmissions from animals to humans have led to recent crises such as COVID-19, Ebola, Nipah, and Monkeypox (Morens & Fauci, 2020). These events highlight significant gaps in surveillance, rapid diagnostics, and global coordination. In an interconnected world, the potential for new or re-emergent microbes and the risk of AMR to spread quickly across borders underscores the importance of robust and quick international response, collaboration, data sharing, and development of counter-measurements.

1.1 Antimicrobial Resistance: The Silent Pandemic

Often referred to as a "silent pandemic", AMR is increasingly recognized as one of the most pressing global threats to public health and sustainable development. Defined as the capacity of bacteria to withstand the effects of antibiotics, AMR renders treatments less effective, prolongs illness, and raises the risk of death. The World

Health Organization (WHO) has increasingly emphasized AMR as a critical challenge to public health, highlighting that it poses significant risks not only to individual patients but also to broader health systems and economies worldwide (Organization, 2022a). With the potential to undermine decades of medical advances, AMR threatens essential medical procedures, including surgeries, chemotherapy, and care for premature infants, thereby jeopardizing sustainable development goals. Without urgent and coordinated global action, these burden is expected to rise dramatically by 2050 (Aljeldah, 2022).

1.1.1 Discovery and Introduction of Antibiotics

Antibiotics are defined as microbial secondary metabolites whose primary function is to inhibit the growth of competing microorganisms. While these compounds are natural products synthesized by various microbes, their biosynthesis is a remarkably sophisticated and energetically demanding process. Advances in our understanding of microbial physiology reveal that microorganisms tend to prioritize the production of metabolites that directly contribute to their survival and proliferation. Consequently, the synthesis of antibiotics is tightly regulated and typically induced only under specific environmental conditions, such as nutrient limitation or intense microbial competition (Stuttard & Vining, 2014). Despite the metabolic cost, the ability to produce antibiotics is a widespread and evolutionarily conserved trait across diverse microbial taxa, including genera such as *Streptomyces*, *Penicillium*, *Cephalosporium*, and *Bacillus* (Abrudan et al., 2015). This suggests that antibiotic production confers significant ecological advantages, enabling organisms to out-compete rivals within complex microbial communities. Moreover, the evolutionary origins and diversification of antibiotic biosynthetic pathways underscore the adaptive value of these compounds in microbial life history, shaping the dynamics of microbial ecosystems over millions of years.

For most people however, antibiotics, recognized as one of the greatest achievements in the world's medical history, are medications specifically designed to fight bacterial infections in humans and animals. The discovery of penicillin from the mold *Penicillium rubens* in 1928 by Sir Alexander Fleming marked a pivotal moment in his-

tory, as it demonstrated for the first time the ability to effectively eliminate the pathogenic bacteria *Staphylococcus* (Tan & Tatsumura, 2015).

However; the modern antibiotic era is often traced back to the discovery of salvarsan and its derivative, neosalvarsan, by Paul Ehrlich in 1910 for the treatment of syphilis caused by *Treponema pallidum*. This was soon followed by the identification of prontosil, a sulfonamide prodrug discovered by Gerhard Domagk, which gradually supplanted salvarsan as a standard therapeutic agent. During the 1930s, Selman Waksman, an American microbiologist and biochemist, pioneered the systematic exploration of soil microorganisms, particularly filamentous *Actinomycetes*, and their capacity to produce antimicrobial compounds. His research led to the discovery of several antibiotics, including streptomycin, an essential drug in the treatment of tuberculosis, and to the formulation of the modern definition of antibiotics as "compounds produced by microbes to eliminate other microbes". The period from the 1940s to the 1960s witnessed the development of most of the antibiotic classes that are still in use today and widely regarded as a cornerstone of 20th-century medical progress, supporting numerous clinical and surgical innovations (Katz & Baltz, 2016). Antibiotics quickly earned the nickname "magic bullets" because of their potent bactericidal properties and remain one of the most transformative medical breakthroughs of the modern era (Salam et al., 2023). Their widespread adoption has significantly altered therapeutic paradigms and continues to prevent millions of deaths from bacterial infections annually. Beyond human medicine, antibiotics have been extensively utilized in agriculture, particularly in livestock farming and aquacultural practices as preventive agents, especially in Low and Middle-Income Countries (LMICs) (Williams-Nguyen et al., 2016; Hedberg et al., 2018).

1.1.2 Evolution and Emergence of Antimicrobial Resistance

Although antibiotics have become vital tools of modern medicine, the global rise of Multidrug Resistant (MDR) bacteria now threatens to diminish these medical advances, leading to an era of potentially untreatable infections. Infections caused by resistant organisms are often more difficult to treat, posing increased risks of mor-

bidity and mortality for the hosts. Therefore, the proliferation of "superbugs" organisms that are resistant to most antimicrobial agents has prompted international alarm (B. M. M. Uddin, Yusuf, & Ratan, 2017). Antimicrobial resistance (AMR) occurs when germs (bacteria, viruses, or fungi) that cause infections resist the effects of the medicines used to treat them by evolving mechanisms to survive exposure to antimicrobial agents. Among these medicines, antibiotics were the most commonly used and therefore the most susceptible to resistance. On its own, antimicrobial resistance is a bacterial natural evolutionary response to selective pressures, driven by genetic mutations that enhance bacterial survival (for Disease Control & Prevention, 2023). There were cases of resistance observed almost simultaneously with the introduction of antibiotics, such as penicillin-resistant strains of *Staphylococcus* were identified even before penicillin's therapeutic use began in the 1940s (Hutchings, Truman, & Wilkinson, 2019).

The trajectory of antibiotic discovery reveals that the pharmaceutical industry was most productive between 1960 and 1980, with a marked decline in the development of novel antibiotic classes thereafter (Iskandar et al., 2022). This growing imbalance between the proliferation of drug-resistant pathogens and the stagnation in antibiotic innovation has led many experts to warn of a looming post-antibiotic era, in which previously treatable infections may become increasingly difficult or impossible to manage. This alarming trend is summarized in the timeline of major antibiotic discoveries as illustrated in Figure 1 (Shuval, 2003). Methicillin, developed in 1959 as the first semisynthetic penicillin resistant to penicillinase, was followed just a year later by reports of methicillin-resistant *Staphylococcus aureus* (MRSA) (T. M. Uddin et al., 2021). In response to methicillin resistance, vancomycin, a glycopeptide antibiotic, was introduced in 1958 as a critical agent against resistant *Staphylococcus* infections. However, vancomycin resistance emerged within two decades. By 1979, vancomycin-resistant coagulase-negative *staphylococci* (CoNS) had been identified, and vancomycin-resistant *Enterococcus* (VRE) was detected ten years later. Further reductions in vancomycin efficacy were marked by the appearance of vancomycin-intermediate *Staphylococcus aureus* (VISA) in 1997 and vancomycin-resistant *Staphylococcus aureus* (VRSA) in 2002 (Parmar et al., 2018). Cephalosporins, a class of beta-lactam antibiotics discovered in

1945, were introduced into clinical practice in 1964 as alternatives to penicillin. While initially effective, particularly against extended-spectrum beta-lactamase (ESBL)-producing Gram-negative bacteria, successive generations of cephalosporins up to the fourth generation have encountered substantial resistance, necessitating the development of fifth-generation agents. Carbapenems, another class of beta-lactam antibiotics introduced in the 1980s, were initially reserved for treating cephalosporin-resistant bacterial infections. However, widespread use between the 1990s and 2000s precipitated the global emergence of carbapenem-resistant organisms, with confirmed cases reported since 2006 (Suay-García & Pérez-Gracia, 2021). Tetracycline, discovered in 1950, proved effective against various common infections, including gastrointestinal disorders. Nevertheless, resistance emerged rapidly and by 1959, *Shigella* strains resistant to tetracycline had been reported. Similarly, levofloxacin, a third-generation fluoroquinolone introduced in 1996, faced resistance from *Streptococcus pneumoniae* within the same year (Zaman et al., 2017). Despite its nature, the high burden of antibiotic use worldwide has created favorable conditions for resistance to proliferate, resulting in heightened public health risks (Tenover, 2006; G. Zhou, Shi, Huang, & Xie, 2015; Khameneh, Diab, Ghazvini, & Bazzaz, 2016).

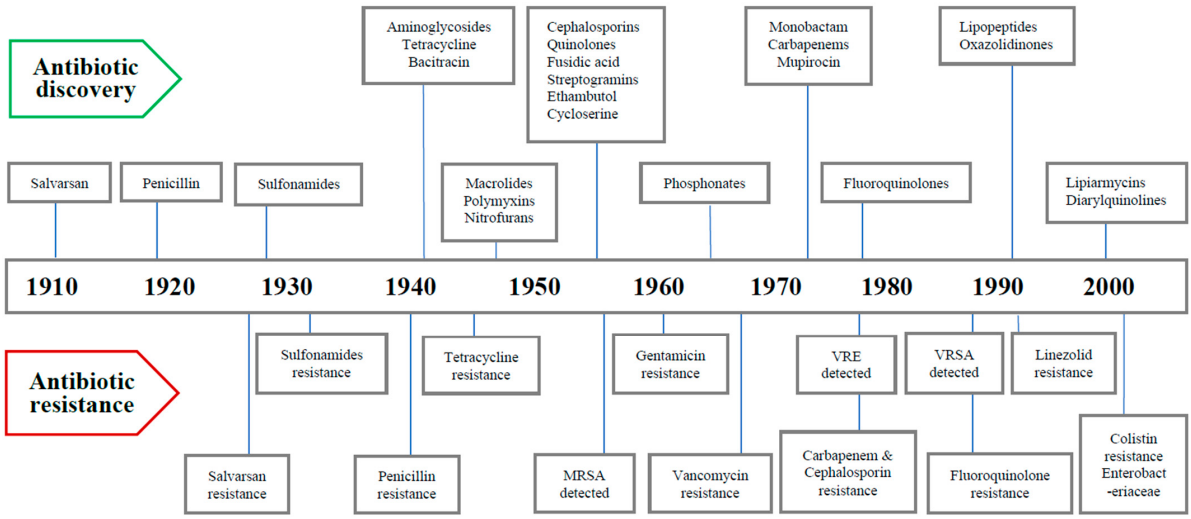

Figure 1: Timeline of the discovery of major antibiotics and antibiotic resistance (Shuval, 2003)

1.1.3 Global burdens and Impacts of Antimicrobial Resistance

Antimicrobial resistance (AMR) is one of the most pressing global health threats of the 21st century. This has led to growing burdens on healthcare systems, on food security, on global economy and biodiversity, increasing mortality from previously treatable infections, significant economic and ecological disruptions. Recognizing and quantifying these burdens is a critical first step toward mobilizing resources and political will to address this global crisis.

The global incidence of antimicrobial-resistant infections has escalated dramatically in the 21st century, prompting public health experts to characterize AMR as a "silent pandemic" (Read & Woods, 2014). The World Health Organization (WHO) lists AMR among the top three global health threats, reporting that in 2019, antimicrobial-resistant infections ranked third among causes of death worldwide, with 1.27 million deaths directly attributable to AMR and nearly 5 million associated deaths (C. J. Murray et al., 2022). This escalating threat is fueled by the widespread availability of over-the-counter antibiotics, which not only drives resistance in common infections such as urinary tract infections, pneumonia, and bloodstream infections (Aslam et al., 2024), but also leads to the resurgence of diseases previously under control, exemplified by the emergence of Extensively Drug-Resistant Tuberculosis (XDR-TB) (Goldman, Plumley, & Laughon, 2007). In 2023, 3.5% of new and 18% of previously treated tuberculosis cases were classified as multidrug-resistant, with XDR-TB remaining a growing concern (Organization, 2023). The crisis is further demonstrated by the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) (Gajdács, 2019), as well as drug-resistant organisms like carbapenem-resistant *Enterobacteriaceae* and *Pseudomonas aeruginosa*, which increase morbidity and strain hospital resources (Jonas, Irwin, Berthe, Le Gall, & Marquez, 2017; Cassini et al., 2019). Treatment failures lead to the reliance on other costlier, less effective therapies that can cause more side effects (Organization, 2022a) and compromise the safety of medical procedures such as surgeries, organ transplants, and cancer chemotherapy (O'Neill, 2016). The impact is most severe in LMICs, where weak healthcare infrastructure and limited access to effective antibiotics amplify the mortality burden, particularly among vulnerable groups like new-

borns, the elderly, and immune-compromised individuals (C. J. Murray et al., 2022). Infections with extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* are increasingly fatal in intensive care settings and neonatal wards (Laxminarayan et al., 2016). Projections suggest that annual AMR-related deaths could reach 10 million by 2050, surpassing the mortality rates of cancer, HIV/AIDS, and malaria in several regions (O'neill, 2014; Aljeldah, 2022). Figure 2 illustrates the current and projected global mortality burden of AMR and other leading causes of death (Aljeldah, 2022).

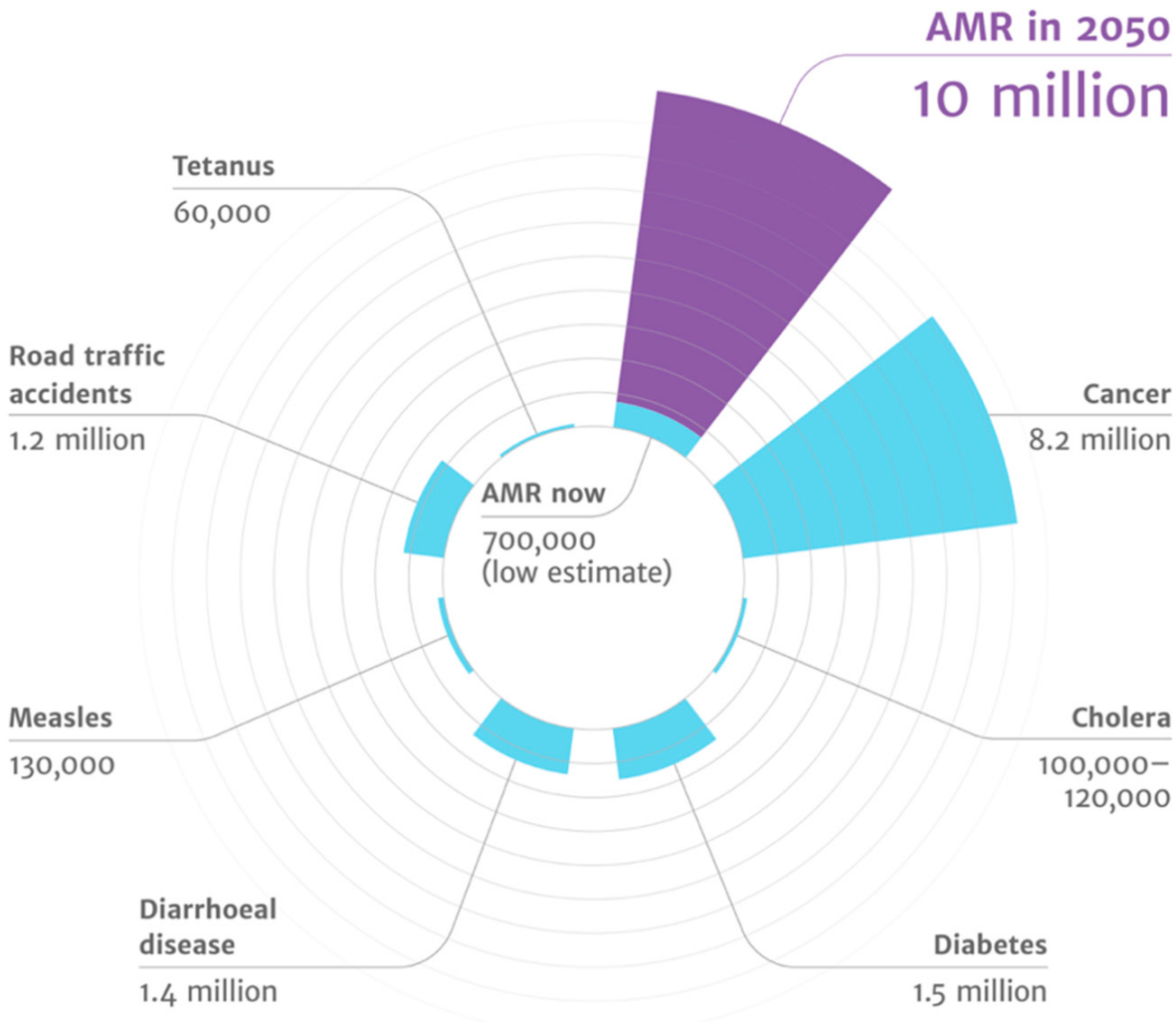

Figure 2: Estimation of human deaths caused by diseases (Aljeldah, 2022).

The economic impact of AMR is also a staggering and growing problem. It manifests in both direct costs, such as increased healthcare expenditures, and indirect costs, including productivity losses, long-term disability, and premature death (Aslam et al.,

2024). Patients infected with drug-resistant pathogens often require longer hospitalizations, additional diagnostic tests, and more expensive treatment regimens. These factors lead to significant cost escalations for individuals, families, healthcare providers, and insurance companies. In the United States, AMR is estimated to cause more than 20 billion USD annually in direct healthcare costs, and an additional 35 billion USD in lost productivity (for Disease Control & Prevention, 2019). In Europe, resistant infections account for an estimated 1.5 billion euros in healthcare and productivity losses each year (Cassini et al., 2019). The situation is even more critical in LMICs, where out-of-pocket expenses for healthcare can push households into poverty and limit access to life-saving treatments. Globally, if left unchecked, AMR could reduce annual global gross domestic product (GDP) by 2–3.5% by 2050, according to a 2016 economic analysis by the Review on Antimicrobial Resistance. The report predicted a cumulative global cost of up to 100 trillion USD over the next few decades (O'Neill, 2016).

Antimicrobial resistance (AMR) also threatens global food security and trade. The use of antibiotics in animal agriculture contributes significantly to the development of resistance, and resistant strains can spread from animals to humans through the food chain, water, and the environment (on Biological Hazards (BIOHAZ) et al., 2021). According to Schar and partners, global antimicrobial consumption is projected to increase by 30% from 2017 to 2030, with antibiotics usage in different sectors such as human's health, terrestrial livestock farming and aquaculture accounted for 20.5%, 73.7% and 5.7%, respectively (Schar, Klein, Laxminarayan, Gilbert, & Van Boeckel, 2020). Consequently, countries face trade restrictions and declining consumer confidence in food safety, resulting in economic losses in the agriculture and export sectors. The World Bank has warned that the global poverty rate could rise by an additional 28 million people by 2050 due to the economic shocks caused by AMR (WBank, 2024). LMICs are projected to suffer the most, with declines in livestock production, reduced labor efficiency, and deteriorating health systems leading to long-term economic stagnation (Jonas et al., 2017).

Beyond human burdens, antimicrobial resistance (AMR) also poses significant ecological challenges by disrupting microbial communities and altering ecosystem func-

tions (Rillig, Lehmann, Orr, & Rongstock, 2024). Antibiotic residues and resistant bacteria are increasingly detected in soil, freshwater, and marine environments due to agricultural runoff, wastewater discharge, and improper disposal of pharmaceuticals (Karkman, Do, Walsh, & Virta, 2018; Larsson et al., 2018). These environmental reservoirs may facilitate the horizontal transfer of resistance genes among diverse microbial populations, amplifying the spread of AMR across ecological boundaries. The proliferation of resistance genes in natural environments can also impact nutrient cycling, degrade soil fertility, and threaten the stability of ecosystems by compromising beneficial microbial processes (Allen et al., 2010). Furthermore, the dissemination of AMR in wildlife and through environmental vectors raises concerns about the re-entry of resistant pathogens into human and animal populations, further complicating our control efforts (Wellington et al., 2013). As a result, the ecological burden of AMR extends well beyond clinical settings, representing a critical component of the global AMR crisis that requires integrated, multisectoral surveillance and intervention strategies. One of the outcomes of AMR are the emergence and re-emergence of infectious diseases which is profoundly influenced by pathogens affecting humans, animals, and plants, each presenting unique characteristics and impacts. Human diseases caused by pathogens significantly impact global health, economy, and societies (Figure 3) (K. E. Jones et al., 2008). Pathogens such as bacteria, viruses, fungi, and parasites cause illnesses ranging from common colds to severe and deadly conditions like tuberculosis, HIV/AIDS, malaria, and COVID-19 (Morens & Fauci, 2020). Viral diseases are especially challenging due to rapid mutation rates, facilitating evasion of immune responses and complicating vaccine development. Respiratory viruses, such as influenza and SARS-CoV-2, highlight the ability of pathogens to cause widespread pandemics through airborne transmission (Parrish et al., 2008). Meanwhile, among all of the 335 emerging infectious disease events listed in the study of Jones and colleagues, more than 54% were caused by bacteria, reflecting a high prevalence of multidrug-resistance microbes (K. E. Jones et al., 2008). Bacterial infections like tuberculosis remain persistent health threats, exacerbated by antibiotic resistance, driven by misuse and overuse of antibiotics in human and veterinary medicine (Van Boeckel et al., 2015). Socioeconomic disparities further aggravate disease burden, especially in resource-limited regions where healthcare infrastructure and public health measures are inadequate

(Bloom & Cadarette, 2019). On the other hand, animal diseases, both domesticated and wild, pose significant risks to global agriculture, biodiversity, and human health. Zoonotic diseases, which originate in animals before infecting humans, include Ebola, rabies, and avian influenza. These diseases often result from direct or indirect contact between wildlife, livestock, and humans, commonly driven by habitat disruption, wildlife trade, and intensive farming practices (Karesh et al., 2012). Animal pathogens also threaten food security and economic stability by causing devastating outbreaks among livestock populations, exemplified by diseases such as foot-and-mouth disease and African swine fever. These outbreaks lead to extensive economic losses and necessitate significant control measures, including mass animal culling and trade restrictions. Moreover, animal pathogens serve as reservoirs for emerging human pathogens, underlining the interconnectedness of animal and human health and highlighting the critical importance of surveillance and biosecurity measures to mitigate disease emergence risks (K. E. Jones et al., 2008). Therefore, in this thesis, we focus our study around bacteria, as they are the leading pathogens responsible for most of the emerging infectious diseases.

1.2 Emerging and Re-emerging Pathogens in A Changing World

1.2.1 Emergence of Zoonotic and Environmental Pathogens

The emergence of zoonotic and environmental pathogens represents a growing threat to global health, driven by complex interactions between humans, animals, and the environment. Zoonotic pathogens, which originate in animals, are capable of transmitting to human and are responsible for more than 60% of emerging infectious diseases (EIDs) (K. E. Jones et al., 2008). Zoonotic diseases, called "zoonoses", result from various zoonotic pathogens such as: bacteria, viruses, eukaryotic parasites and fungi (Rahman et al., 2020). The majority of zoonotic diseases are reported to be transmitted from animals to human with some minor researches suggested the other way around (Cerdà-Cuéllar et al., 2019). The transmission of various diseases to humans is often driven by the emerging of pathogens from wild and domestic animals, pets, aquatic animals, edible insects and even food-borne pathogens. Vertebrate animals

like cattle, sheep, goats, dogs, cats, horses, pigs, and other domestic animals can act as reservoirs of zoonoses, which accounted for 60% of the human infectious diseases. Environmental factors such as landscapes changes, deforestation, intensive agriculture activities, and expanding urbanization disrupt natural habitats and increase direct contact between wildlife, livestock, and humans, also facilitating the transmission of these pathogens. Furthermore, the widespread use of antimicrobials in agriculture and aquaculture accelerates the evolution and dissemination of resistant pathogens in environmental reservoirs, increasing the risk of transmission to humans through food, water, and direct contact (Van Boeckel et al., 2015). Climate change further exacerbates these risks by altering the distribution and ecology of both hosts and pathogens, leading to the emergence of infectious diseases in previously unaffected regions (Semenza & Suk, 2018). As a result, monitoring the dynamics of zoonotic and environmental pathogens has become an essential component of global health security. Although the most recent and notable examples of zoonotic diseases were due to viruses (avian influenza, Ebola virus, and coronaviruses like SARS-CoV and SARS-CoV-2), Bacteria represent the pathogens more often (54.3%) involved in emerging ID events in the last decades (Figure 3) (K. E. Jones et al., 2008). Among them, *Escherichia coli* and *Klebsiella* species are of particular concern due to their increasing prevalence in both hospital and community settings and their ability to acquire and disseminate antimicrobial resistance genes. Extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* are now widespread, not only in clinical environments but also in livestock, wildlife, and aquatic systems, posing a risk of transmission through food, water, and direct contact (Mathers, Peirano, & Pitout, 2015). Both *Escherichia coli* and *Klebsiella pneumoniae* are also known for their antibiotic resistances in aquatic environments, raising concerns about their potential role as reservoirs and vectors for AMR transmission (H. S. Tran et al., 2025). On the other hand, *Vibrio* species, such as *Vibrio cholerae* and *Vibrio vulnificus*, are known marine and estuarine environments bacteria, but their incidence is increasing globally due to rising sea surface temperatures and altered salinity linked to climate change (Baker-Austin et al., 2013). These pathogens can cause a wide range of diseases, from gastroenteritis and wound infections to life-threatening septicemia, and are particularly problematic in coastal regions and aquaculture systems (Vezzulli et al., 2016). Similarly, *Aeromonas* species, which are

ubiquitous in freshwater and brackish environments, have emerged as significant opportunistic pathogens in both humans and animals, causing gastrointestinal, wound, and systemic infections, especially in immuno-compromised individuals (Fernández-Bravo & Figueras, 2020).

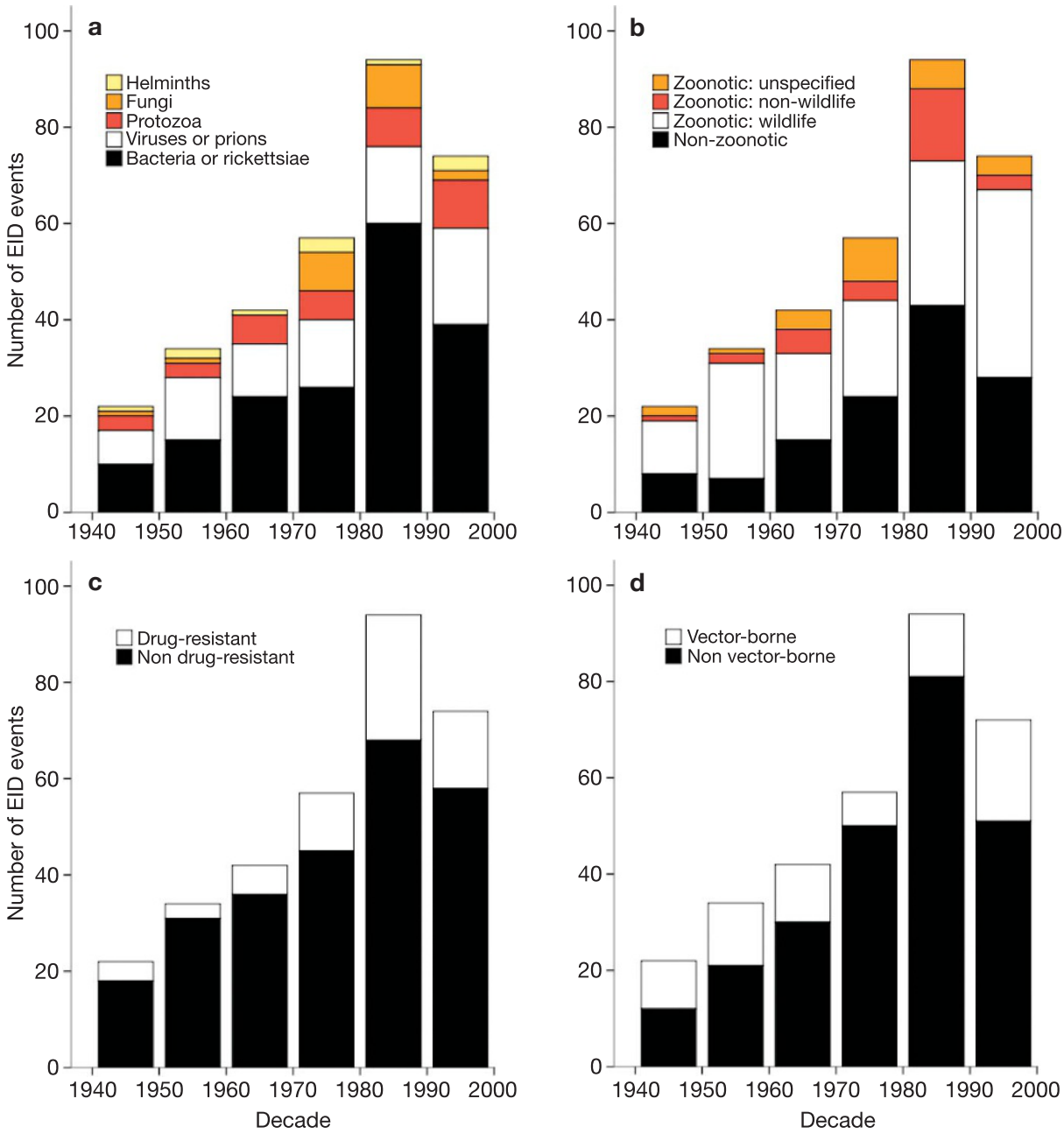

Figure 3: Emerging Infectious Disease (EID) events (defined as the temporal origin of an EID, represented by the original case or cluster of cases that represents a disease emerging in the human population) are plotted with respect to a, pathogen type, b, transmission type, c, drug resistance and d, transmission mode (K. E. Jones et al., 2008).

1.2.2 Transmission Dynamics Across Pathogenic Species and Environments

The transmission dynamics of pathogens across species and environments are shaped by a complex interconnection of ecological, biological, and anthropogenic factors that facilitate the movement of infectious agents within and between pathogenic bacteria populations. Many bacterial pathogens, such as *Escherichia coli*, and *Vibrio cholerae* can survive and persist in diverse ecological environments, enhancing their opportunities for transmission and genetic exchange (H.-S. Xu et al., 1982). Horizontal gene transfer, especially through plasmids and mobile genetic elements, plays an important role in the dissemination of virulence and resistance determinants among bacterial communities in both clinical and environmental settings (Horne, Orr, & Hall, 2023). Human activities, such as intensive agriculture activities, livestock farming, aquacultural practices, and urbanization, have also greatly accelerated the transmission rate of pathogens by creating reservoirs such as wastewater where interspecies circulation is more frequent (Wellington et al., 2013). For example, *Vibrio* and *Aeromonas* species which mostly thrives in aquatic environments can now be transmitted to humans through direct water contact or consumption of contaminated seafood (Fernández-Bravo & Figueras, 2020; Vezzulli et al., 2016). Similarly, the spread of ESBL-producing *Escherichia coli* and *Klebsiella* spp. between animals, humans, and environmental reservoirs underscores the interconnectedness of these systems (Mathers et al., 2015). Wildlife animals and migratory birds can serve as vectors of spreading pathogens and resistance determinants over long distances and across ecological boundaries (Viana, Santamaría, & Figuerola, 2016). Human can also play the role of reservoirs or carrier for the pathogenic species due to symptomatic and asymptomatic infections. The coronaviruses pandemics were proofs of how fast and severe the pathogens transmission can affect the entire world population (Morens & Fauci, 2020). Climate change and extreme weather events further influence transmission dynamics by changing pathogens survival, host distributions, and the frequency of cross-species contacts (Semenza & Suk, 2018). All together, these factors highlight the necessity of adopting a One Health approach that integrates surveillance and control efforts across human, animal, and environmental domains.

1.3 One Health Framework: A Strategy for Combating Antimicrobial Resistance and Pathogens

1.3.1 Conceptual Foundation of One Health Paradigm

The One Health paradigm recognizes the intricate correlation of human, animal, and environmental health. This approach originated from the original "One Medicine" concept, which integrated human and veterinary medicine together to treat zoonoses (Zinsstag, Schelling, Waltner-Toews, & Tanner, 2011). In 2004, the "One World – One Health" action further expanded this vision by incorporating ecosystem health, including wildlife, and calling for holistic, transdisciplinary strategies involving multiple sectors and expertise (Figure 4A). Accelerated global changes, mostly driven by the growth of human population, industrialization, and geopolitical issues, have led to biodiversity loss, ecosystem degradation, and increased migration of humans and other species. These rapid environmental shifts have been linked to the emergence and re-emergence of both infectious and non-infectious diseases (Figure 4B) (Destoumieux-Garzón et al., 2018). Recent zoonotic outbreaks such as avian influenza, Ebola, Zika and Corona virus epidemics dramatically demonstrated the interconnectedness of human, animal, and ecosystem health.

The concept of One Health can be defined at three primary levels: individual, population, and ecosystem, as proposed by Lerner and colleagues (Lerner & Berg, 2015). At the individual level, One Health is typically distinguished as animal health and human health. On the other hand, terms such as population health, public health, and herd health are often used at the population level, but they mainly function as statistical tools for disease monitoring and food safety, rather than as true concepts. Population level becomes particularly significant in the context of zoonotic diseases and environmental contamination, where multiple species are affected and health becomes a measure of disease absence or presence within a group (S. Krebs, Danuser, & Regula, 2001). Lastly, ecosystem health serves as an operational definition that reflects the functionality and service provision of ecosystems, emphasizing the interconnect-

edness of human, animal, and environmental health (Jakobsson, 2012). Overall, while several levels of One Health definitions exist, the choice of definition is subjective and context-dependent. Notably, the One Health as a concept is most robustly applied at the individual level, with population and ecosystem health serving as tools for surveillance and system monitoring.

The One Health approach is increasingly important as our world becomes more interconnected. With both human and animal populations have expanded into new geographic areas, people are brought into closer contact with wild and domestic animals, providing more opportunities for diseases to transmit between species (Bauerfeind et al., 2020). The movement of people, animals, and animal products due to international travel and trade even accelerates the global dissemination of diseases. Animals, whether for food, or companionship, play significant roles in our lives, further increasing the risk of zoonotic transmission. Animals themselves can also serve as early warning systems for human health threats, as seen with birds and West Nile virus (Manore et al., 2014). Moreover, environmental disruptions, such as climate change, deforestation, and intensive agriculture, create new opportunities for pathogens to emerge and spread (Wellington et al., 2013). To address these issues, the One Health approach includes a wide spectrum, including emerging, re-emerging, endemic zoonotic diseases, neglected tropical diseases, vector-borne diseases, antimicrobial resistance, food safety and security, environmental contamination, and climate change (Destoumieux-Garzón et al., 2018). For example, One Health approach can monitor antimicrobial-resistant organisms which are rapidly spreading across communities, healthcare settings, food supplies, and the environment (Lerner & Berg, 2015) and vector-borne diseases that are increasing as climate change expands the range of mosquitoes and ticks (Semenza & Suk, 2018). Importantly, the One Health approach is important beyond just infectious diseases, even offering benefits in areas such as chronic disease, mental health, occupational health, and injury prevention, through interdisciplinary collaboration and integrative surveillance.

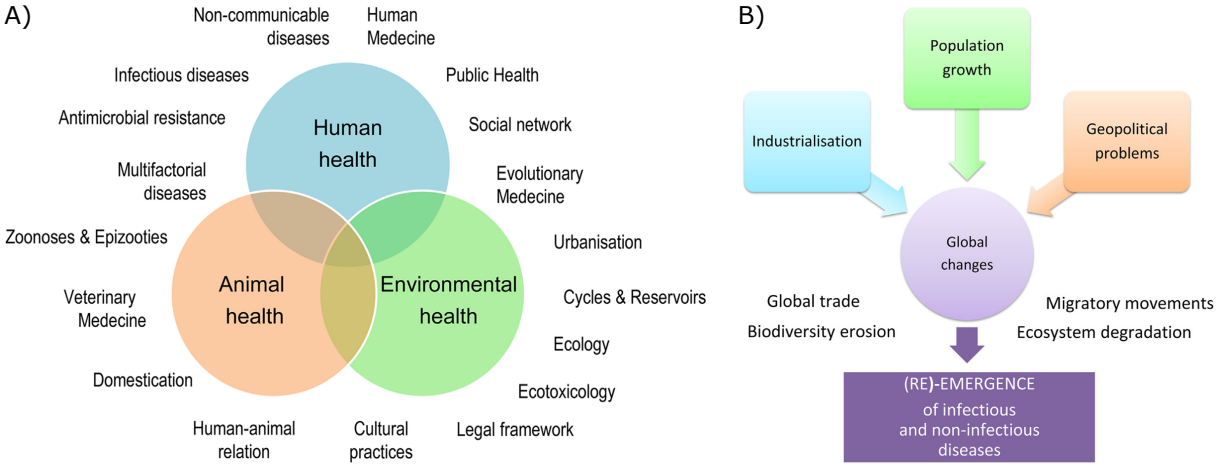

Figure 4: A: The One Health concept: a holistic, transdisciplinary, and multisectoral approach of health. B: Global changes favors the re-emergence of infectious and non-infectious diseases (Destoumieux-Garzón et al., 2018).

1.3.2 Rationale for Integrative, Cross-sectoral Surveillance and Research Approaches

Antimicrobial resistance (AMR) and pathogens represent a pressing global health threat that extends beyond human populations, affecting animals, plants, and ecosystems. Like humans, animals can act as reservoirs for MDR pathogens, with transmission occurring through direct contact or the consumption of animal-derived food products. Given its complex and interconnected nature, the AMR crisis cannot be effectively managed by any single government or organization. A coordinated, multisectoral approach is essential that unites diverse fields such as human and veterinary healthcare, agriculture, pharmaceutical regulation, finance, trade, education, and civil society organizations, both nationally and internationally (Getahun & Balkhy, 2018). This collaborative framework must operate on both horizontal and vertical levels. Horizontal collaboration involves integration across different sectors and stakeholders within a country, such as multistakeholder platforms, while vertical collaboration spans local, regional, national, and international levels. Both forms of collaboration are crucial for the successful implementation of AMR and pathogen control strategies (Salam et al., 2023). Local actions are particularly critical and should be tailored to local contexts, where specific constraints, such as limited infrastructure, shortages of trained person-

nel, and weak surveillance systems, can seriously undermine control efforts.

1.3.2.1. Measures to Combat Antimicrobial Resistance

Effective international action to combat antimicrobial resistance (AMR) requires coordinated collaboration among global agencies, governments, non-governmental organizations (NGOs), and professional groups (Portillo, 2020). Key measures include establishing worldwide surveillance networks to monitor antimicrobial usage and resistance trends, and enhancing laboratory capacities globally to detect, identify, and promptly report AMR pathogens of international health concern. International support for programs guided by essential drug lists and standardized treatment guidelines should be prioritized. Additionally, implementing international tracking systems is critical for rapidly identifying and responding to emerging resistant pathogens and outbreaks (L. Zhang, Guo, & Lv, 2024). Strengthening efforts to monitor and control counterfeit antimicrobial is also essential, as it provides dedicated funding to support research into new drugs, vaccines, and diagnostics. On the other hand, the development of accessible and rapid diagnostic tests is vital for accurately identifying pathogens and monitoring resistance patterns, facilitating informed antibiotic use decisions. Additionally, implementing and enforcing robust antibiotic policies that promote responsible antimicrobial use within both healthcare settings and agriculture sectors is crucial. Moreover, national surveillance efforts should also be enhanced through improved collaboration between public health and veterinary sectors.

Monitoring Antibiotic Resistance Genes (ARGs) is a central component of the Global Antimicrobial Resistance Surveillance System (GLASS), initiated by the WHO in 2015. This global initiative aims to standardize AMR data collection, analysis, and sharing, providing a structured framework to assess existing and emerging surveillance systems. It promotes a shift from isolate-level data to more comprehensive epidemiological and clinical data, enabling nations to generate standardized, comparable information. This facilitates policy-making, tracks the emergence and spread of ARGs, and supports resource allocation to manage AMR threats effectively (Organization, 2017; Grundmann & Gelband, 2018). In recognition of the urgency of the AMR cri-

sis, the World Health Organization has endorsed five strategic objectives as part of its Global Action Plan on AMR (Organization, 2022b):

• Raise awareness and understanding of AMR through education and effective communication;

• Strengthen the evidence base through surveillance and research to inform infection prevention and control measures;

• Improve hygiene, sanitation, and infection prevention practices;

• Optimize antimicrobial use in both human and animal health;

• Encourage sustainable investment in the development of new antimicrobials, diagnostics, and vaccines

1.3.2.2. Measures to Combat Pathogens

Combating and preventing the spread of infectious pathogens is also a substantial task requires multiple approaches that integrates government and international cooperation, policies, surveillance, along with raising awareness about microbes, and development of new treatments (Drexler et al., 2014). Firstly, robust surveillance systems including real-time genomic and metagenomic monitoring are essential for early detection of emerging pathogens (Hendriksen et al., 2019; Besser, Carleton, Gerner-Smidt, Lindsey, & Trees, 2018). Secondly, improving Infection Prevention and Control (IPC) measures in healthcare, agricultural and aquacultural practices, and social settings is vital, including hand hygiene, sanitation, vaccination, and appropriate use of personal protective equipment (Organization, 2022c). Thirdly, investment in research and development of novel diagnostics, therapeutics, and drugs particularly targeting high-priority pathogens is necessary (Piddock et al., 2024). Strengthening regulatory frameworks for the use of agricultural and aquacultural practices, reducing environmental contamination, and improving waste management also play crucial roles in limiting the spread of zoonotic and environmental pathogens (Van Boeckel et al., 2015). Finally, cooperation and policies that encourages collaboration between nations and organizations are key to addressing the complex, interconnected nature of pathogen emergence and transmission (Organization, 2017). Infectious diseases from pathogens are an unavoidable part of life, but there are many strategies to help protect ourselves

from infections and treat diseases once they arise. Some of these strategies are simple steps that individuals can take, while others involve national or global methods for detection, prevention, and treatment. These coordinated strategies, when combined, can substantially reduce the global burden of infectious diseases and mitigate future public threats, maintaining the health and security of communities, nations, and populations worldwide.

2 Antimicrobial resistance: A rising global threat

2.1 Mechanisms of Antimicrobial Resistance and Dissemination

Antimicrobial resistance (AMR) is known fundamentally as an evolutionary strategy that enables bacteria to survive under the selective pressure imposed by antimicrobial agents. Although this phenomenon occurs naturally, it has been significantly intensified by human activities such as the widespread and often inappropriate prescription of antibiotics, the routine use of antibiotics as growth enhancers in animal agriculture, and a significant decline in the discovery of novel antibiotics. These practices have collectively fueled the global AMR crisis.

Newly introduced antibiotics are initially effective against their target pathogens. However, prolonged usage leads to the development of bacterial resistance. Evolutionarily, bacteria can adapt to antibiotic pressure through two principal mechanisms: chromosomal mutations and the acquisition of resistance genes via Horizontal Gene Transfer (HGT). These mutations typically affect genes involved in antibiotic targets, transport mechanisms, or regulatory functions, such as those controlling the expression of efflux pumps or antibiotic-modifying enzymes (J. Davies & Davies, 2010). Environmental and commensal bacteria are recognized as important reservoirs of resistance genes, which may be transferred to pathogenic bacteria through HGT. Notably, many environmental microorganisms naturally produce antibiotics and must therefore harbor corresponding resistance genes to avoid autotoxicity (Koch, Islam, Sonowal, Prasad, & Sarma, 2021).

Antibiotic resistance occurs when bacteria acquire physiological adaptations that neutralize or evade the effects of antimicrobial agents. These mechanisms include structural modifications of the bacterial cell envelope, production of inactivating enzymes, reduced permeability to the drug, increased efflux, and alterations or destruction of the antibiotic's molecular target (Reygaert, 2018). While resistance can emerge naturally, current practices, particularly the indiscriminate use of broad-spectrum antibiotics, greatly intensify the problem (Victor, 2011). There are three main mechanisms for AMR: intrinsic, acquired, or adaptive (phenotypic) (Figure 5) (J.-H. Lee, 2019; Olivares et al., 2013).

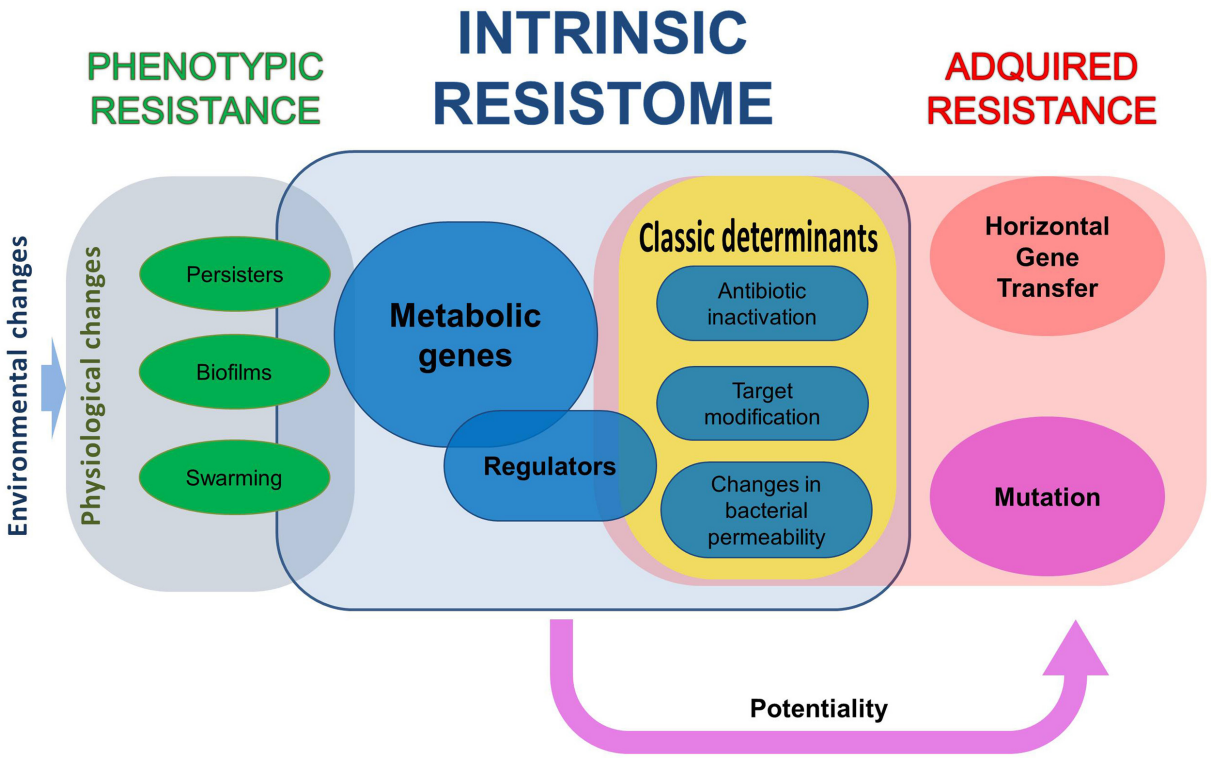

Figure 5: Different mechanisms in bacterial resistance to antibiotics (Olivares et al., 2013)

2.1.1 Intrinsic resistance

This resistance refers to the innate ability of bacteria to resist specific antibiotics due to inherent chromosomal features, without requiring prior exposure or genetic changes. This form of resistance is typically mediated by restricted drug permeability, active efflux systems, and the structural characteristics of bacterial cell envelopes. For

instance, the lipopolysaccharide (LPS)-rich outer membrane of gram-negative bacteria significantly limits antibiotic uptake, serving as a natural barrier to many antimicrobial agents (E. Marti, Variatza, & Balcazar, 2014; Cox & Wright, 2013). Additionally, chromosomally encoded efflux pumps, such as those belonging to the ATP-binding cassette (ABC) and major facilitator superfamily (MFS) in gram-positive bacteria, and the resistance–nodulation–division (RND) superfamily in gram-negative bacteria, actively expel antibiotics, thereby lowering intracellular drug concentrations (Poole, 2005; Nikaido & Pagès, 2012; Blair, Richmond, & Piddock, 2014; Reygaert, 2018).

2.1.2 Acquired resistance

Acquired resistance occurs when susceptible bacteria gain resistance through genetic changes such as chromosomal mutations or horizontal gene transfer (HGT). The primary modes of HGT include transformation (where bacteria incorporate free extracellular Deoxyribonucleic Acid (DNA)), transduction (which involves the introduction of DNA into the cell via a vector such as a bacteriophage), and conjugation, with the latter being the most common via acquisition of genetic material transferred through direct contact between bacterial cells (Holmes et al., 2016; Munita & Arias, 2016). These resistance genes are often carried by Mobile Genetic Elements (MGEs), including transposons, plasmids, and genomic islands. Phages can also serve as vectors for the horizontal transfer of ARGs. These elements may harbor multiple resistance genes, enabling bacteria that acquire them to express multi-drug resistance.

2.1.2.1. Plasmids

Plasmids are non-chromosomal DNA molecules, ranging in size from a few thousand to several million base pairs. They can carry multiple resistance genes and be transferred from one bacterium to another via conjugation, i.e., horizontal gene transfer through direct contact between bacterial cells. Conjugative plasmids contain the genetic elements necessary for their own transfer, whereas mobilizable plasmids rely on host cell mechanisms to be transferred. The spread of plasmids carrying ARGs is enhanced by the selective pressure exerted by the use of antibiotics in both human

and veterinary medicine (Bennett, 2008). A plasmid's ability to transfer to a bacterium depends on its host range, which is experimentally defined. Some plasmids can be transferred to a broad range of bacterial hosts, such as plasmid RK2, which appears to be compatible with most Gram-negative bacteria (Blatny, Brautaset, Winther-Larsen, Karunakaran, & Valla, 1997). Furthermore, a plasmid's persistence potential in a host cell is linked to its incompatibility group, which determines whether multiple plasmids can coexist in the same host. Plasmids from the same incompatibility group cannot be stably maintained together.

2.1.2.2. Transposons

Transposable elements or transposons were first discovered by Barbara McClintock during her work on maize chromosomes, where some chromosomal regions were observed to change position during cell division (McClintock, 1953). These are DNA sequences capable of moving within the genome of a single cell or between different cells. In prokaryotes, transposons are classified into composite and non-composite types. Composite transposons consist of a transposase-encoding gene flanked by inverted repeat sequences, which also surround the genes to be mobilized. This entire unit makes up a transposon. The transposase, encoded by the insertion sequences, is an enzyme responsible for catalyzing the excision and reintegration of the transposon at another genomic location. Non-composite transposons, on the other hand, lack insertion sequences. Instead, they are flanked by inverted repeat sequences, with a terminal sequence recognized by the transposase, enabling transposition. These transposons also contain a gene encoding a resolvase, which represses the transposase and facilitates transposon replication. In the context of antibiotic resistance gene transfer, transposons play a crucial role. They allow resistance genes to move between different DNA molecules (for example, from a plasmid to a bacterial chromosome, or among various mobile genetic elements). As such, transposons can mobilize chromosomal resistance genes onto plasmids or, conversely, integrate plasmid-borne resistance genes into chromosomes, allowing them to persist across bacterial generations (L. S. Frost, Leplae, Summers, & Toussaint, 2005). For instance, the non-composite transposon Tn1696 confers resistance to gentamicin, streptomycin, chloramphenicol,

and sulfamethoxazole in *Pseudomonas aeruginosa* (Rubens, McNeill, & Farrar Jr, 1979).

2.1.2.3. Genomic Islands

Genomic islands are elements or sections of the genome that can be excised and integrated into bacterial chromosomes or plasmids. They are key drivers of horizontal gene transfer, contributing to the genomic adaptability of bacteria. Genomic islands are broadly classified into two categories of Integrative and Conjugative Elements (ICEs), and Integrative and Mobilizable Elements (IMEs) (Bellanger, Payot, Leblond-Bourget, & Guédon, 2014). ICEs possess a complete set of genes required for their excision, transfer, and integration, including a conjugation module that enables their autonomous, self-directed mobility between bacterial cells. In contrast, IMEs lack some of these essential mobility functions and thus rely on the conjugative machinery of co-resident mobile elements for their mobilization. These genomic islands often carry genes that help the host cell adapt to its environment, including metabolism, virulence and notably, antibiotic resistance genes. For example, ICE Tn5397, found in *Clostridioides difficile*, harbors the tetracycline resistance gene tetM (Roberts, 2002). Another example is IME SGI1, identified in *Salmonella*, which contains multiple genes conferring resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (Doublet, Boyd, Mulvey, & Cloeckaert, 2005). The acquisition and dissemination of such elements play a significant role in the evolution of multidrug-resistant bacterial strains, posing a major challenge for public health. Furthermore, the dynamic nature of genomic islands enables them to reshape bacterial genomes rapidly in response to environmental pressures, such as antibiotic exposure, thereby promoting adaptation and persistence in diverse ecological environment.

2.1.2.4. Integrons

Integrons are unique genetic elements that although not inherently mobile themselves, play a pivotal role in the acquisition and dissemination of adaptive genes among bacteria. Their primary function is to facilitate the capture and expression of gene cassettes (small and non-replicative DNA elements), thereby promoting rapid genomic

evolution in response to environmental pressures such as antibiotic exposure. A typical integron is composed of several key components: the *intI* gene, which encodes an integrase enzyme; a specific recombination site known as attI; a variable region capable of incorporating one or more gene cassettes; and a Pc promoter that drives the transcription of integrated genes (Kovalevskaya, 2002).

The process of gene cassette integration involves the recognition of the attI site by the integrase, followed by the excision of a gene cassette from a donor molecule and its subsequent insertion into the integron. Each gene cassette contains its own recombination site (attC) and typically carries genes that can confer selective advantages, such as antibiotic resistance. Once integrated, the expression of these genes is facilitated by the Pc promoter located within the integron structure, ensuring that captured traits are readily available to the host cell (Kovalevskaya, 2002). Although integrons themselves lack the mechanisms for self-mobilization, their ability to assemble and express diverse gene cassettes makes them crucial contributors to bacterial adaptability, especially when they are embedded within mobile genetic elements such as transposons or plasmids. This collaboration between integrons and other mobile elements accelerates the spread of advantageous traits across bacterial populations, contributing significantly to the emergence and persistence of multidrug-resistant strains.

2.1.2.5. Hierarchical arrangement of mobile genetic elements

Resistance genes, integrons, and mobile genetic elements can be nested within one another like matryoshka dolls, forming mosaic structures (Gillings & Stokes, 2012). The R100 plasmid, which carries multiple resistance genes, is a good example. On one side, the tetracycline resistance genes *tet*(R), *tet*(A), *tet*(C), and *tet*(D) are located on a Tn10 transposon embedded in the plasmid. On the other side, the sulfonamide resistance gene *sul*1 and the aminoglycoside resistance gene *aad*A1 are found within a class 1 integron, itself carried by a Tn402 transposon. This transposon, along with a mer operon conferring mercury resistance, is nested within another transposon, Tn21, which is in turn integrated into Tn9, carrying the cat gene that confers resistance to phenicol. This entire structure is carried by the plasmid (Figure 6).

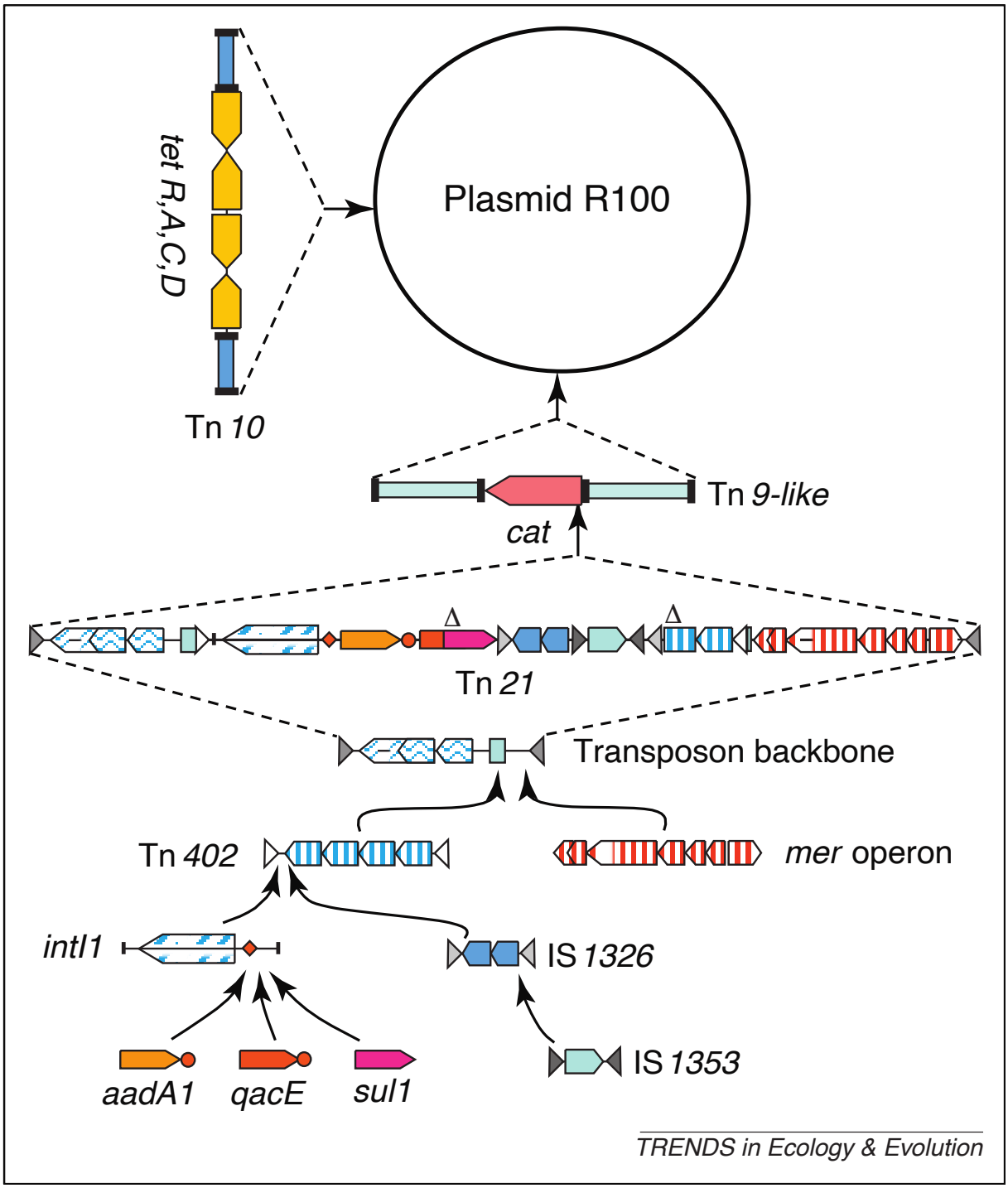

Figure 6: Mosaic structure of the R100 plasmid. Tetracycline resistance genes are shown in yellow, aminoglycoside resistance gene (*aad*A1) in yellow-orange, disinfectant resistance gene (*qac*E) in orange, sulfonamide resistance gene (*sul*1) in bright pink, phenicol resistance gene (*cat*) in light pink. Mercury resistance operon (*mer*) is represented in vertically striped brown. Various shades of blue indicate the different genetic structures carrying the resistance genes (Gillings & Stokes, 2012).

2.1.3 Adaptive resistance

Adaptive resistance constitutes a transient, reversible response to environmental stimuli, including sub-inhibitory antibiotic concentrations, nutrient levels, pH changes, and ionic stress. Though temporary, this resistance poses significant challenges in both clinical and agricultural contexts due to its dynamic nature. Mechanisms associated with adaptive resistance may involve biofilm formation, increased mutation rates, gene amplification, and activation of efflux systems (L. Fernández & Hancock, 2012; Rizi, Ghazvini, & Noghondar, 2018). Biofilms, in particular, protect bacteria like *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* by forming a dense matrix of exopolysaccharides that impedes antibiotic diffusion and reduces drug efficacy (Dutt et al., 2022; Van Acker, Van Dijck, & Coenye, 2014).

Beyond structural defenses and drug inactivation, modification of drug targets is another vital resistance mechanism. Mutations in genes encoding essential bacterial enzymes can reduce drug binding affinity. For example, fluoroquinolone resistance is frequently linked to mutations in the Quinolone Resistance-determining Regions (QRDRs) of DNA gyrase and topoisomerase IV (Ashley et al., 2017). Furthermore, target site methylation, such as by erm genes affecting ribosomal binding sites, confers resistance to macrolides, lincosamides, and streptogramin B (MLS class of antibiotic), while methylation via the cfr gene extends resistance across multiple species, including *Staphylococcus* spp., *Escherichia coli*, and *Proteus vulgaris* (Saha & Sarkar, 2021). Additionally, the expression of alternative penicillin-binding proteins like PBP2a, encoded by *mec*A and *mec*C genes in *Staphylococcus aureus*, is a hallmark of methicillin resistance (Foster, 2017; Wendlandt et al., 2015). Together, these multifaceted mechanisms spanning innate traits, acquired elements, and adaptive responses highlight the complexity of AMR and underscore the importance of comprehensive strategies for mitigation and treatment.

2.2 Key Drivers and Factors Contributing to Antimicrobial Resistance

Antimicrobial resistance (AMR) arises from a complex interplay of microbial characteristics and environmental, behavioral, and systemic human factors (Figure 7). Antibiotics can inevitably enter the environment following their uses in health-care treatment or agricultural practices, where their concentrations also depend on multiple complex factors. According to Kookana, environmental pharmaceutical concentrations, including antibiotics, are influenced by population density, local demographics, healthcare accessibility, pharmaceutical manufacturing presence, wastewater treatment connectivity, ecological characteristics of receiving environments, and local regulatory policies (Kookana et al., 2014). The intricate interplay among these factors explains why environmental antibiotic concentrations vary substantially across different regions globally (Aus der Beek et al., 2016). These contributing elements can be broadly classified into four categories: environmental factors (e.g., ecology, population density, poor sanitation, global dissemination), drug-related factors (e.g., consumption products, counterfeit, substandard, and/or over-the-counter medicines), patient-related factors (e.g., self-medication, poverty, poor compliance, and low education), and physician-related factors (e.g., inappropriate prescribing practices and lack of updated clinical knowledge) (Reygaert, 2018; Abushaheen et al., 2020).

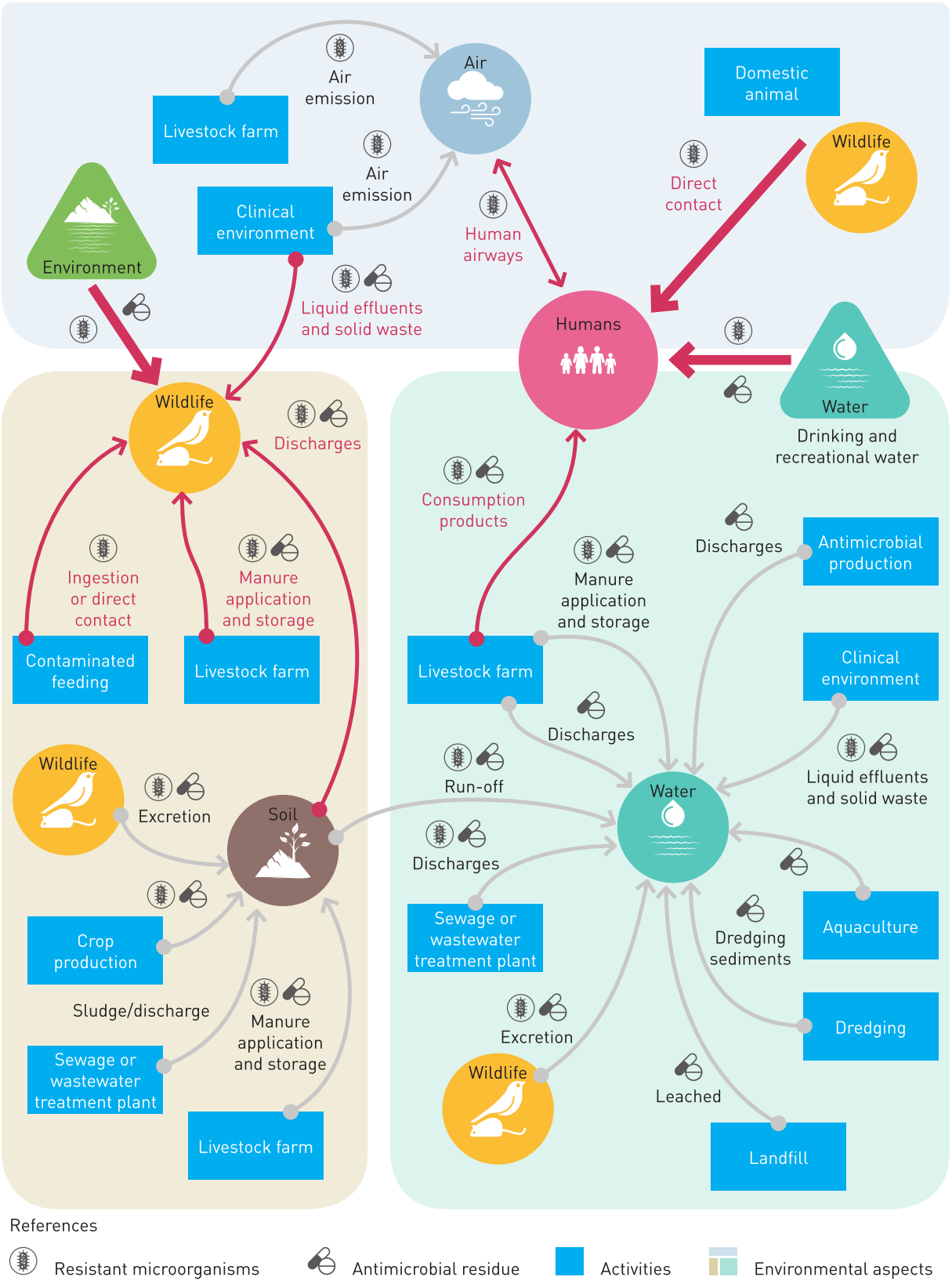

Figure 7: Environmental complexities of AMR drivers and transmission (Coque et al., 2023)

2.2.1 Misuse and Overuses of Antibiotics

The situation of antimicrobial resistance (AMR), although a natural aspect of microbial evolution, has experienced a notable acceleration in recent decades and is primarily driven by the improper and excessive use of antibiotics in both human healthcare and livestock production. Numerous epidemiological investigations have established a robust and consistent association between the over-utilization of antibiotics and the proliferation of resistant microbial strains (Chaw, Höpner, & Mikolajczyk, 2018). Despite increasing global awareness and public health campaigns, the inappropriate consumption and misuse of antibiotics persists, particularly in resource-limited settings where constrained access to diagnostic tools and healthcare infrastructure necessitates reliance on empirical treatment approaches (Chokshi, Sifri, Cennimo, & Horng, 2019).

A key reason for antibiotic misuse is widespread misconceptions, such as the belief that antibiotics are effective against viral infections like the common cold or influenza, which persist, especially in communities with lower education. In many developing countries, antibiotics are frequently prescribed without appropriate diagnostic justification due to the lack of adequate laboratory infrastructure (Chokshi et al., 2019). In addition, the unregulated sale of over-the-counter antibiotics for both human and veterinary use exacerbates resistance risks, as these medications are often obtained without prescriptions or medical oversight.

Rising gross domestic product (GDP), particularly in LMICs, has been associated with increased access to antibiotics and higher consumption rates. Between 2000 and 2015, global antibiotic consumption rose by 65%, a trend closely linked to improvements in socioeconomic conditions (Vikesland et al., 2019). Rising income levels have driven dietary changes, notably for a greater demand in animal-derived protein, which in turn has intensified the use of antibiotics in livestock production (Van Boeckel et al., 2015). Notably, in 2013, approximately 131,000 tons of antibiotics were administered to food-producing animals worldwide, a figure projected to reach 200,000 tons by 2030 (Figure 8) (Van Boeckel et al., 2017; Vikesland et al., 2019). Antibiotics are used not only for disease treatment but also as growth promoters and prophylactic agents to enhance

feed efficiency (Food & Organization, 2022). For instance, a study conducted in Vietnam determined that 84% of the antibiotics used in chicken farms were for prophylaxis, with only 12% for disease treatment, and 3.8% for a combination of prophylaxis and disease treatment (Carrique-Mas et al., 2015). This widespread use, especially in countries with weak regulatory frameworks, contributes significantly to the AMR burden in humans due to the presence of antibiotic residues in animal meats and products such as milk and eggs (Castro-Sánchez, Moore, Husson, & Holmes, 2016). As the economies of LMICs further advance and food animal consumption rises, it is expected that antibiotic use within these countries will increase until it exceeds that within present-day High-Income Countries (HICs) (Blaskovich, 2018). Alarmingly, approximately 70% of medically important antibiotics in the United States are sold for animal use (I. Frost, Van Boeckel, Pires, Craig, & Laxminarayan, 2019). The similarity in the types and mechanisms of antibiotics used in veterinary and human medicine further aggravated resistance transmission between compartments. Weak policies and the absence of standardized treatment guidelines further intensify the issue. Furthermore, substandard and counterfeit antibiotics infiltrate the supply chain, reducing treatment efficacy and promoting resistance. Over prescription, prolonged treatment courses, and improper dosing, often driven by financial incentives or patient pressure, further accelerate the problem (Michael, Dominey-Howes, & Labbate, 2014; Pulia et al., 2018).

In clinical settings, inappropriate prescribing remains a pervasive issue. Research indicates that nearly half of hospitalized patients receive at least one antibiotic unnecessarily, and in roughly one-third of cases, antibiotics are administered without diagnostic confirmation and continued beyond recommended durations (M. Woolhouse, Waugh, Perry, & Nair, 2016). In long-term care facilities, such as nursing homes, up to 75% of antibiotic prescriptions are deemed inappropriate, particularly regarding dosage and duration (for Disease Control & Prevention, 2022). Prescriptions should be guided by culture and susceptibility testing, yet such practices are inconsistently implemented in many healthcare systems.

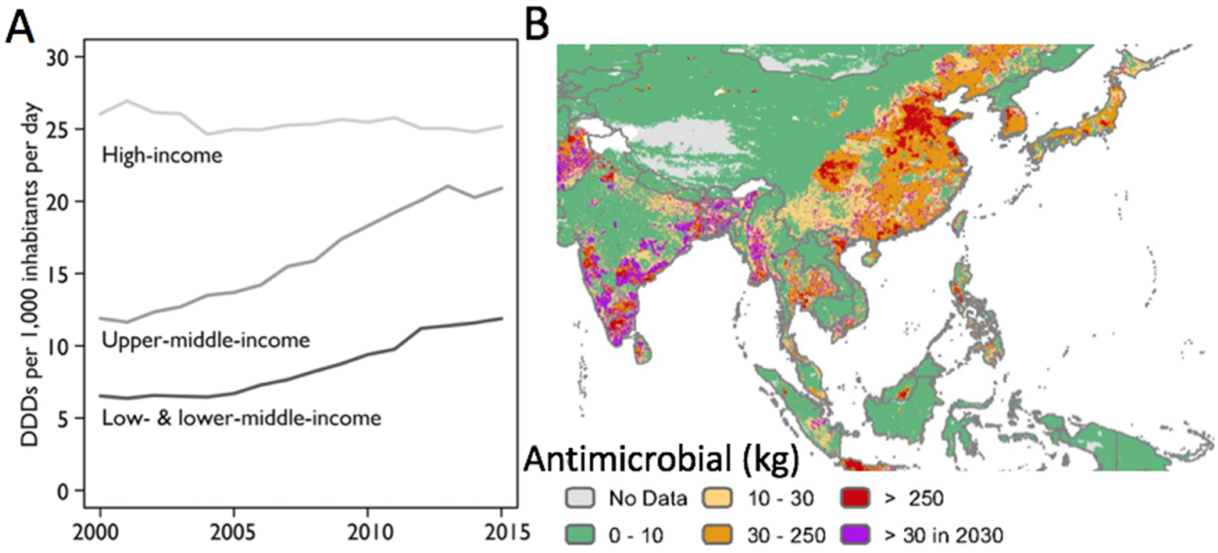

Figure 8: (A) Global consumption of antibiotics in terms of Defined Daily Doses (DDDs). (B) Antimicrobial consumption in chickens in 2010. The purple areas reflect areas where consumption will be >30 kg per 10 km^2 in 2030 (Vikesland et al., 2019).

2.2.2 Limited Innovations and Development of New Antibiotics

Current efforts to develop new antibiotics are falling short, posing a serious threat to global strategies against antimicrobial resistance (AMR). Despite repeated international appeals for innovation, the pharmaceutical industry has made limited progress in discovering novel antibiotic classes. According to the World Health Organization, this stagnation in antibiotic development is undermining global efforts to combat drug-resistant infections (WHO, 2020). A major barrier to progress lies in the economic disincentives associated with antibiotic research and development. Although companies invest heavily in creating new drugs, they often struggle to recover their costs or generate sufficient profit. Most newly developed antibiotics are reserved as "last-resort" treatments for multidrug-resistant pathogens, which means their use is intentionally limited to prevent resistance development. Consequently, low sales volumes and the comparatively low prices of antibiotics reduce financial returns, making antibiotics far less lucrative than drugs for chronic conditions or lifestyle diseases. As a result, many large pharmaceutical companies have withdrawn from antibiotic research altogether. In the 1980s, there were 18 multinational companies actively engaged in antibiotic development, but only a few remain today (Rex, 2023). Among the 51 antibiotics devel-

oped recently, only eight are considered novel in terms of their mechanisms against resistant bacteria (DiMasi, Grabowski, & Hansen, 2016). Most are derivatives of existing drugs, increasing the likelihood of rapid resistance development. The lack of effective new treatments has severely limited options for managing infections caused by resistant pathogens, such as drug-resistant tuberculosis, urinary tract infections, pneumonia, and gram-negative bacterial infections (Q. Chang, Wang, Regev-Yochay, Lipsitch, & Hanage, 2015). Even when scientific breakthroughs occur, they rarely translate into marketable treatments without substantial financial support. The process of clinical testing, regulatory approval, and commercialization is costly and time-consuming, often requiring early-stage funding to attract further investment. Without adequate support during these critical early phases, promising antibiotic candidates frequently stall before reaching patients. Given the rapid emergence of resistance to newly developed antibiotics and the difficulties in creating effective alternatives, a comprehensive strategy that includes both vaccine development and novel antibiotic research has become essential. Development initiatives can be directed toward several key areas: (1) optimizing current antibiotic classes through structural refinement, (2) designing novel antibacterial agents with unique chemical scaffolds, and (3) investigating the therapeutic potential of alternative approaches, such as bacteriophage therapy or nanotechnological approaches (Natan & Banin, 2017).

2.2.3 Global Dissemination and Mobility of Resistance Genes

Modern travel infrastructure has significantly accelerated the global spread of antibiotic resistant organisms. International travelers may unknowingly acquire resistant bacteria during visits to high-abundance regions and carry them, contributing to transnational AMR transmission (Arcilla, 2017). Studies indicate that resistant organisms can persist in the gut for up to 12 months post-travel, posing a risk for onward transmission within communities and healthcare settings (McCubbin et al., 2021).

Collignon and colleagues outlined antimicrobial resistance (AMR) dissemination as a two-step process with the first step involves the emergence of antibiotic-resistant bacterial phenotypes through genetic mutations or HGT and the second step involves

the subsequent spread or contagion of these resistant strains via vectors, such as humans and animals, and environmental vehicles, including water, food, and sediment (Collignon, Beggs, Walsh, Gandra, & Laxminarayan, 2018). This framework effectively distinguishes between factors that initially drive resistance development and those responsible for its spread. Interestingly, resistance rates in LMICs are generally higher compared to HICs, despite LMICs having lower per-capita antibiotic consumption (Klein et al., 2018). This discrepancy supports the hypothesis that, once resistance emerges (as in Step 1), environmental, socioeconomic, and infrastructural factors predominantly determine the extent and pace of AMR dissemination. Consequently, global disparities in healthcare spending, poverty, sanitation infrastructure, education, and governance significantly influence the magnitude of AMR spread and have become focal points of international interventions (Figure 9).

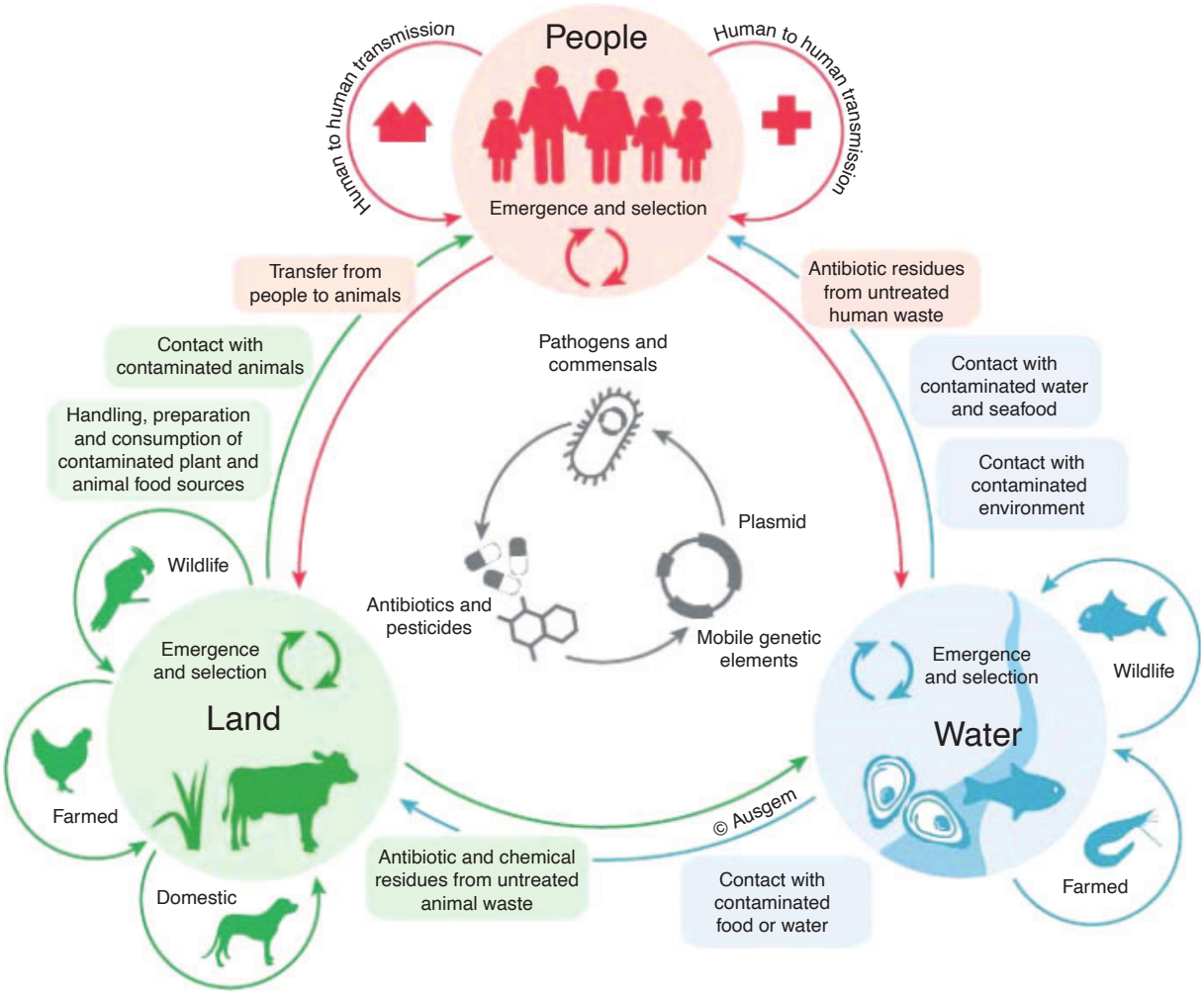

Figure 9: One Health system diagram showing the spread of AMR (Djordjevic & Morgan, 2019)

2.2.4 Environmental and Ecological Factors

A growing body of research highlights that ecological elements and physicochemical parameters in aquatic environments also play critical roles in shaping the distribution, persistence, and dissemination of antimicrobial resistance (AMR) (Larsson & Flach, 2022; Calero-Cáceres, Marti, Olivares-Pacheco, & Rodriguez-Rubio, 2022). Factors such as pH, temperature, heavy metal concentrations, and Dissolved Organic Matter (DOM) levels can significantly affect the abundance and transfer dynamics of antibiotic resistance genes (ARGs) and resistant bacteria. These environmental factors interact with microbial communities, influencing how resistance traits evolve and spread (Bengtsson-Palme, Kristiansson, & Larsson, 2018). For instance, metal pollutants including copper, zinc, and mercury are known for promoting horizontal gene transfer and maintaining ARGs via co-resistance mechanisms on mobile genetic elements (Baker-Austin, Wright, Stepanauskas, & McArthur, 2006; Seiler & Berendonk, 2012). When wasted water streams contain compounds such as ammonium, other biocides, and certain heavy metals, they can negatively impact aquatic and land species. These substances can create co-selective pressure when bacteria have multiple resistance genes for these chemicals and other antimicrobials. If these resistance genes are present in the same microorganisms, exposure to one substance can lead to resistance to another, even if the second substance is absent (Pal, Bengtsson-Palme, Kristiansson, & Larsson, 2015). This co-selection of antimicrobial resistance often occurs in environmental systems. Zinc and copper are widely used in animal feed to improve nutrition and control diseases, which can promote the survival of drug-resistant bacteria in environments with these metals, such as the gastrointestinal tract of animals consuming them (Bearson et al., 2020). Similarly, elevated temperatures may enhance microbial growth rates and metabolic activity, thereby accelerating the proliferation and exchange of ARGs among environmental microbes (Martinez, 2009; Andersson & Hughes, 2014). A study by Reverter and colleagues reported antimicrobial resistance (AMR) in aquaculture, revealing that the average Multiple Antibiotic Resistance (MAR) index calculated from bacteria found in aquaculture across 40 countries was positively correlated with rising temperatures. This trend was also particularly evident in LMICs (Reverter et al., 2020). pH fluctuations can also influence bacterial commu-

nity structure and the stability of both antibiotics and ARGs in sediments and water (Zhu et al., 2017). Additionally, high DOM levels presented in water can inhibit the absorption rates of antibiotics, indirectly influencing resistance selection (Z. Chen et al., 2015). Collectively, these ecological factors interact with anthropogenic pressures, such as antibiotic use and pollution, to drive the complex landscape of AMR in aquatic and soil ecosystems.

2.2.5 Identified Knowledge and Infrastuctural Gaps

Lack of awareness and understanding about antibiotic resistance among the public and healthcare professionals significantly undermines global efforts to promote responsible antibiotic use and implement effective public health interventions. Many studies show that misconceptions about antibiotics remain widespread, fueling the development and spread of antimicrobial resistance (McCullough, Parekh, Rathbone, Del Mar, & Hoffmann, 2016). It was observed that a lack of understanding and awareness regarding the use of antibiotics, self-treatment with leftover antibiotics, using antibiotics to treat viral infections or as pain killer, anticipating an antibiotic prescription as the outcome of a consultation, and the credibility of received information are challenges present in various countries (Antwi, Stewart, & Crosbie, 2020). Recent research emphasizes the importance of implementing Antimicrobial Stewardship Programs (ASP) toward agricultural practices, society's health and particularly within medical education, to instill responsible antibiotic prescribing practices among all professionals. These initiatives aim to prepare future clinicians to effectively confront AMR as a major public health concern (Majumder et al., 2020; Razzaque, 2021).

Beside the gaps in our understanding of antimicrobial resistance (AMR), insufficient numbers of infrastructure and facilities, especially in LMICs, resulted in a lack of clean water, sanitation and hygiene, and wastewater treatments, which has caused a significant prevalence of infectious diseases (Pokharel, Raut, & Adhikari, 2019). Several studies report that nutrient-rich environments with high bacterial densities, such as untreated wastewater, provide optimal conditions and greatly influence the development and dissemination of Antibiotic-Resistant Bacteria (ARB) and AMR emer-

gence. Wastewater has been widely identified as a significant reservoir for antibiotic resistance genes and resistant pathogens (Di Cesare, Fontaneto, Doppelbauer, & Corno, 2016; Q.-B. Yuan, Guo, Wei, & Yang, 2016). Specifically, fecal contamination from open defecation, inadequately treated sewage, or illegal waste discharges serves as a key mechanism through which resistance enters aquatic ecosystems. Unfortunately, only 39% of the global population currently has access to safely managed sanitation systems, leaving the remaining 61% mostly residing in LMICs exposed to poorly managed sanitation practices. Consequently, ARGs and AMR are frequently detected in pit latrine wastes and contaminated waterways within both urban and rural LMICs settings (Beukes, King, & Schmidt, 2017; Q.-Q. Zhang, Ying, Pan, Liu, & Zhao, 2015). Furthermore, solid waste management practices significantly impact environmental exposure to antibiotics and resistant organisms. Annually, approximately 1.85 billion tons of Municipal solid Waste (MSW) are generated globally, with LMICs producing about 62% and HICs accounting for 38% (Kaza, Yao, Bhada-Tata, & Van Woerden, 2018). Although per capita waste generation remains much higher in HICs, waste production rates in LMICs continue to increase. Waste disposal practices vary significantly by region, ranging from highly organized landfills and incineration systems to indiscriminate open dumping. In many Low-Income Countries (LICs), only about 40% of solid waste is formally collected, compared to nearly 100% in HICs. Additionally, collected waste in LMICs often ends up in open dumps rather than engineered landfills or incineration facilities. This disparity results in closer, more frequent interactions between LMICs populations and solid waste, potentially increasing exposure to antibiotic residues and resistant organisms.

Improper disposal of antibiotics, both from household use and clinical or hospital settings, also introduces antibiotic residues into solid waste, creating localized selective pressures for resistance development. Studies confirm that landfill leachates often contain ARGs and ARB, and groundwater near MSW landfills can also become heavily contaminated (M. Sun et al., 2016). Although evidence suggests higher antibiotic contamination in environmental compartments like rivers and soils in LMICs compared to HICs, it remains unclear whether this difference truly reflects greater contamination or merely sampling biases and confounding factors (Q.-L. Chen, Li, et al., 2017).

2.3 Surveillance and Diagnostic: Technologies and Approaches

The dynamic nature of the AMR also highlights the need for advanced technologies to explore microbial diversity, especially the pathobiome, and antibiotic resistance genes and mechanisms (J. A. Perry, Westman, & Wright, 2014). Tools such as Next-Generation Sequencing (NGS), bioinformatics pipelines, and expansive public databases have significantly advanced research in antimicrobial resistance, facilitating in-depth genomic analysis and enabling improved surveillance strategies (Crofts, Gasparrini, & Dantas, 2017). In addition to sequencing-based methods, high-throughput Quantitative Polymerase Chain Reaction (qPCR) systems like the SmartChip platform (Takara, Shiga, Japan) have gained traction for ARGs investigations across diverse environmental microbiomes (F.-H. Wang et al., 2014; Q.-L. Chen, An, et al., 2017; K. Lee et al., 2020)

2.3.1 Bacterial Isolation and Antibiotic Susceptibility Testing

Bacterial isolation remains a fundamental step in the detection, monitoring, and study of antimicrobial resistance. By cultivating bacterial isolates from clinical, animal, or environmental samples on selective and differential media, pure cultures can be obtained for Antibiotic Susceptibility Testing (AST) to determine their resistance patterns (Gajic et al., 2022). The most widely used AST methods include disk diffusion (Kirby-Bauer), which assess bacterial growth in the presence of standardized concentrations of antibiotics. The results are interpreted according to internationally accepted guidelines (e.g., Clinical & Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST)), classifying bacteria as susceptible, intermediate, or resistant to specific drugs (Cockerill et al., 2012; on Antimicrobial Susceptibility Testing et al., 2018). These phenotypic resistance profiles are crucial not only for informing clinical therapy but also for regional, national, and global AMR surveillance systems (Organization, 2017). By regularly performing AST, clinical institutions and laboratories can contribute essential data for the tracking of resistance trends, detecting the emergence of multidrug-resistant, and informing public health interventions (van Belkum et al., 2020). Combined, bacterial isolation and susceptibility

testing remain the gold standards for diagnosing AMR and remain vital components of integrated AMR monitoring programs.

2.3.2 Whole Genome Sequencing

Whole-genome sequencing (WGS) is often the preferred method for identifying antimicrobial resistance determinants in resistome studies, due to its technical practicality, cost-effectiveness, and capacity to yield clinically actionable data relevant to infection control (Schürch & van Schaik, 2017). Compared to traditional resistance testing, WGS offers additional benefits such as critical microbial identification, elucidation of phylogenetic relationships, detection of mutations, discovery of potential novel genes, and prediction of phenotypic antibiotic resistance. Genomic data also provide essential insights into antibiotic resistance traits, which are invaluable for outbreak investigations, real-time surveillance, and monitoring the spread of ARGs (Hendriksen et al., 2019; Alghoribi, Balkhy, Woodford, & Ellington, 2018). However, WGS also has technical limitations, notably the requirement for pure cultures, and the fact that many bacterial and archaeal species cannot be cultured in laboratory settings (Steen et al., 2019).

One of the advantages of whole-genome sequencing is the foundational method for detecting ARGs, both chromosomal and plasmid-borne. Results from WGS data, such as gene presence, absence, and abundance, are increasingly being used to develop machine learning models that predict AMR in clinical and environmental samples. WGS has been utilized to consistently identify genes conferring resistance to multiple major antibiotic classes, including beta-lactams, aminoglycosides, macrolides, tetracyclines, sulfonamides, quinolones, and glycopeptides (e.g., *van*A, *van*B). For instance, a survey on clinical *Pseudomonas aeruginosa* genomes from two tertiary hospitals showed the presence of multiple ARGs, such as *sul*1, *bla*PAO, *bla*GES-1, *bla*OXA-50, *aph*(3')-IIb, *aad*A6, *fos*A, *tet*(G), *rmt*F (Ahmed, 2022). In the United Kingdom, two studies on antimicrobial resistance surveillance of methicillin-resistant *Staphylococcus aureus* and commensal *Escherichia coli* genomes using WGS revealed widespread transportation of ARGs including *mec*A (methicillin resistance), as well as beta-lactam, tetracycline

and sulphonamides/trimethoprim resistance genes (Holden et al., 2013; Stubberfield et al., 2019). Notably, global spread of carbapenemase genes such as *bla*KPC, *bla*NDM, *bla*OXA-48, particularly in *Klebsiella pneumoniae* and *Acinetobacter baumannii* (Logan & Weinstein, 2017) as well as the emerging of *mcr*-1 gene (colistin resistance), often located on plasmids and detected in *Enterobacteriaceae* from both clinical and food animal sources (Y.-Y. Liu et al., 2016), highlighted the revolution of ARGs detection, identification by WGS.

One of the most interesting advantage of whole genome sequencing is AMR prediction independently of known ARGs databases. Antimicrobial resistance prediction has been achieved using genome assembly and BLASTn in *Staphylococcus aureus* (N. Gordon et al., 2014). Similarly, the web-based tool PointFinder identifies resistance-associated point mutations through database matching, further analyzed when the sequence identity exceeds 80% (Zankari et al., 2017). Another notable tool, PhyResSE, is tailored for *Mycobacterium tuberculosis* and uses a reference-based mapping strategy via BWA-MEM to determine lineages and antibiotic resistance profiles based on a curated catalog of 92 known lineages (Feuerriegel et al., 2015; H. Li, 2013). The Mykrobe Predictor offers an alignment-f2ree approach using de Bruijn graphs to analyze resistance profiles in *Mycobacterium tuberculosis* and *Staphylococcus aureus*, also identifying species, phylogenetic relationships, and virulence factors. A significant outcome of large-scale WGS efforts was the CRyPTIC consortium's study involving over 10,000 clinical *Mycobacterium tuberculosis* isolates from 16 countries. The findings demonstrated that WGS could accurately characterize susceptibility to first-line tuberculosis drugs, making it suitable for clinical decision-making (Consortium & the 100, 2018).

Another approach to identify AMR mechanisms independent to known ARGs is by comparing the complete genomes of different bacterial strains with distinct susceptibility profiles. One study demonstrated the effectiveness of "stability selection" in understanding genotype-phenotype relationships in *Mycobacterium tuberculosis* and *Staphylococcus aureus*. Using k-mer-based genotyping (alignment-free) method that captures various genetic variations including Single Nucleotide Polymorphisms (SNPs) and indels, linear regression models were built to predict antibiotic responses with accuracy

comparable to Mykrobe (Mahé & Tournoud, 2018). Further research has leveraged machine learning and pan-genome analysis to identify AMR-associated genetic signatures. By mapping these to 3D protein structures, scientists were able to locate mutation hotspots likely tied to antibiotic resistance (Kavvas et al., 2018). Another study compared classification and regression tree models and found them to be highly accurate in AMR prediction, revealing both known and potentially novel resistance mechanisms across 12 bacterial species and 56 antibiotics (Drouin et al., 2019). Although this pan-genomic approach is resource-intensive, requiring extensive sequencing and computational infrastructure, it enables the exploration of entire genomes beyond known ARG regions. This is crucial for determining whether genomic associations with resistance are merely correlative or causative. However, large and diverse strain collections are needed to draw meaningful conclusions, adding to the logistical challenges.

2.3.3 Metagenomics

Metagenomics has emerged as a pivotal tool in understanding and combating antimicrobial resistance (AMR) by providing comprehensive insights into antibiotic resistance genes (ARGs) across diverse ecosystems, including human, animal, and environmental microbiomes. Over the past decade, metagenomic methods have significantly advanced the detection and characterization of ARGs, circumventing limitations associated with traditional culture-dependent techniques. Two primary metagenomic approaches as shotgun metagenomics and functional metagenomics, are widely employed and recommended in AMR research.

Shotgun metagenomics involves sequencing all genetic material directly extracted from environmental or biological samples, providing unbiased insights into the microbial community composition and functional potential, including the resistome - the collection of all ARGs within a given microbiome (Pehrsson, Forsberg, Gibson, Ahmadi, & Dantas, 2013). This descriptive approach is particularly valuable for analyzing microbial community dynamics and responses to environmental pressures such as antibiotic exposure, enabling researchers to track ARG abundance, diversity, and potential mobility across microbial taxa and ecological niches. For example, Moore and

colleagues utilized metagenomic recombinant libraries derived from pediatric gut microbiota, uncovering extensive resistance to 14 antibiotics spanning eight drug classes (A. M. Moore et al., 2013). The identified ARGs included clinically significant resistance mechanisms such as dihydrofolate reductases, chloramphenicol acetyltransferases, multidrug efflux pumps, and multiple beta-lactamases, many of which were demonstrated to be potentially mobilizable, underscoring the threat posed by ARG dissemination within microbial communities.

Functional metagenomics complements shotgun sequencing by cloning fragmented environmental DNA into surrogate hosts, typically *Escherichia coli*, followed by phenotypic screening for antibiotic resistance. This strategy enables not only the detection but also the functional characterization of previously unknown ARGs, facilitating the discovery of novel resistance mechanisms and their evolutionary contexts (Garmendía, Hernandez, Sánchez, & Martinez, 2012; Chistoserdova, 2009). Additionally, functional metagenomics has revolutionized molecular taxonomy by establishing a culture independent classification system based entirely on genetic and functional attributes of microbes, significantly expanding our understanding of microbial ecology and taxonomy (Schloss & Handelsman, 2003)). The method has also been effectively employed in screening natural and engineered environments for novel antimicrobial compounds, proteins, and biosynthetic gene clusters, thereby offering opportunities to identify potential new therapeutic agents to counteract AMR (De, 2019).

Integrating advanced sequencing technologies, robust ARG databases (e.g., CARD, ResFinder, etc..), and sophisticated bioinformatics pipelines has significantly enhanced our ability to quantify and contextualize ARG abundance and distribution on a global scale. For instance, structured databases and bioinformatic tools allow for accurate resistome annotation, facilitating the rapid identification of ARG hotspots and informing targeted mitigation strategies (Alcock et al., 2023; Bortolaia et al., 2020). However, despite the transformative impact of ARG databases, most existing databases are heavily biased toward antibiotic resistance genes characterized from commensal or pathogenic bacteria relevant to human, primarily reflecting data from hospitals, human-associated microbiomes, with an underrepresentation of ARGs from other environments (C. Lee

et al., 2023).

In conclusion, metagenomics offers an unparalleled framework for revealing AMR dynamics at a resolution previously unattainable through traditional microbiological techniques. By combining shotgun, functional, and targeted approaches, researchers can achieve a comprehensive understanding of the resistome across various ecosystems, identify emerging resistance threats promptly, and inform evidence-based strategies to mitigate global AMR proliferation.

2.3.4 Quantitative Polymerase Chain Reaction

Quantitative Polymerase Chain Reaction (qPCR) is a highly sensitive and specific molecular technique widely used for the detection and quantification of genes such as antibiotic resistance genes (ARGs) in a variety of samples, including clinical, environmental, agricultural, and food settings (S.-Y.-D. Zhou et al., 2020; Srathongneam et al., 2024; Al Asad et al., 2024). Its crucial role in ARGs surveillance arises from its ability to provide fast, precise, and high-throughput measurement of target genes, even when they are present in low quantities. qPCR enables the precise detection of specific ARGs by amplifying targeted DNA sequences with gene-specific primers and measuring the accumulation of amplified product in real-time using fluorescent probes or dyes. This allows researchers to not only confirm the presence of ARGs, but also to quantify their absolute or relative abundance within microbial communities (Looft et al., 2012; H. S. Tran et al., 2025). By employing standard curves generated from known concentrations of plasmids or synthetic DNA fragments, qPCR can determine gene copy numbers per unit of DNA, sample, or per cell, making it especially valuable for comparative studies across time points, treatments, or geographic locations.

Quantitative Polymerase Chain Reaction is widely adopted in ARGs surveillance due to its high throughput, reproducibility, and cost-effectiveness, especially for monitoring key resistance genes in environmental reservoirs such as wastewater, surface water, soils, and sediments (Waseem et al., 2019). For example, qPCR has been extensively used in monitoring the spread of sulfonamide resistance and *intI*1 genes

as indicators of anthropogenic pollution and antibiotic resistance dissemination from wastewater treatment plants (WWTPs) into river systems (Haenelt et al., 2023). In hospital settings, qPCR enables routine surveillance of ARGs to assess the impact of antibiotic use and inform risk management strategies in early stage of pandemic (C. Wang et al., 2022). Recently, high-throughput qPCR platforms (e.g., microfluidic qPCR arrays) have enabled parallel screening of dozens to hundreds of ARGs across numerous samples, greatly enhancing large-scale surveillance efforts (Stedtfeld et al., 2018). Despite the undeniable advantages, qPCR is still limited to known ARG targets and cannot identify novel or unexpected resistance genes, its quantitative power makes it an indispensable tool for validating metagenomic findings and tracking trends in ARG prevalence over time. When used alongside sequencing-based methods, qPCR provides a robust, quantitative layer to ARG surveillance and risk assessment frameworks.

2.3.5 Transcriptomics and Metabolomics

Another valuable method for predicting AMR involves analyzing gene expression changes in bacterial isolates after exposure to antibiotics. A study by Suzuki et al. (2014) revealed that acquiring resistance to one antibiotic can influence susceptibility to others. Transcriptomic analysis uncovered adaptive mechanisms, including compensatory gene expression changes (Suzuki, Horinouchi, & Furusawa, 2014). Teixobactin, a novel antimicrobial agent effective against *Staphylococcus aureus* and *Enterococcus faecalis*, was studied for its transcriptional impact. Findings indicated that *Enterococcus faecalis* could develop intrinsic tolerance at high drug concentrations via deletion of the croRS system, a possible precursor to resistance (Darnell et al., 2019). Beyond known resistance pathways, such as efflux pump expression or altered antibiotic targets, emerging transcriptomics research is uncovering regulatory networks and stress response mechanisms. However, clinical applications are limited by the complexity of experimental design, lengthy data processing, and incomplete functional annotation of many clinically important pathogens and their transcripts.

Metabolomics is a rapidly growing field that involves the detailed and compre-

hensive analysis of all metabolites and small molecules present within a biological sample. Metabolomics offers a complementary perspective in AMR research by analyzing metabolic changes in bacterial cells. Yang et al. (2019) introduced an integrated method combining biochemical screening, metabolic network modeling, and machine learning (termed a "white-box" approach) to explore antibiotic action mechanisms (J. H. Yang et al., 2019). Their study examined *Escherichia coli* responses to three antibiotics as ampicillin, ciprofloxacin, and gentamicin, by simulating the bacterium's metabolic state using genome-scale models. This approach quantified how various metabolic pathways contributed to antibiotic lethality. In parallel, Zampieri et al. (2017) profiled over 500 intracellular and extracellular metabolites across 190 evolved *Escherichia coli* populations (Zampieri et al., 2017). They discovered that carbon and energy metabolism significantly influenced the speed and mode of resistance development. The study also uncovered multiple bacterial strategies to survive antibiotic pressure, including changes in efflux pump expression and mutation acquisition mechanisms that varied depending on the metabolic state and carbon source available. This metabolomics-based perspective expands our understanding of bacterial responses to antibiotics, offering insights into the interplay between metabolism and resistance evolution.

3 Pathogens and Infectious Diseases in the Context of Global Change

3.1 Definition and Characteristic of Pathogens

The concept of a pathogen was first explained by Casadevall & Pirofski in 2002 with definition focused on a pathogen-centered perspective, describing virulence factors based on microbial traits (Casadevall & Pirofski, 2002). However, virulence must be expressed in a vulnerable host, so it could not exist independently. This means that the idea of a pathogen was always linked to a host, highlighting the interaction between host and microbe. After the acceptance of germ theory, pathogenic microbes were thought fundamentally distinct from non-pathogenic organisms due to intrinsic virulence factors. Early classifications divided microbes into pure saprophytes, pure

parasites, and half-parasites, based on pathogenic potential (Zinsser, 1914). Observations like attenuation and increased virulence through animal passage supported the pathogen-centered view of microbial pathogenicity, emphasizing microbial features like toxins and capsules (Casadevall & Pirofski, 2001). Modern research continues this focus by associating virulence with pathogenicity islands, bacteriophage, specific virulence factors, and growth-related gene expression. Nonetheless, the pathogen-centered view cannot fully explain microbial pathogenesis in cases involving host susceptibility. For instance, organisms like *Candida albicans* and *Staphylococcus epidermidis* become pathogenic only when host defenses are compromised, and distinct virulence determinants are often unclear in immunocompromised scenarios (Casadevall & Pirofski, 2001). Furthermore, this view fails to account for changes in host immunity, as pathogens can lose their pathogenic potential following immunization. Host-centered approaches, highlighting the role of host susceptibility, also face limitations. Defining pathogens solely by their ability to cause disease in impaired hosts neglects scenarios where the same microbes can cause disease in both healthy and immune-compromised individuals, as seen with *Candida albicans* and *Aspergillus fumigatus*. Changes in host immunity, such as immune reconstitution in HIV/AIDS patients, further challenge singular pathogen definitions.

Given these complexities, the "damage framework" has been proposed, defining pathogenicity based on damage to hosts resulting from the host-microbe interaction (Casadevall & Pirofski, 1999, 2000, 2001). This outcome-based classification overcomes limitations of pathogen- and host-centered views, simplifying microbial characterization by the presence or absence of host damage. This framework categorizes microbes into groups based on host response and damage over time, avoiding problematic terms like "commensal" or "opportunistic". Emphasizing outcomes such as infection, colonization, commensalism, and disease provides clarity for research and potential clinical applications, encouraging the development of more sensitive host-damage assessments.

An emerging pathogen is defined as an infectious disease agent showing increasing incidence either in a newly invaded host population or in an existing population due

to epidemiological changes (M. E. Woolhouse, 2002). Emerging or novel pathogens frequently appear in human, domestic animal, wildlife, and plant populations, yet the dynamics of their initial introduction and spread remain inadequately understood. These pathogens, often originating from host species jumps, commonly share characteristics such as being directly transmitted Ribonucleic Acid (RNA) viruses. Host species jumps have historically triggered severe epidemics, including HIV/AIDS, myxomatosis in rabbits, rinderpest in African ruminants, and distemper in North Sea seals (M. E. Woolhouse, Haydon, & Antia, 2005). Additionally, major human diseases like measles, tuberculosis, influenza, and smallpox are believed to have originated from domestic animals over thousands of years. Crop diseases like potato blight and chestnut blight in American chestnuts further illustrate significant impacts of host jumps (Anderson et al., 2004; Milgroom, Wang, Zhou, Lipari, & Kaneko, 1996). However, not all species jumps lead to severe epidemics. Examples include BSE/vCJD and Ebola virus, which despite their seriousness, have not produced widespread epidemics comparable to HIV/AIDS. Pathogens like the rabies virus regularly jump between species without triggering major outbreaks (M. E. Woolhouse et al., 2005). Understanding the epidemiological and evolutionary differences behind these varied outcomes is essential for managing emerging infectious diseases.

3.2 The Pathobiome Concept in the Omics Era

Recent studies highlight that the "one microbe–one disease" paradigm has often overlooked the complex microbial networks of bacteria, protists, fungi, viruses, and phages that influence diseases (Chow, Tang, & Mazmanian, 2011). In the gut microbiome, commensals can gain virulence through horizontal gene transfer or when antibiotics disrupt community balance (Stecher et al., 2012). On the other hand, arthropod symbionts can also play a role in pathogen spread or symbiotic *Wolbachia* species can disrupt the dengue and Chickungunya viruses in mosquito salivary, thus limiting their transmission. Therefore, the term "pathobiome" was introduced to describe a pathogen within its microbial environment. Studying this concept, using meta-omics requires profiling microbial communities, demonstrating pathogenic effects, and understanding factors that disrupt disease dynamics (Figure 10 (Vayssier-Taussat et al.,

2014).

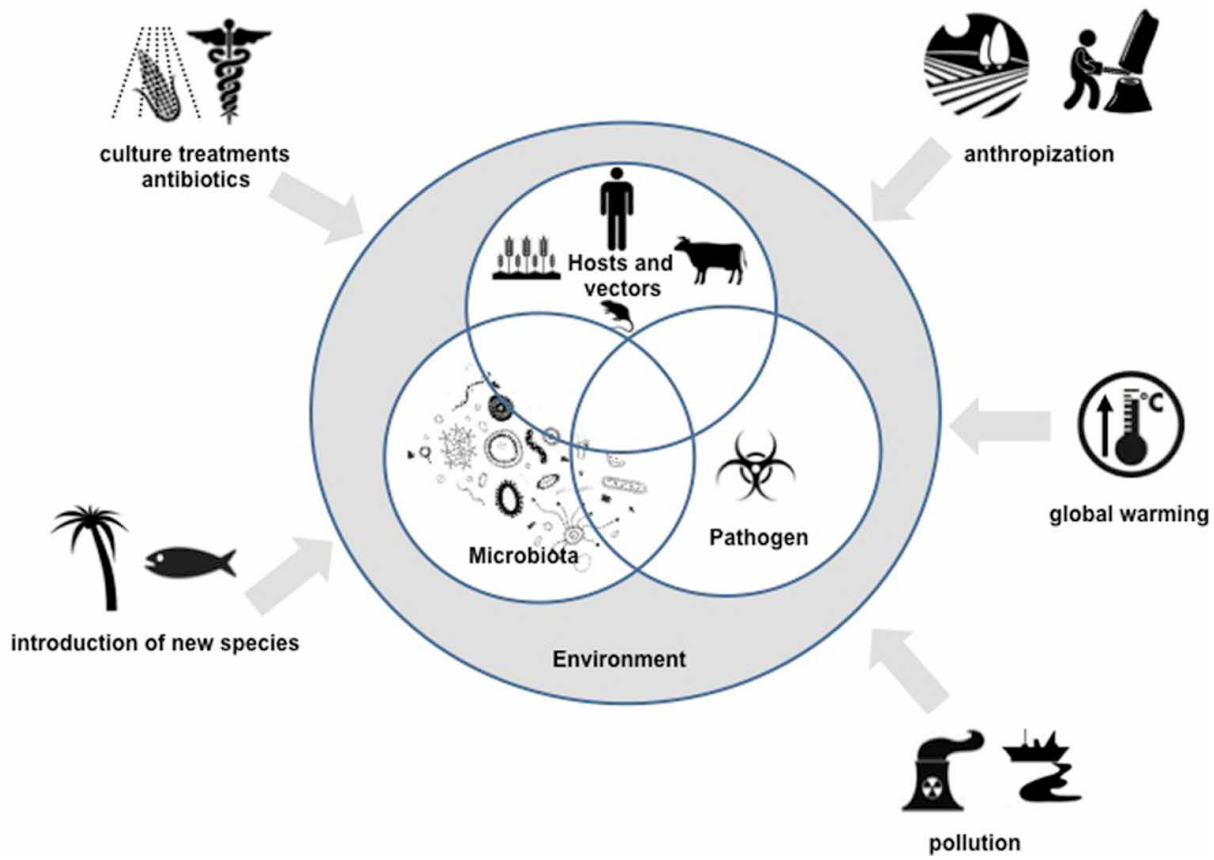

Figure 10: Overview of the pahobiome concept and challenges for the future (Vayssier-Taussat et al., 2014)

The pathobiome concept originated from observations in humans, where a disruption of the stable, health-promoting gut microbiome leads to dysbiosis, a low-diversity community with altered metabolism that allows pathogen invasion and overgrowth and potentially weaken immune function and cause further harm to the hosts (Defazio, Fleming, Shakhsheer, Zaborina, & Alverdy, 2014; Krezalek, DeFazio, Zaborina, Zaborin, & Alverdy, 2016). Unlike a 'normal' community which likely spans a range of structures, a pathobiome refers to a pathogenic group that must be distinguished from healthy baselines. Definitions in the literature vary, from a single pathogen and its surrounding abiotic and biotic environment to the effects of entire microbial communities on host health (Vayssier-Taussat et al., 2015; Sweet & Bulling, 2017). But in the end, an inclusive approach was taken where the pathobiome must include multiple symbionts, spanning bacteria, archaea, eukaryotes, viruses, and phages, that interact with each other and the host. Following the term of pathobiome, symbiome was used

to refer to the full host-associated community (excluding the host) and symbiont for individual members. This aligns with the broad spectrum of symbiosis, from neutralism to mutualism and antagonism, recognizing that interactions may be temporary or long-term (Martin & Schwab, 2013).

Advances in environmental and host-associated DNA/RNA sequencing have uncovered a much greater microbial diversity than previously understood, including protists, bacteria, and viruses found in tissues, gut contents, skin epibionts, water, sediment, and soils. Many of these lineages can become part of pathobiomes as 'cryptic' or emerging pathogens, which include: (i) new clades related to known pathogens; (ii) previously unidentified pathogens from free-living groups; and (iii) opportunists or commensals that shift to pathogenic lifestyles. As our understanding of these complex communities expands, so will our knowledge and definition of what makes a pathogen (Bass, Stentiford, Wang, Koskella, & Tyler, 2019).

The term "**potential pathogen**" will be preferred in this thesis to refer to identified species for which cases of infection have already been reported in the literature, as virulence has not been confirmed genetically or phenotypically in our experiments.

3.3 Contributing Factors of Infectious Diseases

Emergence of infectious diseases is a complex phenomenon influenced by a spectrum of interconnected factors. One primary driver is ecological disruption, often resulting from anthropogenic activities such as deforestation, urbanization, and agricultural expansion, which alter natural habitats and facilitate increased interactions between wildlife, livestock, and human populations (K. E. Jones et al., 2008). Habitat fragmentation can lead to biodiversity loss, reducing the ecological barriers that previously limited pathogen transmission, thus enhancing transmission events of novel pathogens from animals to humans (Keesing et al., 2010). Additionally, climate change significantly contributes to disease emergence by altering the geographical distribution of vectors, pathogens, and hosts. Warmer temperatures and changing precipitation patterns can expand the habitat range of disease-carrying organisms like mosquitoes

and ticks, leading to the spread of vector-borne diseases into previously unaffected areas (Lafferty, 2009).

Globalization and increased international travel further amplify disease emergence risks, enabling rapid pathogen dissemination across continents and exposing naive populations to novel infectious agents (Tatem, Rogers, & Hay, 2006). The speed and scale of modern transportation networks significantly enhance the potential for pandemics, as demonstrated by recent outbreaks like the H1N1 influenza pandemic in 2009 and COVID-19 in 2019 (Morens, Daszak, Markel, & Taubenberger, 2020). Moreover, socioeconomic factors, including poverty, urban overcrowding, and inadequate sanitation, create optimal conditions for infectious disease spread, fostering outbreaks and epidemics, particularly in vulnerable populations lacking adequate healthcare infrastructure (Bloom & Cadarette, 2019).

Human behaviors, notably the consumption and trade of wildlife, significantly elevate zoonotic disease risks. Markets dealing in wildlife, especially those involving live animal sales, present critical points for cross-species pathogen transmission, as observed with SARS and Ebola virus outbreaks (Karesh et al., 2012). Additionally, intensive livestock production and antimicrobial misuse in agriculture have led to increased antimicrobial resistance, complicating disease management and enhancing the potential emergence of resistant pathogenic strains, posing substantial public health threats (Van Boeckel et al., 2015).

Evolutionary pressures, including pathogen adaptation and genetic mutation, play pivotal roles in disease emergence. RNA viruses, in particular, have high mutation rates, facilitating rapid evolution and adaptation to new hosts and environments. This genetic flexibility underscores their significant potential for causing outbreaks when they successfully adapt to human hosts (Parrish et al., 2008). Furthermore, weakened public health systems and inadequate surveillance capabilities hinder early detection and rapid response to emerging diseases, enabling pathogens to spread unchecked and escalate into large-scale outbreaks or pandemics (Morens & Fauci, 2020). Collectively, these factors illustrate that infectious disease emergence is a multifaceted issue requir-

ing integrated, multidisciplinary approaches for effective surveillance, prevention, and control (Figure 11).

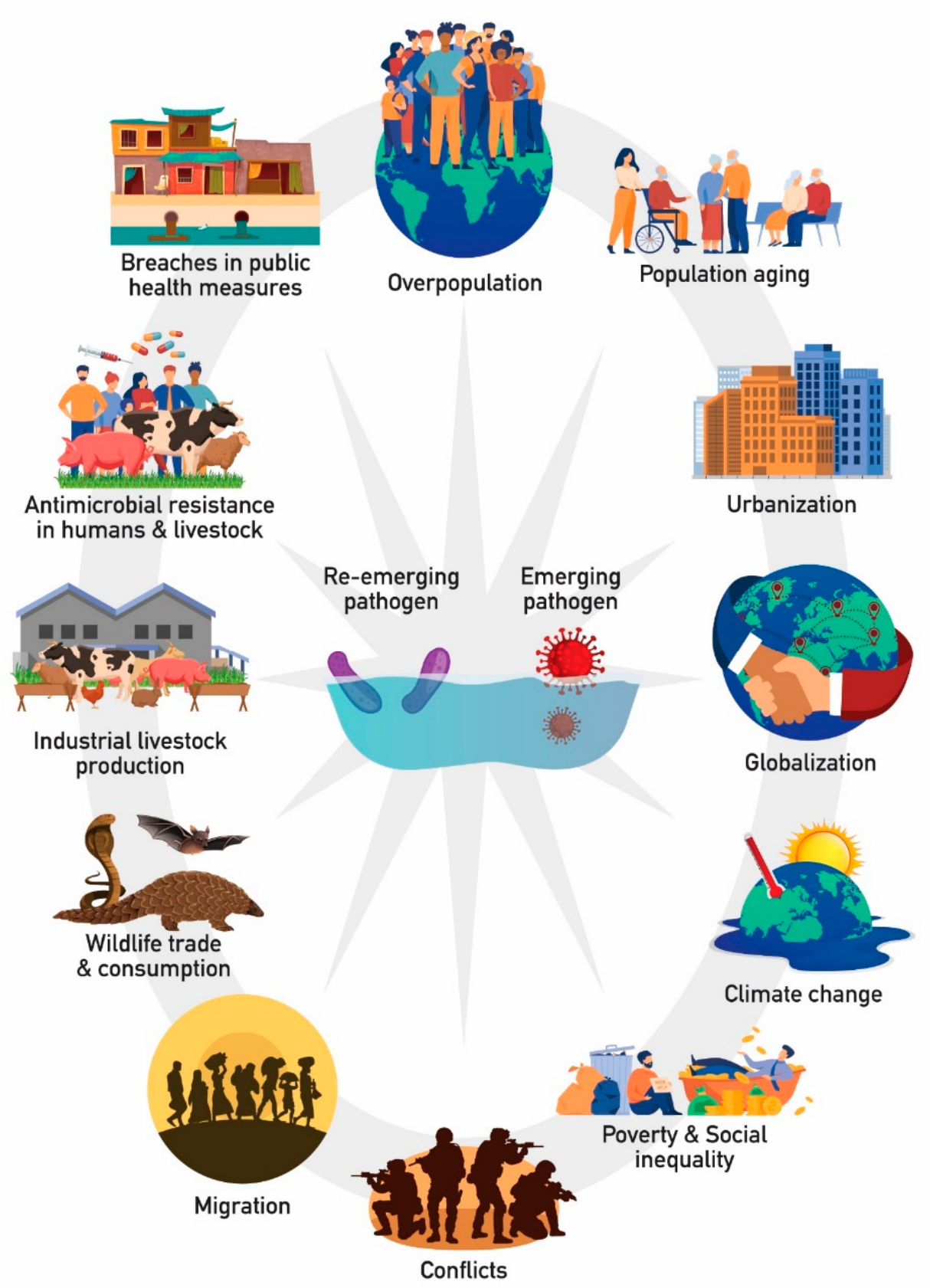

Figure 11: Factors contribute to emergence of infectious diseases (Spernovasilis et al., 2022).

3.4 Pathogens Detection and Characterization Approaches

The precise detection and characterization of pathogenic microorganisms play a vital role in the fields of disease diagnosis, outbreak investigation, and epidemiological surveillance. These processes are essential for understanding the spread and impact of infectious diseases. A variety of methodologies are utilized for these processes, ranging from traditional culture-based techniques, such as bacterial culturing on selective media or Polymerase Chain Reaction (PCR) for DNA amplification, to advanced molecular and sequencing approaches like NGS that allow for comprehensive genomic analysis (Rajapaksha et al., 2019).

Each of these methods has its own strengths and limitations. For instance, culture-based techniques, while being the gold standard for isolating culturable organisms, are time-consuming and unable of detecting microorganisms that are present in low numbers or are difficult to culture (S.-O. Kim & Kim, 2021). On the other hand, molecular techniques offer rapid results and higher sensitivity but may require specialized equipment and expertise. Sequencing approaches provide detailed insights into the genetic makeup of pathogens, facilitating the identification of variants and resistance mechanisms, but they can also be resource-intensive (Sloots, Nissen, Ginn, & Iredell, 2015). As such, the choice of method often depends on the specific context of the investigation, the resources available, and the particular pathogens of interest. Understanding the landscape of these methodologies is critical for improving public health responses to infectious diseases.

3.4.1 Culture-based Methods for Pathogens Detection

Culture-based methods have long been regarded as the gold standard for the detection and identification of pathogens in various environments, including clinical, agricultural, and environmental settings (S.-O. Kim & Kim, 2021). These techniques typically involve the cultivation of microorganisms on selective or differential media, which are specifically formulated to promote the growth of target organisms while inhibiting others. Once the microorganisms have been cultured, they undergo pheno-

typic characterization, where various observable characteristics, such as colony morphology and growth patterns, are assessed. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-ToF MS) has recently become a widely used tool in microbiology for the rapid, sensitive, and cost-effective identification of microorganisms (Singhal, Kumar, Kanaujia, & Virdi, 2015). By analyzing intact cells or cell extracts, MALDI-ToF MS provides quick and accurate microbial identification, making it valuable for applications such as epidemiological surveillance, detection of biological threats, identification of food- and water-borne pathogens, and diagnosis of infections from blood or urine samples. The technology is popular for its speed, low material requirements, and ability to handle diverse sample types. However, it is limited by the need for comprehensive spectral databases, as new isolates can only be identified if reference mass of bacteria are available. Recent advances in software and computer science have further improved the efficiency and depth of analysis, supporting high-throughput workflows that includes MALDI-ToF MS.

Culture-based methods have many advantages, including detection of viable bacteria, isolation of pure cultures with low equipment costs (Figdor & Gulabivala, 2008). One significant advantage of culture-based methods is that it confirms detected organisms are alive and reproductive. This method provide actual living organisms, which can be further characterized for potential infectious capacity, such as antimicrobial resistance or virulence factors. For example, once isolated, pathogens can undergo AST to guide effective therapy (McLain, Cytryn, Durso, & Young, 2016). Moreover, traditional culture-based methods require relatively low cost compared to molecular methods, making them suitable for small laboratories with limited funds.

Despite their established reliability, culture-based techniques come with some notable limitations. The process can be very time-consuming, often from 24 hours up to several weeks to yield results, particularly for slow growing organisms (S. V. Gordon & Parish, 2018). Additionally, these methods can struggle to detect hard-to-cultivate organisms, including bacteria that require specific growth conditions or those classified as Viable-but-non-culturable (VBNC), which may not grow on standard media but can still pose health risks (Ayrapetyan & Oliver, 2016). Furthermore, culture techniques

may exhibit limited sensitivity when dealing with samples where the presence of multiple organisms can complicate detection and identification efforts. These challenges underscore the need for complementary methods that can overcome the inherent limitations of culture-based approaches.

3.4.2 New Generation Sequencing Approach for Bacterial Taxonomic Profiling

Next Generation Sequencing (NGS) technologies, such as 16*S rRNA* gene amplicon sequencing, whole-genome sequencing (WGS), and metagenomic sequencing, have transformed bacterial detection and taxonomic profiling (Besser et al., 2018). NGS allows for the high-throughput identification of both culturable and unculturable organisms, comprehensive characterization of microbial communities, and the discovery of novel pathogens.

3.4.2.1. 16*S rRNA* Gene Amplicon Sequencing

The 16S ribosomal RNA (rRNA) gene is a highly conserved genetic marker present in all prokaryotes, encoding a component of the small subunit of the bacterial ribosome. It is about 1500 base pairs (bp) long contains both conserved and hypervariable regions, making it suitable for phylogenetic studies and bacterial taxonomic classification (Clarridge III, 2004). The conserved segments of the gene provide universal priming sites for PCR amplification, with the nine hypervariable regions (V1–V9) occupied sequences useful for distinguishing between bacterial taxa (Johnson et al., 2019). Because of its slow evolutionary change, and presence in most bacteria, the use of 16*S rRNA* had been first introduced by Carl Woese and George E. Fox in 1977 (Woese & Fox, 1977) and it has become a gold standard for microbial community profiling and taxonomy (Janda & Abbott, 2007).

16*S rRNA* gene amplicon sequencing is a culture-independent technique that involves amplifying specific hypervariable regions of the 16*S rRNA* gene using univer-

sal primers, followed by high-throughput sequencing. Commonly targeted regions include V1-V2, V1-V3, V3-V4, V4, V4-V5, V6-V8, and V7-V9 with the choice of regions affecting outcomes (Abellan-Schneyder et al., 2021). 16*S rRNA* sequencing is typically performed on platforms such as Illumina MiSeq or Ion Torrent, which generate millions of short-read sequences. After amplification and sequencing, the resulting reads are quality-filtered, clustered into Operational Taxonomic Units (OTUs) and Amplicon Sequence Variants (ASVs), and compared against curated bacterial databases (such as SILVA) or pathogenic bacteria databases (PHI-base; Host-pathogen and related species interactions database) for taxonomic assignment (Quast et al., 2012; Wardeh, Risley, McIntyre, Setzkorn, & Baylis, 2015a; Urban, Irvine, Cuzick, & Hammond-Kosack, 2015).

This approach has become a cornerstone of microbial ecology and clinical microbiology, enabling high-throughput, cost-effective profiling of complex microbial communities from diverse environments such as soil, water, the human gut, and built environments (Johnson et al., 2019). 16S amplicon sequencing bypasses the need for cultivation, allowing detection of both culturable and unculturable bacteria. It is commonly used to characterize bacterial diversity, study changes in microbial communities in response to environmental or clinical interventions, and identify bacterial taxa in diagnostic settings (Revetta, Pemberton, Lamendella, Iker, & Santo Domingo, 2010; Patel, 2001; Janda & Abbott, 2007). However, 16*S rRNA* sequencing is highly limited in distinguishing closely related species. The results are also influenced by primer selection, where products of targeted regions sequencing only yield single short-reads of 100–500 nucleotides compared to the entire 1500 base pairs of the gene (Bailén, Bressa, Larrosa, & González-Soltero, 2020).

3.4.2.2. Whole Genome Sequencing (WGS)

Unlike targeted sequencing methods (e.g., 16*S rRNA* gene sequencing), WGS captures all genetic elements, including chromosomal, plasmid, and mobile genetic elements, offering entire nucleotide resolution (Khachatryan et al., 2020). WGS also starts from the extraction of high quality DNA from a pure bacterial culture. Sequencing libraries are prepared and loaded onto high-throughput sequencing platforms such

as Illumina, Nanopore, or PacBio, producing millions of both short or long sequence reads (Phan et al., 2018). These reads are either assembled de novo to reconstruct the entire genome or aligned to a reference genome. High-quality databases, such as NCBI RefSeq, enable robust comparisons for identification and characterization (O'Leary et al., 2016).

WGS has become the gold standard for bacterial identification, strain typing, and outbreak investigation (Quainoo et al., 2017). It can clearly distinguish between closely related strains and reveal microevolutionary changes, such as SNPs, insertions, deletions, and gene acquisitions (Schürch, Arredondo-Alonso, Willems, & Goering, 2018; Sarwal et al., 2022). Additionally, public health agencies are now using WGS to track transmission chains during hospital and community outbreaks, to confirm sources of foodborne illness, and to monitor the spread of antimicrobial resistance (Nadon et al., 2017). Moreover, WGS has also transformed our understanding of bacterial evolution, population structure, and adaptation. It can reveal the presence of novel or emerging pathogens, and, when combined with metagenomic samples, provides insights into the structures and dynamics of complex microbial communities (Forbes, Knox, Ronholm, Pagotto, & Reimer, 2017). Despite its advantages, WGS requires considerable infrastructure, including advanced sequencing platforms, computational resources, and bioinformatics expertise. Even though the time and costs are decreasing, WGS remains less accessible for some laboratories compared to targeted sequencing (16*S rRNA* sequencing). The need of high-quality, up-to-date reference databases are also critical for accurate interpretation of WGS data.

3.4.2.3. Metagenomics

Metagenomic sequencing is a powerful, culture-independent approach that provides insights into the entire microbial community, including bacteria, archaea, viruses, and fungi (Quince, Walker, Simpson, Loman, & Segata, 2017). One of the major advantages of metagenomic sequencing is its ability to identify and quantify microbial taxa with remarkable precision, often at the species and even strain level. This capability significantly surpasses the resolution typically offered by 16*S rRNA* gene sequencing,

which is limited to assessing bacterial communities. Metagenomics can detect not only the most abundant organisms present in a sample but also rare taxa that might otherwise be overlooked. Furthermore, this approach allows for the discovery of novel or unexpected microorganisms, expanding our understanding of microbial diversity and ecology in various environments (Abellan-Schneyder et al., 2021).

Similar to antibiotic resistance genes, metagenomics has also revolutionized our understanding about bacterial pathogenicity by enabling the direct study of virulence genes within complex microbial communities taxa (Belda-Ferre, Cabrera-Rubio, Moya, & Mira, 2011; Gawande et al., 2024). To support metagenomic studies of bacterial virulence, several specialized databases have been developed that curate and annotate known virulence factors from diverse bacterial pathogens. The Virulence Factor Database (VFDB) is among the most widely used, offering a comprehensive, manually curated resource of experimentally verified virulence genes from major bacterial species, complete with functional classification and literature links (B. Liu, Zheng, Jin, Chen, & Yang, 2019). Pathosystems Resource Integration Center (PATRIC) also provides extensive virulence, resistance, and metabolic data (J. J. Davis et al., 2020). Genomic prediction tool like PathoFact enables identification of virulence factors, bacterial toxins, and antimicrobial resistance genes with high accuracy (De Nies et al., 2021). These resources, often used in conjunction with metagenomic analysis pipelines, enhance the detection and interpretation of bacterial virulence signatures in complex samples.

Metagenomics has greatly advanced the field of microbial genomics by enabling the assembly of Metagenome-Assembled Genomes (MAGs) directly from complex samples, without the need for cultivation. This approach allows the reconstruction of partial or near-complete genomes from mixed microbial communities, providing access to the genetic contexts of bacterial taxa, including many pathogenic species that are somtime difficult or impossible to culture using traditional techniques (Parks et al., 2017). Importantly, MAGs also enable the identification and characterization of pathogens with precise accuracy and exploration of genomic contexts such as antibiotic resistance genes, virulence factors, and metabolic pathways (Quince et al., 2017). Furthermore,

the high-resolution of genomic data provided from MAGs can be used for epidemiological tracking, phylogenetic analysis, and enhancing our capacity to monitor and respond to public health threats (Gauthier et al., 2025).

3.4.3 Knowledge Gaps in Pathogenic Curated Datasets

Despite the rapid growth in sequencing technology and the establishment of large reference databases (NCBI RefSeq, SILVA, GTDB-tk, etc. . .), significant knowledge gaps still persist in curated datasets of pathogenic microorganisms (National Academies of Sciences, Medicine, et al., 2025). These gaps can arise from several interconnected issues, such as geographic bias and incomplete metadata, taxonomic discrepancies or contamination, and insufficient data on novel and emerging pathogens (Chorlton, 2024; Vashisht et al., 2023).

Firstly, the majority of reference databases are currently biased toward pathogens from first world or HICs, often ignoring LMICs and other region-specific pathogens (Di Bari et al., 2023). Some zoonotic diseases are often overlooked, even within the category of neglected diseases, especially when they are from the underdeveloped regions. Secondly, multiple databases annotation and classification on the same bacteria can result in inconsistent or outdated taxonomic assignments within reference databases. Synonyms, basionyms, mislabeling, and incorrect lineage assignments can mislead bioinformatics pipelines and downstream analyses, affecting the accuracy of pathogen identification and surveillance (Ceccarani & Severgnini, 2023). Contamination of different species also influence the consistency of reference sequence databases (V. Lupo et al., 2021). The pace of new pathogen discovery also exceeds the ability of databases to curate and include novel genome sequences. As a result, unknown, uncultured, or newly emerging pathogens are often missing, making their detection difficult or even impossible (Deneke, Rentzsch, & Renard, 2017). And last but not least, curated datasets frequently lack standardized and detailed metadata, such as geographic origin, isolation source, or clinical context. This limits comparative analyses, epidemiological studies, and the tracking of pathogen evolution or outbreak sources (Sheldon, Moran, & Hollibaugh, 2002).

These knowledge gaps together can lead to false positives (incorrect identification), false negatives (missed detection of pathogens), or limited information at the species or strain level. Such limitations have significant consequences for infectious disease diagnostics, outbreak investigations, and pathogens surveillance (Bradford, Carrillo, & Wong, 2024; Quainoo et al., 2017). Continuous curation and expansion of pathogen reference databases are crucial to ensure accurate, rapid detection and effective response to infectious disease threats in an increasingly interconnected world (J. M. Marti et al., 2025). To address these gaps, the development of a a comprehensive full-length 16*S rRNA* gene dataset for detecting potentially pathogenic bacteria in humans, fish, and crustaceans has been an important part of this PhD work. This dataset serves as a key component in improving manual curation and developing more accurate automated annotation tools.

4 Tropical Coastal Zones as Hotspots of Microbes Circulation

Coastal areas, are among the most dynamic and biodiverse ecosystems on the planet, encompassing estuaries, mangroves, coral reefs, seagrass beds, and sandy shores (Ray, 1991; Rashid, 2019). These environments function as interfaces between terrestrial and marine habitats, influenced by intense anthropogenic pressures and natural variability. As such, coastal environments can function as transmission foci for Potentially Pathogenic Bacteria (PPB) and/or AMR because of the concentrated aggregations of bacteria from different sources, both marine and terrestrial, where environmental, human, and/or animal related bacteria can coexist, at least temporally. This mixing and the highly altered condition of coastal marine ecosystems (i.e. anthropisation, contaminants, pharmaceutical residue) can affect species interactions, select antibiotic resistance and trigger disease emergences (Patz et al., 2004).

4.1 Coastal Ecosystems under pressure

Coastal ecosystems worldwide offer significant value through various ecosystem services to humanity. Although they cover less than 20% of the land surface, coastal zones currently serve as a crucial source of food (including the majority of croplands

and much of global agriculture, as well as most fisheries and aquaculture practices). They are also centers for transportation and industrial growth, provide minerals and geological resources like oil and gas, are popular tourist destinations, and vital for their rich biodiversity and ecosystems (Crossland & Kremer, 2001; Wong, Boon-Thong, & Leung, 2006; Neumann, Vafeidis, Zimmermann, & Nicholls, 2015). Over the past decade, advances in research and environmental management have significantly expanded our understanding of how global environmental changes and human activities affect coastal zones, shaping their ecology and functions. Among these coastal ecosystems, tropical and temperate coastal regions differ significantly in their climatic characteristics, ecological dynamics, and susceptibility to pressures (Vernberg, 1981; Saenger & Holmes, 2018). Understanding the differences between these coastal ecosystems, especially in tropical zones, is essential to investigate their responses to global warming, sea-level rise, and anthropogenic stressors, particularly in underdeveloped regions like Southeast Asia (Islam & Khan, 2020).

Tropical climates are typically located surrounding the equator and characterized by persistently high temperatures throughout the year. Monthly average temperatures in these regions rarely fall below 18°C, and they often experience distinct dry and wet seasons with annual rainfall ranging between 1,500 and 4,000mm depending on regional geography and topography. Therefore, tropical coastal areas are generally warmer and more humid, supporting ecosystems such as coral reefs, mangroves, and seagrass meadows. Tropical coastal ecosystems are affected by the shifting climate in various ways. Climate change exacerbates these pressures through rising sea surface temperatures, rising sea levels, shift in salinity levels, ocean acidification and intensified storms (Doney et al., 2012; Santojanni, Miner, Hain, & Sutton, 2023).

When examining vulnerability to global warming, tropical coastal zones exhibit a higher degree of ecological and socio-economic exposure. Sea surface temperatures in these areas often remain between 25–30°C throughout the year (Huber & Sloan, 2000). The relatively stable thermal profile supports high biodiversity but also makes these systems particularly vulnerable to even slight temperature changes. One of the most pressing issues is coral bleaching, which occurs when prolonged thermal stress causes

corals to expel their symbiotic algae, leading to loss of color and function. The frequency and severity of coral bleaching events were significant. For example, during the 1982-1983 El Nino, approximately 70% of coral reefs in the eastern Pacific Ocean and the Caribbean Sea were lost due to bleaching, with only around 20% reefs recovered after 20 years (Guzman & Cortés, 2007). Furthermore, increased temperatures due to global warming are also making tropical cyclones more intense. A rise of +2°C in temperature can lead to a maximum wind speed increase of up to 10%, resulting in a greater potential for damage from each storm (Knutson et al., 2021).

Another key environmental concerning climate changes is sea-level rise, driven by global warming and glacial melt. Satellite data over a 20-years period (1993-2014) indicates that the average sea level in the East Sea increased by 4.05 ± 0.6 mm/year, while the average water level in coastal areas of Vietnam has risen by 3.5 ± 0.7 mm/year (Thuc et al., 2016). Due to the elevation of sea level, tropical coastal zones, especially in low-lying delta regions like the Mekong Delta in Vietnam and the Ganges Delta in Bangladesh, are highly susceptible to coastal flooding, increased saltwater intrusion, and damage to coastal infrastructure (Vu, Yamada, & Ishidaira, 2018; Alam, Momtaz, Bhuiyan, & Baby, 2019). The impacts of sea level rise also include permanent inundation of low-lying areas, increased frequency of tides, and cause most sandy beaches to recede and erode. Therefore, all coastal infrastructure will be adversely affected by rising sea levels (Magnan et al., 2022). An additional example of sea-level rise effect on the tropical coastal area is the significant influence on salinization patterns, posing risk to water security and agriculture (Gibson, Barnes, & Atkinson, 2002; Feist, Hoque, Islam, Dewan, & Fowler, 2025).

One of the rising burdens tropical coastal ecosystems are facing is the wide range of human activities, including rapid coastal urbanization and development, intensive agricultural and aquacultural practices, and accelerated tourisms (Culhane et al., 2024). Throughout history, human has always been connected to coastal regions, motivated by trade and abundant resources. Currently, there is a trend of migration from rural areas to urban coastal cities, with many of the world's megacities located along coasts (Barragán & De Andrés, 2015). However, variations in population sizes across regions

can impact the speed and scale of urban development, and possible habitat destruction, and pollution, leading to different effect of coastal biodiversity and habitat health (Mahtta et al., 2022). Tropical coastal areas, especially in underdeveloped regions like Southeast Asia, often struggle with lack of transportation networks, insufficient access to clean water and sanitation facilities. Additionally, poor waste management systems intensify environmental issues, leading to pollution in water streams and beaches that negatively impact both local the communities and marine ecosystems (Herrera-Franco et al., 2024). High population densities in these tropical coastal zones also further complicate the pressures. A research conducted by Hooijer and Vernimmen indicates that in 2020, between 197 and 347 million people lived in coastal regions less than 2 meters above sea level, with 59% located in tropical coastal area in Asia (Hooijer & Vernimmen, 2021). Increasing number of tourists and population growth has also brought substantial changes and immense pressures on the coastal ecosystems. Significant demand for resorts and buildings requires transformation and deforestation of coastal lands (Wong, 1998). Unregulated tourism activities also damage multiple coral reefs in Southeast Asia (Chou, Tuan, Philreefs, Cabanban, & Suharsono, 2002). Moreover, to meet increasing demand of both tourisms and local populations, intensive aquaculture production has negatively impacted the environment, particularly through the conversion of mangroves into aquaculture ponds, altered hydrologic regimes, and increased organic waste in coastal waters. This has led to declining water quality, harming aquaculture production and profitability (Eng, Paw, & Guarin, 1989).

4.2 Pathobiome and Resistome ecology in Tropical Coastal Environments

In general, researches on pathogens and antimicrobial resistance has been mostly focusing on inland systems with comparatively lower efforts directed towards marine habitats. Recently, there has been an increased number of studies indicated that pathogens and antibiotic resistance genes are also prevalent in marine environments. This has shifted the world attention into gaining more knowledge about the pathobiome and resistome ecology in marine environments.

One recent study is a comprehensive review by Zheng and colleagues compiled a total of 145 records regarding antibiotics and antibiotic resistance genes in estuarine and coastal settings around the world (Figure 12) (Zheng et al., 2021). Results indicate that antibiotics and antibiotic resistant genes (ARGs) in coastal marine environments are mainly attributed to human activities including aquacultural practices. Higher antibiotic resistance gene concentrations, notably from tetracycline, fluoroquinolones, aminoglycosides, and MLS were observed in LMICs compared to HICs. This study also raises serious concern about the potential transfer of ARGs from the environment to marine organisms and the human body, highlighting the One Health impacts of long-term antibiotic exposure (Figure 13). Several researches have also proven that aquaculture are hotspots for ARG contamination in estuarine and coastal ecosystems (Gao et al., 2018; H.-Y. Kim, Lee, & Oh, 2017; S. Liu, Zhao, Lehmler, Cai, & Chen, 2017; J. Wu et al., 2019). The high prevalence of ARGs in aquaculture contributes to antibiotics ineffective, and antibiotic exposure alters gut's microbes, increasing the vulnerability of aquaculture species to diseases (S. K. Singh, Ekka, Mishra, & Mohapatra, 2017; Zhao, Liu, Wang, & Li, 2019). This situation has resulted in significant economic losses within the aquaculture sector (Mohamad et al., 2019; Reverter et al., 2020). For humans, especially aquaculture farmers, ARGs can be transmitted through both direct (swimming, diving activities) and indirect (consumption of seafood) interactions with the environment (Figure 13). Another investigation by Cuadrat and colleagues, which examined the abundance and distribution of antibiotic resistance genes in ocean samples, highlighted the necessity of monitoring coastal waters for human impacts, as the influx of antibiotic-resistant bacteria from wastewater may spread ARGs through HGT to environmental microbiome (Cuadrat, Sorokina, Andrade, Goris, & Davila, 2020). Antibiotic classes such as quinolone, bacitracin, and fosmidomycin are suggested to be under selective pressure from the influence of aquacultural practices in coastal zones. The resistomes are also subjected to change and sensitive to seasonal variation, as proposed by a study in surface waters from a coastal lagoon of Southern Brazil under the impact of anthropogenic activities (Leite, Chaves, Nunes, Jank, & Corção, 2019). Similarly, higher rainfall in rainy season was shown to be correlated with the increasing of pathogens concentrations and total ARGs in Newport Beach coastal water (Suarez et al., 2025). It is noteworthy that most studies on the resistome have been conducted in

developed regions of Asia (Figure 12). However, similar research is also underway in less developed areas, such as Southeast Asia, including countries like Malaysia, Thailand, and Vietnam, where aquaculture plays a significant role in people's livelihoods (Siri, Precha, Sirikanchana, Haramoto, & Makkaew, 2023). Another study on Singapore's coastal environments, focusing on marine ecosystems and aquaculture sites, observed low ARGs levels in sea waters but elevated concentrations at aquaculture sites (Q. Yuan et al., 2025). The high abundant of multidrug-resistant pathogens, such as *Acinetobacter baumannii* and *Escherichia coli*, were identified in aquaculture sites, posing serious food safety and public health challenges.

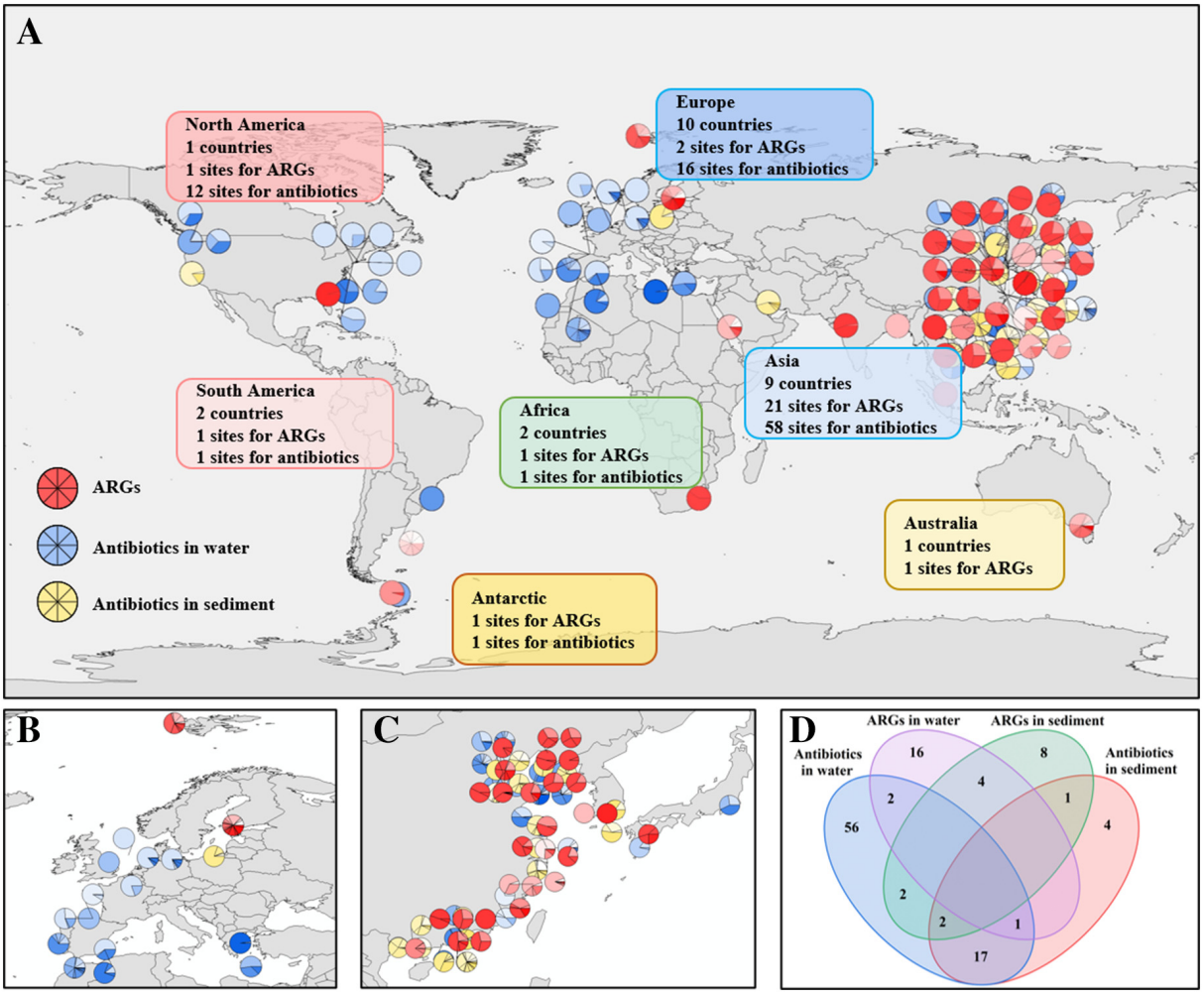

Figure 12: Geographical distributions of antibiotics and ARGs in estuarine and coastal environments of the world (A), Europe (B), and East Asia (C). The number and composition of reported sites on antibiotics or ARGs according to previous studies (D) (Zheng et al., 2021)

A study examines how anthropogenic activity and climate change exacerbate the

spread of bacteria in the environment, detects human pathogenic bacteria from various habitats including animals, plants, soil, and aquatic environments globally (Geng et al., 2025). The identified human pathogenic bacteria were from nine different phyla, with 59% of the species classified under *Pseudomonadota* and *Bacillota*, which include *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. This study also found the diversity and relative abundance of these infections-causing bacteria significantly higher in LMICs compared to those in HICs (Geng et al., 2025). Results of the pathobiome, especially in marine environments of underdeveloped regions provides tremendous insights into marine ecology. Research suggests that approximately 90% of bacteria found in seawater exhibit resistance to one or more antibiotics, while nearly 20% of these bacteria are resistant to at least five different antibiotics, as proposed by Martínez (Martinez, 2003). A recent study highlighted the relationship between the features of microbial communities, pathogenic bacteria, and high-risk antibiotic-resistant genes, focusing on coastal beaches and a variety of hosts (Long et al., 2023). The study detected several pathogens, including *Vibrio cholerae*, *Vibrio vulnificus*, *Campylobacter* spp., *Pseudomonas aeruginosa*, and *Salmonella* spp., as well as carbapenem-resistant *Klebsiella pneumoniae* and key antibiotic resistance genes (ARGs) from the beta-lactamase class (*bla*NDM-6, *bla*NDM-8, *bla*KPC-14, *bla*OXA, *bla*KPC-24), which gathered significant attention. Similarly, a study of potential pathogens causing diseases in marine organisms observed multiple pathogenic bacterial genera such as *Vibrio*, *Pseudomonas*, and *Burkholderia* from *Gammaproteobacteria* (38%); *Mycoplasma*, *Staphylococcus*, and *Corynebacterium* from *Firmicutes* (8%); and *Aquimarina*, *Prevotella*, *Arcobacter* and *Campylobacter* from *Bacteroidetes* (7%) (Jurelevicius et al., 2021). Among these potentially pathogenic bacteria, several genera are capable of affecting multiple marine organisms include *Vibrio*, *Pseudomonas*, and *Nocardia*. Additionally, antibiotic resistance genes (ARGs), specifically *amp*C MOX and *amp*C from class C beta-lactamases, were also presented in all samples from the study. These results indicates that natural marine environment has emerged as a reservoir for many potentially pathogenic bacteria. Moreover, several studies conducted on microplastics in marine environments highlight the specific pathogens composition of plastic biofilms. Due to their persistence and mobility, plastics can serve as vehicles and act as fomites for harmful organisms in the oceans. The presence of high abun-

dant pathogens such as *Arcobacter* spp., and particularly *Vibrionaceae* family, including *Vibrio alginolyticus*, serves as strong biomarkers of the plastisphere compared to seawater samples. This observation also raises health risks associated with microplastics in tropical coastal environments (Curren & Leong, 2019; Naudet, d'Orbcastel, Bouvier, & Auguet, 2025).

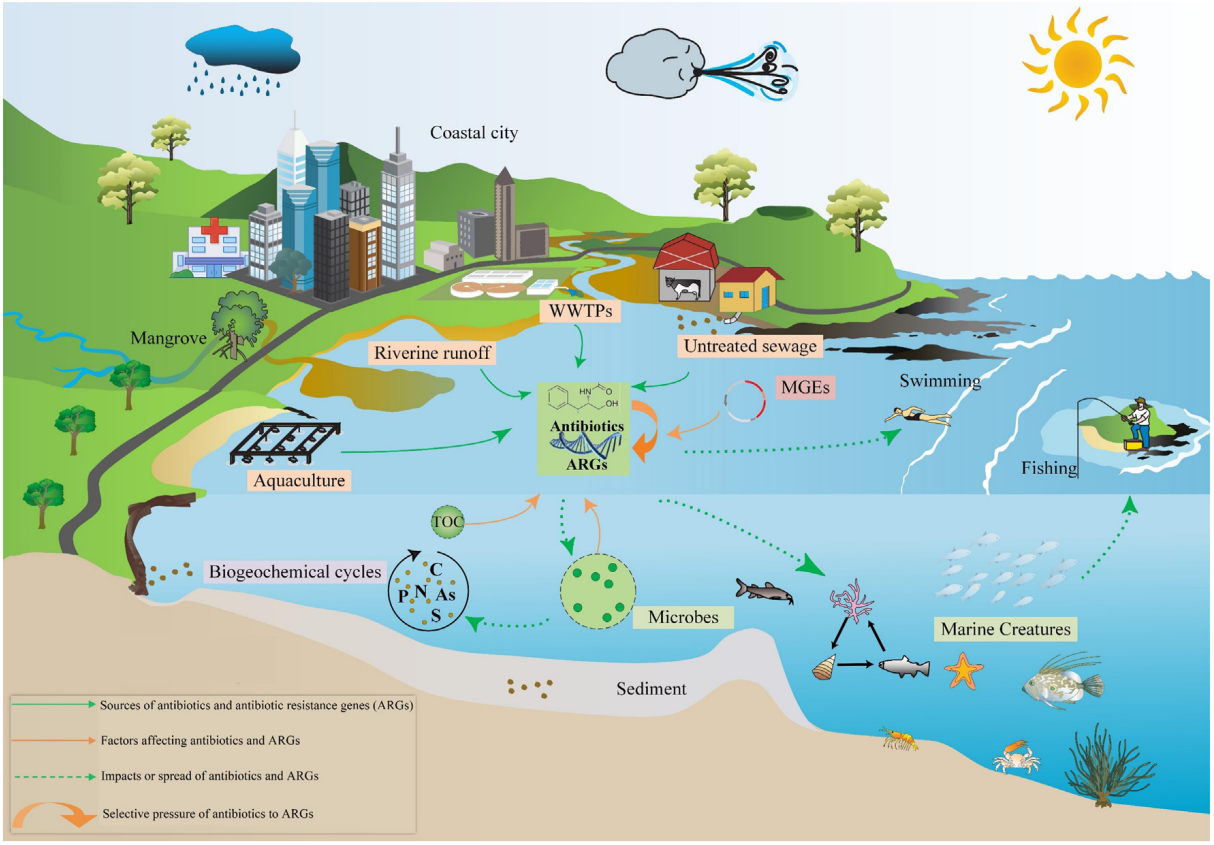

Figure 13: The transport of antibiotics and antibiotic resistance genes (ARGs) in estuarine and coastal environments is influenced by river runoff, coastal wastewater treatment plants, aquaculture effluent, and sewage discharge. Environmental factors and mobile genetic elements affect the abundance of antibiotics and ARGs, creating selective pressure on microbes that leads to ARG enrichment. This disrupts biogeochemical cycles and microbial communities, posing health risks to marine life and potentially transferring to humans through activities like swimming, fishing, or dietary intake (Zheng et al., 2021)

The risk of infection and/or AMR spread linked to the marine habitat or marine products is a major world health challenge. Evidence for microbial and AMR dispersal mechanisms between the different marine and terrestrial biomes have been found in a number of studies (Cabello et al., 2013; Michaud et al., 2018; Nielsen, Wang, & Jiang, 2021). In particularly, a study by Shuval in 2003 estimated 120 million people

contracted gastroenteritis and 50 million developed a severe respiratory infection after having swum or immersed themselves in seawater, resulting in an estimated economic loss of some 12 billion dollars (Shuval, 2003). Similarly, livestock mass mortalities due to the common burden of infectious diseases and AMR challenge the ability of aquaculture (an increasing source of dietary protein worldwide) to feed the growing human population (Leung & Bates, 2013). A co-occurrence network analysis of environmental bacteria in aquatic systems revealed high antibiotic resistance profiles primarily in genera such as *Pseudomonas* and *Vibrio* and antibiotic resistance genes such as *bla*TEM, *sul* and *intI* (Saravanakumar et al., 2023). For virulence factors, 16 genes were also assessed, including adhesion, invasion (*las*B, *nan*I and *inv*A), toxin (*tox*A, *eta*, *ctx*A, *trh*, *tdh*, *hly*A, *stx*) and OVGs (*apr*A and *plc*H), with co-occurrence of antibiotic resistance and virulence found in 43.3% of coastal water samples. The direct transmission of human or marine livestock pathogens from the marine habitats is therefore not negligible but a major risk to public health and aquaculture production is assumed to be the development of a reservoir of resistance genes transferable to human or marine livestock pathogens and the emergence of pathogens with new acquired antibiotic resistance (Heuer et al., 2009; Cabello et al., 2013; Ashbolt et al., 2013). Overall, understanding the ecological dynamics of the pathobiome and resistome in tropical coastal ecosystems is essential for predicting and mitigating the emergence of infectious diseases and spread of AMR, safeguarding biodiversity, and supporting the sustainability of ecosystems. However, although likely significant, health risk related to the marine pathobiome and resistome has not been fully investigated, mainly because the inventory of potential pathogens that occur in marine habitats is astonishingly incomplete.

4.3 Aquaculture Zones as Interfaces of One Health Risks

As Earth's population continues to grow rapidly, the demand for increased agricultural productivity rises accordingly. While agricultural advancements have consistently aimed to improve human well-being, they often come with unintended consequences for the health of humans, animals, and the environment. Aquaculture is one of the most fast-expanding sector of animal agriculture and is forecasted to provide the majority of global food security particularly in tropical and coastal regions by the

year 2050 (Food & Organization, 2022). In this context, aquaculture provides nearly half of the world's total fish amount (Thornber et al., 2020; Nafiqoh et al., 2020), with a production valued at over US$250 billion and a sector forecasted to grow by 62% at the horizon in 2030 (Tacon, 2020). Over 80% of global aquaculture production is currently depended on developing countries, with ASEAN countries accounting for 15% of the production (Phillips, Subasinghe, Kassam, et al., 2016; Chan et al., 2017).

A fundamental One Health risk in aquaculture is the overuse and often misuse of antibiotics for disease prevention and growth promotion. Antibiotics are frequently added to feed or water in intensive fish and shrimp farms, sometimes without veterinary oversight (Hedberg et al., 2018). This practice creates strong selective pressure for the emergence and proliferation of antimicrobial-resistant bacteria (ARB) and the horizontal transfer of antibiotic resistance genes (ARGs) among aquatic microbial communities. Moreover, these resistant bacteria and genes are not only contained within the aquaculture ponds/cages, but also can spread to wild aquatic populations, contaminate seafood products, and affect humans directly through occupational exposure or indirectly via the food chain (Watts, Schreier, Lanska, & Hale, 2017). Furthermore, aquaculture environments are not only reservoirs of ARGs and mobile genetic elements, but also hotspots for the transmission of infectious diseases (E. Marti et al., 2014; Mordecai et al., 2021). Pathogenic bacteria, viruses, and parasites can proliferate in densely stocked aquatic farms, where high animal densities, poor water quality compromise animal health and increase susceptibility to infection (A. G. Murray & Peeler, 2005). Another problematic aspect of antimicrobial resistance is the identification of macroplastic pathobiomes and antibiotic resistance in aquaculture in tropical coastal environments (Naudet et al., 2023, 2025). Since the majority of equipment utilized in aquaculture farms is composed of plastic, biofilms associated with plastics might harbor Potential Human Pathogenic Bacteria (PHPB) and ARB such as *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Photobacterium damselae*, and *Staphylococcaceae* species. Several other potentially pathogenic genera (*Sulfurovum*, *Acidiferrobacter*, *Maritimimonas*), bacteria that can degrade polycyclic aromatic hydrocarbons, and bacteria linked to antibiotic resistance have been found on various microplastics (e.g., polyvinyl chloride, polyethylene, polypropylene, and polystyrene) (De Tender et al., 2017). These

studies have shown that microplastics can also act as carriers for potentially pathogenic bacteria (Jiang et al., 2018), and can transfer pathogens attached to microplastics especially to marine organisms in aquaculture farms after they are ingested (Viršek, Lovšin, Koren, Kržan, & Peterlin, 2017).

Socio-economically, One Health risks in aquaculture are often most acute in LMICs), where regulatory oversight, biosecurity, and access to veterinary care may be limited (Food & Organization, 2022). The lives of small-scale farmers, fishers, and coastal populations are at risk when disease outbreaks or AMR spread render farming unsustainable or reduce market access due to food safety concerns. Addressing the One Health risks of aquaculture zones requires coordinated, multisectoral strategies. These include regulated antimicrobial use, disease management practices, and improved farm biosecurity and waste management (S.-O. Kim & Kim, 2021). Public education, farmer training, and international cooperation are also vital for ensuring that One Health principles are mainstreamed into aquaculture policy and practice. As aquaculture continues to expand, adopting a One Health perspective is essential to balance food production needs with the health of people, animals, and the environment. Despite the discovery of evidence that ARGs could transfer from livestock farms to the human body (X. Sun et al., 2020), our understanding regarding ARGs transmission from estuarine and coastal environments to humans is still lacking.

4.4 Vietnamese Floating Farms: A Case Study

Antimicrobial resistance (AMR) in Vietnam has emerged as a critical public health issue, with studies consistently indicating high prevalence and rising trends of resistant pathogens. A study has demonstrated notably high resistance rates among key bacterial pathogens such as *Escherichia coli* and non-typhoidal *Salmonella*, particularly against critically important antibiotics (Nhung, Phu, Carrique-Mas, & Padungtod, 2024). Also, Vietnam has the highest prevalence of *Streptococcus pneumoniae* resistant to penicillin (74.1%) and erythromycin (92.1%) (J.-H. Song et al., 2004). Several factors have contributed significantly to the rise and persistence of pathogens and AMR in Vietnam and among them is the lack of access to clean and drinkable water. Ur-

ban and industrial wastewater are the largest contributor to water pollution, with only 12.5% of municipal wastewater treated before discharge into water bodies. Much industrial wastewater is discharged without pre-treatment, damaging the environment. By the end of 2018, it was estimated that roughly 71% of industrial wastewater was being processed by centralized wastewater treatment facilities (Chinh, 2018). As a consequence, the Ministry of Natural Resources and Environment estimates that up to 80% of diseases in Vietnam is directly caused by water pollution. It is without doubt that agriculture has a large burden on antimicrobial resistance spread in Vietnam. Antibiotic use is widespread, with estimates indicating that about 72% of Vietnam's total antibiotic usage is attributed to animal husbandry, primarily pigs and aquaculture (Carrique-Mas, Choisy, Van Cuong, Thwaites, & Baker, 2020). Farmers use between 5-7 times higher levels of antibiotics to raise a chicken than in Europe and most antimicrobial usage (84%) aims at disease prevention rather than treatment (Carrique-Mas et al., 2015). The presence of major resistant pathogens has been documented across agricultural and environmental settings. Monitoring programs from 2017 to 2022 have further confirmed extensive antimicrobial resistance in animal-derived pathogens, notably *Escherichia coli* and *Salmonella*, originating from pig and poultry farms where small-scale farmers commonly resort to prophylactic antibiotic applications without professional veterinary guidance (Cuong et al., 2019; Phu et al., 2022).

In Vietnam, the aquaculture sector experienced a mean annual growth of 12.77% over the period 1995-2018 and in 2018 the overall aquaculture production represented 4.15 million tons, reporting Vietnam as the fourth most important aquaculture producer worldwide (Tri, Tu, Van Tu, et al., 2021; Tacon, 2020). However, many aspects of marine aquaculture production and waste treatment systems in Vietnam may facilitate the transmission of pathogens and the spread of AMR in coastal ecosystems. These issues include the prevalence of mixed-species farms, inadequate biosecurity measures, poor sanitary conditions on floating farms, the frequent misuse of antibiotics, often for prophylactic purposes, and a lack of infrastructure for wastewater treatment plants. The extensive use of antibiotics to combat or prevent pathogen spread, or as substitutes for proper hygiene on farms, poses a significant health risk associated with intensified aquaculture (Carrique-Mas et al., 2015; Hedberg et al., 2018). Between 2014 and

2015, for instance, nearly 32,000 tons of aquaculture products exported from Vietnam were rejected and returned due to antibiotic concentrations exceeding permitted limits. Bacterial contamination and veterinary drug residues were among the top reasons for Vietnamese fisheries products being rejected in United States, European and Japan markets (Quyen, Hien, Khoi, Yagi, & Karia Lerøy Riple, 2020). Another example in 2011, an estimated 106 tons of antimicrobials were used in Vietnam to produce 1.14 million tons of *Pangasius* catfish, resulting in 29 tons of active compounds released into the Mekong Delta (Rico et al., 2013). Catfish microbiotas have also been reported to act as a reservoir of antibiotic resistance from which MGEs can readily be transferred intra and interspecies, including foodborne pathogens such as *Salmonella* spp. (Budiati et al., 2013).

Floating sea-cage is one of the most popular aquacultural technique utilized widely in Vietnam. Various types of cages, including fixed, floating, and submerged, are utilized for lobster farming. In 2010, there were 51,797 lobster cages, which rose to 56,942 units by 2015 (of Agriculture & Development, 2015). Therefore, lobster production saw an increase, rising from 1,397 tons in 2010 to 1,657 tons in 2015. Additionally, between 2010 and 2015, the number of marine fish cages also increased significantly, growing from 30,031 units in 2010 to 172,119 units by 2015. The main cultured fish species in these floating cage settings include cobia (*Rachycentron canadum*), grouper (*Epinephelus species*), Asian sea bass (*Lates calcarifer*) and pompano (*Trachinotus species*). Most small-scale marine farms in Vietnam use traditional wooden cages, while the industrial-scale farms tend to use floating, circular, high-density polyethylene (HDPE) net cages. The construction of wooden cages is straightforward, featuring standard rectangular dimensions of 4 by 4 meters. These cages are frequently grouped into "rafts" consisting of four or more units. Temporary shelters can be built on the rafts to facilitate daily activities of the farmers. These setups are very appropriate for family-run operations, and the cages can only be placed in the sheltered bays on the calm sides of the islands. A very informative and comprehensive study from Hedberg had covered the usage of antimicrobial substances in Vietnamese floating cage in aquaculture (Hedberg et al., 2018). This study reported that many farmers did not know the name and/or the dose of the antibiotics incorporated in fish and lobster feeds used. The study addressed

another problem where four "Critical for human use" antibiotics, seven "Highly important" antibiotics, and only one "for Veterinary use only" antibiotic, as ranked by WHO, were used in the farms setting for animals production (Hedberg et al., 2018). Regulations on the use of antibiotics in aquaculture have existed in Vietnam since 2002 (Van Tai, 2012), with several substances banned or controlled since then (Table 1), mainly due to import restrictions from export markets. This has been relatively effective for export products like shrimp and *Pangasius* (Pham et al., 2015). However, enforcement of these regulations remains weak in small-scale cages farming, where most products are for local consumption.

Table 1: Antimicrobials used in marine aquaculture cage farms in Vietnam (Hedberg et al., 2018)

Active substance	WHO classification	Fish (n=81 of 109 farms)				Lobster (n=45 of 109 farms)			
		% farm using	mean (g/ton)	± sd (g/ton)	avg. (g/ton)	% farm using	mean (g/ton)	± sd (g/ton)	avg. (g/ton)
Ampicillin Trihydrate*	Critically important	1.2	145	NA	1.8	0	0	0	0
Chloramphenicol**	Highly important	0	0	0	0	2.2	193	NA	4.3
Ciprofloxacin HCl***	Critically important	0	0	0	0	2.2	197	NA	4.4
Colistin Sulfate*	Critically important	0	0	0	0	11.1	72	±250	8.0
Doxycycline	Highly important	0	0	0	0	2.2	78.8	NA	1.8
Enrofloxacin***	Veterinary use only	0	0	0	0	11.1	48	±328	5.3
Nifuroxazide = Nitrofuran**	Important	0	0	0	0	24.4	1152	±1074	281
Oxytetracyclin HCl*	Highly important	0	0	0	0	11.1	72	±250	8.0
Rifampicine	Critically important	0	0	0	0	11.1	72	±250	8.0
Sulfamethoxazole	Highly important	3.7	10401	±367	385	28.8	9255	±15117	2665
Sulfadimidine	Highly important	3.7	17	±0.6	0.6	0	0	0	0
Tetracycline*	Highly important	7.4	1233	±174	91	26.6	5268	±4902	1403
Trimethoprim*	Highly important	7.4	1041	±147	77	28.8	2443	±3396	705

* approved for limited use in Vietnam (Van Tai, 2012).
** banned for aquaculture use since 2005 (Van Tai, 2012).
*** banned for aquaculture use since 2012 (Rico et al., 2013).

Such intensive and often unregulated use of antimicrobial drugs in aquaculture, combined with land-derived contamination into watercourses, not only threatens local productivity by reducing drug efficacy against pathogens, but also directly contribute to the selection, emergence and spread of antimicrobial resistant (ARB) bacteria and antibiotic resistance genes (ARGs) in surrounding ecosystems, one of the major threats of the twenty-first century according to the WHO (Thornber et al., 2020; Reverter et al., 2020). Crucially, it poses serious risks to human health through direct contact, environmental exposure, or the consumption of contaminated animal products, including

exported seafood. We therefore hypothesize that Vietnam faces a high health risk for human and marine livestock (i.e. fish and crustaceans) related to aquaculture practices and their influence on the circulation and spread of antibiotic microbial resistances and pathogens in floating farm socio-ecosystems. However, although likely significant, this health risk has not been fully investigated, mainly because i) our knowledge of aquaculture practices, particularly the compliance to antibiotics use regulations, and the perception of the related risk, is low, ii) the influence of these practices on the variability of the surrounding environmental, animal and human pathobiome and resistome is unknown, iii) the inventory and dynamics of PPB and ARG circulating between the marine habitats, humans and the marine livestock is poorly documented.

5 Objectives of the study

This introduction has shed light on the impacts of infectious diseases and antimicrobial resistance, emphasizing their harmful consequences for humans, animals, and the environment. We also pointed out that while research on Infectious Diseases (IDs) and AMR has grown significantly, it has largely concentrated on inland ecosystems, with far less attention given to marine environments. Coastal marine environments in particular can act as hubs for the transmission of PPB and AMR due to the convergence of bacteria from diverse marine and terrestrial sources. The co-occurrence of human, animal, and environment-associated microbes, combined with anthropogenic pressures such as pollution and pharmaceutical residues, can alter microbial interactions, promote antibiotic resistance, and facilitate the emergence of new diseases. The spread of infections and antimicrobial resistance (AMR) through marine environments and seafood represents a significant global challenge for public health and food security. However, critical knowledge gaps remain, limiting our ability to fully assess this risk. This includes an incomplete inventory of marine pathogens, limited knowledge of antibiotic use and risk perception in aquaculture, unclear impacts of these practices on surrounding microbial communities, and insufficient evidence on the transmission of PPB and ARGs between marine environments, farmed animals, and humans.

Addressing key questions, the three objectives (Figure 14) of this thesis aim to bet-

ter understand the health risks related to marine pathogens and AMR in contrasted areas of the Vietnamese coast through an interdisciplinary framework integrating ecological, microbiological, and social knowledge.

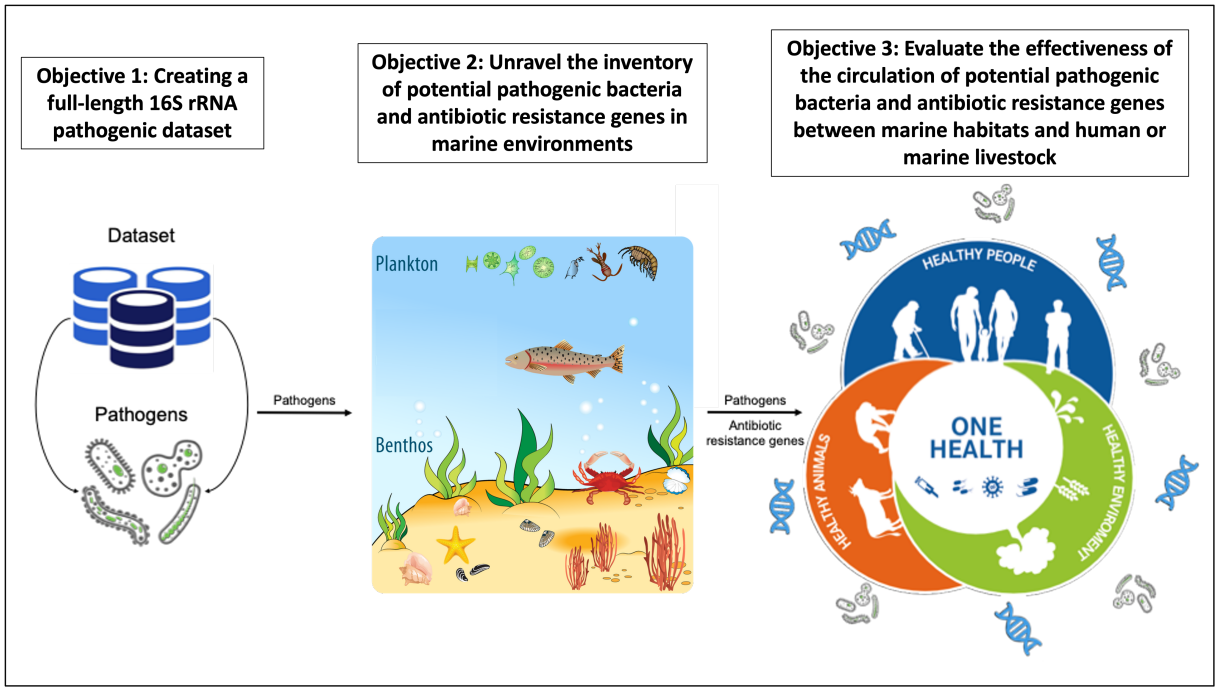

Figure 14: Main objectives of the thesis

The first chapter of the thesis focuses on developing a full-length 16*S rRNA* gene dataset targeting pathogenic bacteria. We first constructed a dedicated reference dataset (NEMESISdb - Tran et al., in minor revision) to enable the identification and tracking of potentially pathogenic bacteria (PPB) in humans, fish, and crustaceans. This dataset was then tested using data from the literature on marine holobionts in Mayotte to identify and characterize potential human pathogenic bacteria (PHPB). The results of these analyses are presented in the form of two scientific articles, both currently under review.

The second chapter investigates the presence of both potentially pathogenic bacteria and antibiotic resistance genes (ARGs) in various environmental compartments. This objective comprises two main analyses. The first examines the environmental drivers of phenotypic and genetic resistance within the microbiome of the anthropogenically impacted bay of Nha Trang, Vietnam. The second combines phenotypic

and genetic approaches to assess the occurrence of multidrug-resistant (MDR) *Enterobacteriaceae* and ARGs in the same region. These analyses are detailed in two scientific articles, with one currently under review, the other already published.

The final chapter aims to evaluate the circulation of PPB and ARGs between marine environments and human or aquaculture hosts. This work, presented in Chapter 3, takes the form of a scientific article that explores the longitudinal dynamics of the pathobiome and resistome within the floating farm socio-ecosystems of Nha Trang Bay.

Chapter 1: Development and utilization of a full length 16*S rRNA* gene dataset for the detection of human, fish, and crustacean potentially pathogenic bacteria

Foreword

Large-scale detection of potentially pathogenic bacteria (PPB) utilizing 16*S rRNA* gene is implemented by recent developments in high-throughput sequencing. However, there are still few tools available that can be used to accurately identify PPB between human, marine animal, and environmental compartments within a One Health framework. Existing datasets often focus on a particular host group, lack of taxonomic consistency, or only include incomplete 16*S rRNA* regions, making cross-system comparisons difficult.

Therefore, the first Chapter of this thesis focuses on designing a curated full-length dataset dedicated to bacterial 16*S rRNA* genes for identification of potentially pathogenic bacteria to humans, fish and crustaceans.

The first objective in this chapter is to develop three curated full-length 16*S rRNA* reference datasets for human, fish, and crustaceans. These datasets support both short- and long-read sequencing across variable regions of the 16*S rRNA* gene and increase the accuracy of PPB identification. The development of the NEMESISdb is submitted as a scientific paper, currently under minor review (Chapter 1-1).

Afterward, the NEMESISdb was also tested on molecular data obtained from the literature in Mayotte to identify and characterize potential human pathogenic bacteria (PHPB) between marine holobionts and the water and sediment of a coral reef ecosystems. The results of this study is presented in the form of a scientific article (Chapter 1-2), also currently under review.

This study is under review by the journal Data in Brief

NEMESISdb: a full length 16*S rRNA* gene dataset for the detection of human, fish, and crustacean potentially pathogenic bacteria

Son-Hoang Tran[1,2,4], Claudia Ximena Restrepo-Ortiz[3], Quang Vu Dinh[2], Marc Troussellier[1], Yvan Bettarel[1], Thierry Bouvier[1], Van Ngoc Bui[4], Nguyen Hieu Minh[5], Trung Du Hoang[5], Quang Huy Nguyen[2], Jean-Christophe Auguet[3],

[1]UMR MARBEC, Univ Montpellier, CNRS, Ifremer, IRD, Montpellier, France;

[2]University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Hanoi, Vietnam;

[3]UMR MARBEC, Univ Montpellier, CNRS, Ifremer, IRD, Sète, France;

[4]Institute of Biology (IB), Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Hanoi, Vietnam;

[5]Institute of Oceanography, Vietnam Academy of Science and Technology (VAST), 01, Cau Da, Nha Trang, Khanh Hoa, Vietnam

1 NEMESISdb: a full length 16*S rRNA* gene dataset for the detection of human, fish, and crustacean potentially pathogenic bacteria

NEMESISdb is a 16*S rRNA* full length sequence curated dataset designed to enable the identification and tracking of potentially pathogenic bacteria (PPB) for human, fish, and crustacean hosts. It addresses the limited focus on marine and coastal environments as key reservoirs for PPB, where bacteria from diverse sources—terrestrial, marine, and animal—can coexist. Leveraging recent advances in high-throughput sequencing, NEMESISdb provides a robust resource for the detection of PPB in 16*S rRNA* gene metabarcoding or metagenomic data. The database comprises three datasets corresponding to human, fish, and crustacean hosts, containing 1757, 223, and 65 PPB species, respectively, with a total of over 435,000 16*S rRNA* full length sequences curated for accuracy. This resource was constructed by extracting sequences from the SILVA database, refining them through a rigorous curation pipeline to ensure taxonomic consistency and eliminate misclassifications. The resulting datasets are optimized for use with popular tools such as BLAST and classifier software, enabling rapid and accurate detection of PPB in metabarcoding and metagenomic data. NEMESISdb supports diverse applications, including pathogen surveillance in aquatic ecosystems, studies on environmental factors influencing PPB dynamics, and the development of targeted strategies for mitigating pathogen impacts in aquaculture. Additionally, it facilitates research within the One Health framework by linking the circulation of PPB across environmental, animal, and human compartments.

1.1 Specifications table

Subject	Microbiology
Specific subject area	Full length 16*S rRNA* gene sequences pathogenic bacteria dataset
Type of data	Information table, Pathogens lists, Filtered fasta files, Python scripts
Data collection	We constructed a list of pathogenic bacteria for humans, fishes, and crustaceans from various studies and pathogen detection pipeline such as 16SPIP, FAPROTAX, MPD and MBPD. Afterward, full length 16*S rRNA* gene sequences of each of the pathogenic bacteria of the list was downloaded from the Silva 138.2 SSU Ref bacterial database in order to obtain three pathogenic reference datasets for humans, fishes, and crustaceans, respectively. Lastly, each dataset was curated with homemade scripts to remove all sequences wrongly assigned at the species taxonomic level in Silva.
Data source location	Raw data for the construction of the pathogen list came from (T. Zhang et al., 2018), (A.-N. Zhang et al., 2021), (Wardeh et al., 2015a), (Blauwkamp et al., 2019), (Urban et al., 2015), (Louca et al., 2016), (Miao et al., 2017) and (X. Yang et al., 2023). Full length 16*S rRNA* gene sequences of each of the pathogenic bacteria of the list were downloaded from the Silva 138.2 SSU Ref bacterial database.
Data accessibility	Repository name: Zenodo. Data identification number: 10.5281/zenodo.13937699. Direct URL to data: `https://doi.org/10.5281/zenodo.13937699`
Related research article	None

1.2 Value of the data

• The NEMESISdb is a set of three curated 16*S rRNA* full length sequence datasets enabling the identification and tracking of potentially pathogenic bacteria (PPB) across human, fish and crustacean hosts and helping reveal factors that influence their dynamics.

• The NEMESISdb can be directly and easily used in blast or in classifier softwares for fast detection of PPB in 16*S rRNA* gene metabarcoding or metagenomic data.

• NEMESISdb could benefit a wide range of stakeholders involved in diseases outbreak prevention and food security (e.g. health agencies, aquaculture and fisheries industries), biodiversity conservation and pathoecology (e.g. researchers and environmental monitoring organizations) and coastal management (e.g. policy makers).

• These datasets can be utilized and reused in several ways to provide further insights in pathogen surveillance by monitoring the dynamics and hotspot of PPB in aquatic environments, in comparative studies aiming to investigate how environmental factors influence pathogen diversity and abundance, in targeted interventions and mitigation strategies by guiding aquaculture management practices, to reduce pathogen impact and in the framework of One health studies by facilitating the identification of PPB circulating within the environmental, animal and human compartments.

1.3 Background

Most research on infection diseases has focused on inland systems with comparatively little efforts directed towards marine habitats. However, marine and particularly coastal environments can function as transmission foci for PPB because of the concentrated aggregations of bacteria from different sources, both marine and terrestrial, where environmental, human, and/or animal related bacteria can coexist. Recent breakthroughs in high-throughput sequencing technologies now allow for the detection of PPB on an unprecedented scale using 16*S rRNA* gene sequencing. However, the dataset needed to precisely identify PPB circulating among the human, marine environment and marine animal compartments in a one health framework remain largely underdeveloped. Here, we constructed NEMESISdb, a set of three curated 16*S rRNA* full length sequence dataset allowing the use of both, long reads sequencing and short read sequencing under different 16*S rRNA* gene variable regions, to accurately detect PPB. NEMESISdb is a convenient tool for the rapid identification of human, fish, and crustacean PPBs in next generation sequencing (NGS) data, supporting key areas such as food safety, epidemic prevention in both livestock and humans, disease detection, and environmental surveillance.

1.4 Data description

The NEMESISdb available with the following link: `DOI10.5281/zenodo.13937699`, is composed of 13 files containing respectively, the three full length 16*S rRNA* gene sequences of human, fish and crustacean datasets in fasta format, three tab-separated text files containing the list of genus-species pairs of PPB used to create each dataset, an excel file containing the information on the sources used to build the dataset, an excel file for each group, in which taxonomic synonyms are given, a respective excel per group with the list of species that compose the curated database and their corresponding synonyms found and a github repository containing the PathoLens python package used to create and curate the databases.

The three files, HUMAN_Pathogen_DB.fasta, Fish_Pathogen_DB, CRUSTACEAN_Pathogen_DB.fasta contain the full-length 16*S rRNA* gene sequence of PPB for humans, fishes and crustacean respectively. Headers of each sequence within the fasta files correspond to the ACC number followed by the Silva taxonomy of the sequence from the kingdom to the species level. The datasets contain 34 481, 80 761 and 196 770 16*S rRNA* gene sequences with an average length of 1150.7 bp, 1151.8 bp, and 1158.8 bp, respectively for crustaceans, fishes and human (Table 1.1. This number of sequences encompasses 65, 223 and 1757 species of PPB for crustaceans, fishes and human, respectively.

Table 1.1: Summary of the dataset of potentially pathogen bacteria for different hosts

	Crustacean	Fish	Human
Species	65	223	1757
Sequences	34,481	80,761	196,770
Length's mean	1150.674	1151.832	1158.806
Length's sd	63.590	66.255	65.689

Overall, PPB sequences from the three datasets mainly belonged to the same two phyla namely Bacillota and Pseudomonadota which represented on average 50.83% and 42.66% of the PPB dataset (Figure 1.1). The diversity of PPB sequences were greater in humans, with twelve phyla represented, compared to four and three phyla

observed in fishes and crustaceans, respectively. Bacillus was the most represented genus in the three datasets and represented up to 45% of all the PPB sequences in the crustacean dataset. As expected, we observed some differences of composition among the 10 most represented genera of each dataset with notably the presence of Vibrio and *Aeromonas* only in fishes and crustacean datasets while the genera *Streptococcus*, *Escherichia-Shigella*, *Mycobacterium* were only present in the human datasets.

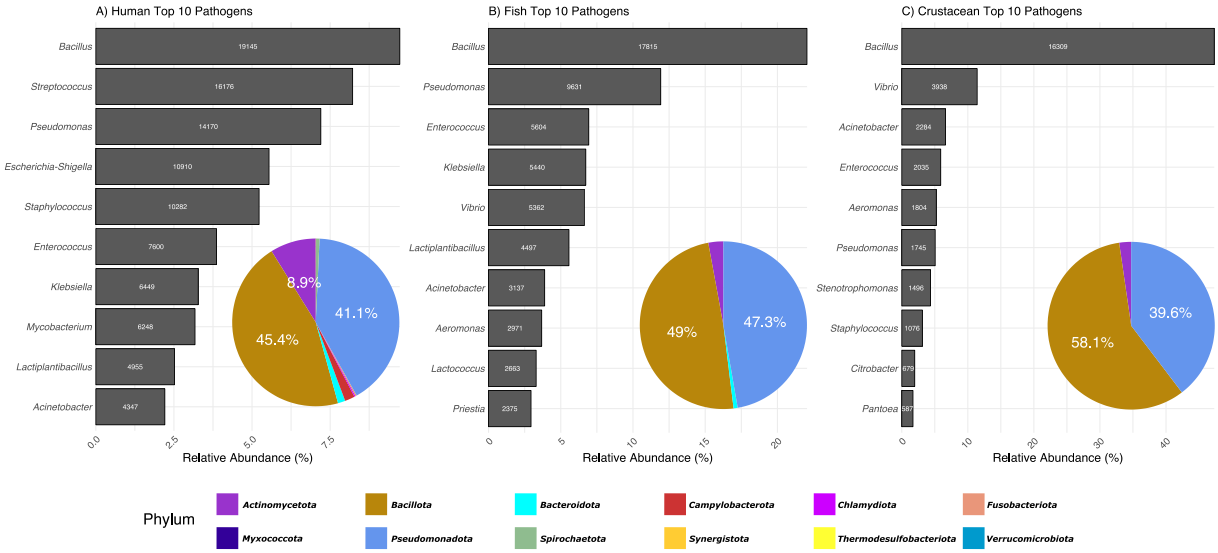

Figure 1.1: Taxonomic composition of the three PPB dataset. Barplot represents the contribution of the top ten genera in each dataset. The number of full length 16*S rRNA* gene sequences in each genus is indicated. Pie chart represents the taxonomic composition of each dataset at the phylum level (The percentage of Phylums higher than 5% is indicated).

The Pathogen_dataset_sources.xlsx file contains 2 sheets indicating the different sources where the PPB derived from (sheet 1) and the list of PPB species extract from each source (sheet 2). The initial list of PPB used to generate extended list and extract the full length 16*S rRNA* gene sequences from the SILVA 138.2 SSU Ref database is contained in three tab-separated text files containing the genus-species pairs of PPB for each host group: Crustacean_sp_pathogens_list.txt (70 species), Fish_sp_pathogens_list.txt (240 species), and Human_sp_pathogens_list.txt (1942 species)

Finally, the Zenodo repository contains a github repository of the PathoLens package, a Python tool designed to filter and curate taxonomic databases. It includes various modules and functions for validating records, which were used in the creation of

the three PPB datasets.

1.5 Experimental design, materials and methods

1.5.1 Data Acquisition and Cleaning

To support the tracking and identification of potentially pathogenic bacteria (PPB) across different hosts, we developed PathoLens, a custom Python 3.10.9 package tailored for this study. PathoLens integrates modular scripts and functions for automated data retrieval, processing, and curation of reference sequences. The package includes configuration files that define all required dependencies, ensuring reproducibility and ease of use.

The primary focus of this work was to build a curated set of 16*S rRNA* datasets enabling the tracking of potentially pathogenic bacteria (PPB) across hosts and their rapid detection using BLAST or classifier software. The human PPB list was constructed using a list of pathogenic bacteria for humans from various studies (A.-N. Zhang et al., 2021; Wardeh et al., 2015a; Blauwkamp et al., 2019; Urban et al., 2015) and pathogen detection pipeline such as 16SPIP (Miao et al., 2017), FAPROTAX (Louca et al., 2016), MPD (T. Zhang et al., 2018) and MBPD (X. Yang et al., 2023) (See Pathogen_dataset_sources.xlsx file for details). The fish and crustacean PPB lists were derived from the study of Wardeh et al. (Wardeh et al., 2015a). Crustacean PPB were not explicitly listed in the Wardeh dataset but were grouped under arthropods. To isolate crustacean pathogens, we used the script ensembl_crustacea.py, included in the PathoLens package. This script queries the Ensembl REST API (Yates et al., 2015), a comprehensive genome browser that provides various tools such as BLAST, BLAT, BioMart, and the Variant Effect Predictor (VEP) for all supported species. The script was designed to check if a given species belongs to the Crustacea class, by querying the Ensembl database for taxonomic information and determines whether the species falls under the "Crustacea" class. If it does, the species is labeled as a crustacean in the output. The script reads the input CSV file SpeciesInteractions_EID.csv, which contains information on host-pathogen interactions (Wardeh et al., 2015a; Wardeh, Risley, McIntyre, Setzkorn,

& Baylis, 2015b). Once the list of PPB for humans, fish, and crustaceans was obtained, three tab-separated text files containing the genus-species pairs of PPB for each host group: Crustacean_sp_pathogens_list.txt, Fish_sp_pathogens_list.txt and Human_sp_pathogens_list.txt were prepared for further analysis. Given the dynamic nature of bacterial taxonomy and the fact that databases such as SILVA are not updated synchronously with taxonomic databases like NCBI Taxonomy, we performed a thorough synonym search for each genus-species pair in these intermediate lists to maximize sequence recovery. This was done using the script get_sp_synonyms.py, which queries the NCBI Taxonomy database via Biopython's Entrez module. For each species name, the script retrieves its currently accepted scientific name along with all known synonyms. In cases where no taxonomic record was found, the script performs a secondary search in the general NCBI database to obtain an accession number—provided the entry is valid and not associated with uncultured or unknown organisms—and uses it to retrieve the correct taxonomic ID and associated name. This process yields an expanded taxonomy that includes all known naming variants for each species. The script generates an Excel file per host group (CRUSTACEAN_Pathogen_TaxSyn_List.xlsx, FISH_Pathogen_TaxSyn_List.xlsx, HUMAN_Pathogen_TaxSyn_List.xlsx) that lists all taxonomic variants (synonyms, basionyms and 'included' names) identified for each pathogenic species. From this, an intermediate file is created with the extended species list including all nomenclatural variants for further query of the SILVA database (CRUSTACEAN_sp_pathogens_list-EXT.txt, FISH_sp_pathogens_list-EXT.txt, HUMAN_sp_pathogens_list-EXT.txt), and a curated list of pathogenic species containing only the currently accepted scientific names, which serves as the final reference for each host group.

1.5.2 Generate SILVA reference pathogens dataset

To generate the SILVA reference pathogen dataset, the database builder module (1_run_database_builder.py) from the PathoLens package was implemented. The process began by filtering the SILVA database to retain only entries corresponding to the taxon Bacteria. At this step, 10.87% (241 878 sequences) of the initial sequences and 25.26% (75 879 taxonomies) of the unique taxonomies were flagged. Next, all sequences

labeled as "uncultured," "unidentified," "unclassified," "uncultivated," "unculturable," or "unicellular" were systematically removed to ensure the quality and relevance of the data. At this step, 67.09% (1,330,364 sequences) of the Bacteria sequences and 9.36% (21,008 taxonomies) of the unique taxonomies were flagged. After cleaning, the Bacteria_filtered.fasta dataset was created and used to extract species matches from the extended species list generated in prior steps. These matches were cross-referenced with the Bacteria dataset for each host group, ensuring that only relevant pathogens were included. Finally, a custom pathogen dataset was generated for each host group (CRUSTACEAN_Pathogen_DB_Unfiltered.fasta, FISH_Pathogen_DB_Unfiltered.fasta and HUMAN_Pathogen_DB_Unfiltered.fasta), which will serve as the basis for the subsequent steps in the analysis pipeline. Most filtering occurred during the removal of unidentified or uncultured entries, resulting in the exclusion of over 1.3 million sequences and 21,000 taxonomies, (Figure 1.2).

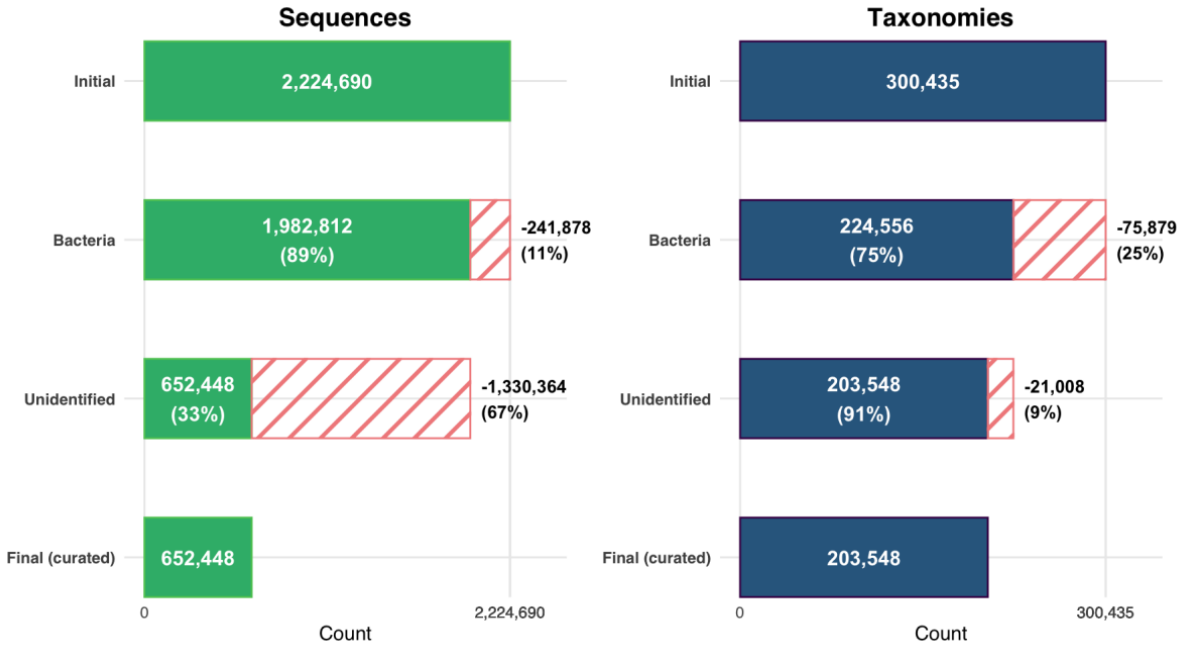

Figure 1.2: Overview of sequence and taxonomy retention across data cleaning steps in the construction of the SILVA reference pathogen dataset. Bars represent the total number of entries retained (solid) and removed (striped) at each stage of the pipeline: Initial, Bacteria filtering, Unidentified/uncultured removal, and Final (curated). For each step, the number and percentage of retained and removed entries are indicated. The left panel shows the evolution of sequence entries, and the right panel displays unique taxonomies.

1.5.3 Data curation

After extracting the sequences from the SILVA database, a comprehensive curation process was applied to each FASTA dataset to ensure the quality of the taxonomy annotations. This step was critical for removing any sequences with taxonomic discrepancies, misclassifications, or incomplete annotations that could negatively impact the correct identification of PPB. The curation process is divided into three key steps, each implemented through specific functions in the database filter module (2_run_db_filters.py):

Genus-Species Correspondence Check- This is the most important step in the curation process. When importing the sequences coming from repositories such as NCBI, Silva curators verify their correct taxonomical assignment. If discrepancies are observed between the original taxonomy and the phylogenetic assignment in the Silva tree, Silva curators correct the taxonomy until the genus level but conserve the original genus-species pair at the species level (see examples in table 1.2). This would result in wrongly affiliated PPB during the detection process or even worse in false positive PPBs. This curation step ensures that not only instances of these discrepancies observed in the MBPD (X. Yang et al., 2023) database are now systematically corrected, but also their sequences are accurately aligned to the pathogenic sequences before being presented in the NEMESISdb dataset.

Table 1.2: Example of discrepancies between the Genus and Species level within the Silva taxonomy. The correct taxonomy goes until the genus level indicating that the sequence belongs to the bacillus genus but the genus-species pair at the species level is incorrect

Acc number	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**
EU146061.1.1484	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*Streptomyces clavuligerus*

Hence, the first step of our curation process involved the identification of discrepancies when the genus in the taxonomy did not match the genus derived at the species

level. The input for this step consisted of the FASTA files produced from the database builder analysis. Discrepancies and unique taxonomies with mismatches are flagged in two Python lists for the subsequent curation step.

Multiple-Genera Check - The second curation step assessed multiple genera mentioned within a single taxonomic description. For example, taxonomies that included multiple genera, such as *Hafnia-Obesumbacterium* or *Shigella-Escherichia*, were reviewed (Table 1.3). If one of the genera of the genus level matched with the genus at the species level, the taxonomy was retained; otherwise, an Excel file, Tax_to_manual-review_group.xlsx, was generated with sequences flagged for further manual review due to ambiguous or missing genera.

Table 1.3: Example of multiple genera within the genus level of the taxonomy

Acc number	Phylum	Class	Order	Family	Genus	Species	Decision
JMPC01000 305.1.1285	Proteobacteria	Gammaproteo -bacteria	Enterobac -terales	Enterobac -teriaceae	Escherichia -Shigella	*Acinetobacter baumannii* 42057_5	Flagged
HG738867 .2611898 .2613439	Proteobacteria	Gammaproteo -bacteria	Enterobac -terales	Enterobac -teriaceae	Escherichia -Shigella	*Escherichia coli* str. K-12 substr. MC4100	Retained

Manual Review - A manual review process was conducted to validate the flagged discrepancies from the ambiguous or missing genera list. This review was essential for finalizing the list of sequences to be removed from the database. Following this manual review, the final set of sequences marked for deletion was established, and these sequences were subsequently removed from the dataset. The input for this stage was the file Tax_to_manual-review_group.xlsx, and the output was Tax_reviewed_group.xlsx, which included the “Retained” column with values of "Yes" or "No" to indicate whether the associated taxonomy (and all sequences with the same taxonomies) would be retained or deleted from the dataset.

1.5.4 The Final Curated FASTA Dataset

To generate the final curated and validated FASTA datasets, the database curation module (3_run_db_curation.py) was implemented. The process begins by reading the input Excel file Tax_reviewed_group.xlsx, which indicates which taxonomic entries should be excluded. For each taxonomy marked as "No", a function retrieves the corresponding sequences from the unfiltered FASTA files produced by the database builder module (CRUSTACEAN_Pathogen_DB_Unfiltered.fasta, FISH_Pathogen_DB_Unfiltered.fasta, and HUMAN_Pathogen_DB_Unfiltered.fasta) to identify and remove the corresponding sequences. As a result, the script outputs the final curated FASTA files—CRUSTACEAN_Pathogen_DB.fasta, FISH_Pathogen_DB.fasta, and HUMAN_Pathogen_DB.fasta—which include only the sequences retained after the curation process. Additionally, at the end of this module, a species-level summary is generated for each group. An Excel file is created (Species_match_CRUSTACEAN.xlsx, Species_match_FISH.xlsx, Species_match_HUMAN.xlsx) listing the currently accepted scientific names along with all synonyms or variant names found in the database that correspond to each accepted species. This provides a reliable reference for analyzing the species composition of the curated dataset. Throughout the entire curation process of the datasets, the number of sequences and unique taxonomies that passed through each filter was meticulously recorded. This tracking allowed for a comprehensive understanding of the sequences and taxonomies to be eliminated for each host dataset (Figure 1.3. Overall, this plot highlights how the filtering process progressively reduces the pool of sequences and taxonomies marked for elimination, leaving only a small set of sequences (i.e.; 584, 1202 and 4194 respectively for Crustacean, Fish and Human) and unique taxonomies (i.e.; 182, 409 and 1057 respectively for Crustacean, Fish and Human) to be removed after the final "Manual Review". Overall, the pipeline for creating and curating the dataset is briefly described in Figure 1.4.

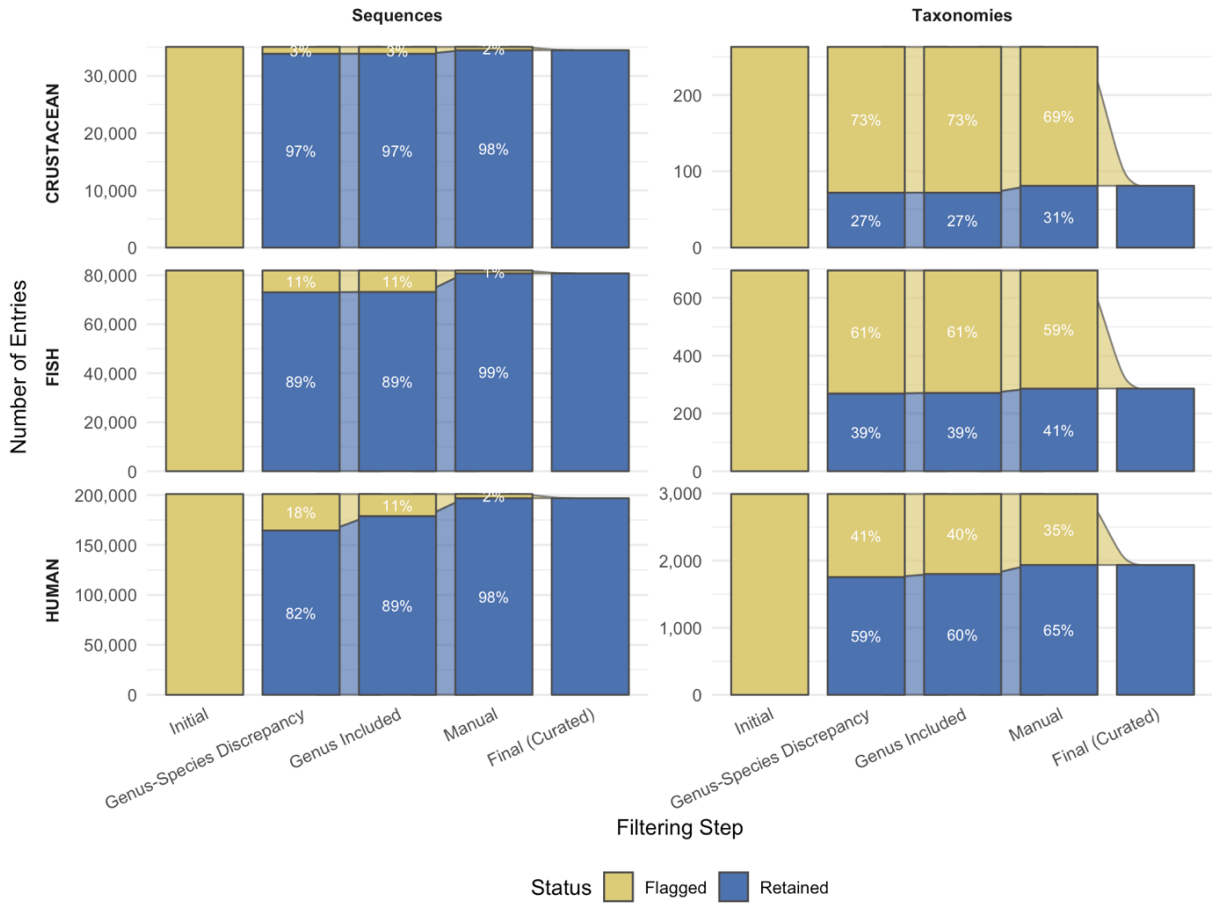

Figure 1.3: Retention and flagging of sequences and taxonomies across curation filters for each host group. Each alluvial plot shows the evolution of the number of entries (sequences or taxonomies) that were retained or flagged during the successive data curation steps. The top panels display results for sequence entries, while the bottom panels show taxonomic entries. Rows correspond to different host groups (CRUSTACEAN, FISH, HUMAN), and Y-axis scales are adapted to each case.

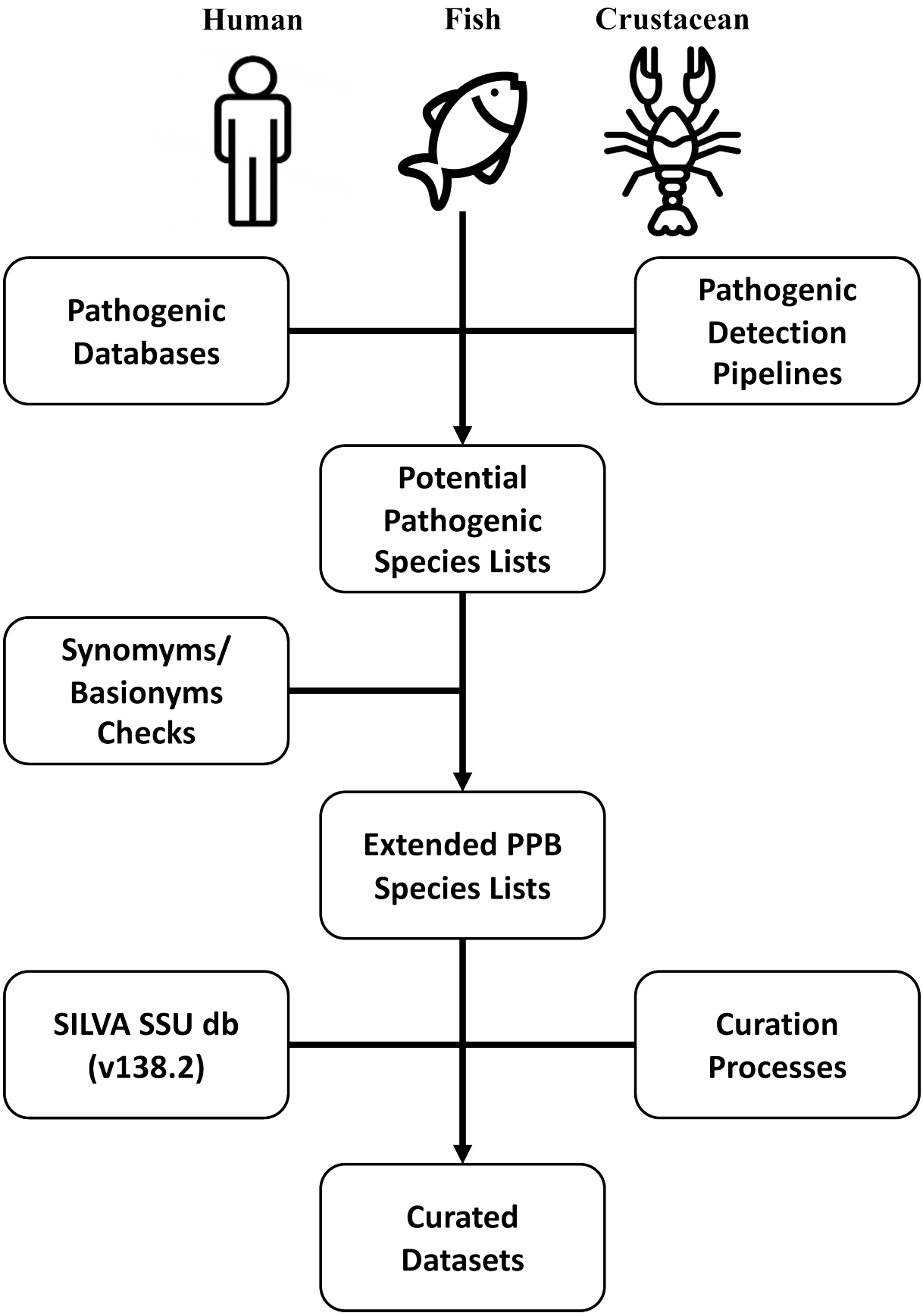

Figure 1.4: Pipeline of the creation and curation of the dataset

Limitations

While amplicon and metagenome sequencing have been used to analyse the composition and risk of pathogen contamination (Naudet et al., 2023), establishing the definitive pathogenicity of a bacteria still demands additional experimental validations.

Ethics statement

Authors have read and follow the ethical requirements for publication in Data in Brief. Authors confirm that the current work does not involve human subjects, animal experiments, or any data collected from social media platforms.

CRediT Author Statement

Son-Hoang Tran: Formal analysis, Investigation, Data Curation, Writing - Original Draft; Claudia Ximena Restrepo Ortiz: Methodology, Software, Data Curation, Validation, Writing - Review & Editing; Quang Vu Dinh: Data Curation; Marc Troussellier: Conceptualization, Writing - Review & Editing; Yvan Bettarel: Writing - Review & Editing; Thierry Bouvier: Writing - Review & Editing, Van Ngoc Bui: Writing - Review & Editing; Nguyen Hieu Minh: Data Curation; Trung Du Hoang: Writing - Review & Editing; Quang Huy Nguyen: Conceptualization, Writing - Review & Editing; Jean-Christophe Auguet: Conceptualization, Methodology, Data Curation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

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influence on the circulation of anTibiotic microbial reSistances and pathogens in floating farm socio-ecosystems" (THREATS) and (3) the NEMESIS project (2021-EST-149) funded by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES).

Declaration of competing interests

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.

This study is under review by the journal Microbiome

Marine holobionts: an overlooked reservoir and vector of rare species and pathogens

Son-Hoang Tran[1,2], Marc Troussellier[1], Marlène Chiarello[1], Sébastien Villéger[1], Yvan Bettarel[1], Thierry Bouvier[1], Fabien Rieuvilleneuve[1], Claudia Ximena Restrepo Ortiz[1], Elliot Sucré[1,3], Thomas Claverie[1,3], Van Ngoc Bui[4], Quang Huy Nguyen[2], Jean-Christophe Auguet[1,2],

[1]UMR MARBEC, Univ Montpellier, CNRS, Ifremer, IRD, Montpellier, France;

[2]University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Hanoi, Vietnam;

[3]Centre Universitaire de Formation et de Recherche de Mayotte, Dembéni, Mayotte, France;

[4]Institute of Biology (IB), Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Hanoi, Vietnam

2 Marine holobionts: an overlooked reservoir and vector of rare species and pathogens

Marine holobionts, comprising macroorganisms such as corals, sponges, and fish and their associated microbiotas, play a critical yet underappreciated role in structuring marine microbial diversity. Our study demonstrates that these holobionts serve as major reservoirs of microbial richness, accounting for up to 98.4% of total genera richness in coral reef ecosystems, with surface microbiotas contributing more than gut communities. Importantly, holobionts disproportionately support rare environmental microbes, validating the "sustaining the rare" hypothesis. We show that 95% of rare genera from the environment are hosted by holobionts, with 33.6% becoming enriched, highlighting their role in promoting conditionally rare taxa (CRTs), including symbionts and opportunistic copiotrophs. However, this enrichment mechanism also fosters potential human pathogenic bacteria (PHPB), some of which transition from rarity to dominance within host microbiotas. Over 48% of total PHPB genera were found exclusively in host-associated microbiotas, and PHPB relative abundances were nearly twice as high in holobionts compared to the environment. These findings reveal a dual ecological role for marine holobionts—as refuges for both beneficial rare microbes and potential human pathogenic bacteria. This duality underscores the need to consider holobiont-microbiota dynamics in both conservation and public health frameworks, particularly in the context of global environmental change.

2.1 Introduction

The term "holobiont" refers to a conceptual framework in ecological and evolutionary biology that challenges the traditional view of individual organisms as discrete entities. First introduced by Margulis and Fester in 1991, the “holobiont” paradigm emphasizes the intricate and dynamic interactions between a host organism and its associated microbial communities (Margulis & Fester, 1991). Many recent studies have evidenced that marine holobionts harbor specific microbial communities which differ from those in the surrounding planktonic and sediment habitats. These studies span various marine organisms: mammals (Bik et al., 2016; Chiarello, Villéger, Bouvier, Auguet, & Bouvier, 2017), fishes (Chiarello et al., 2018; Parris, Brooker, Morgan, Dixson, & Stewart, 2016; Schmidt, Smith, Melvin, & Amaral-Zettler, 2015), mollusks (Pfister, Gilbert, & Gibbons, 2014), sponges (Dupont, Corre, Li, Vacelet, & Bourguet-Kondracki, 2013; Thomas et al., 2016), corals (Bettarel et al., 2018; Sunagawa et al., 2015), zooplankton (De Corte et al., 2018), macrophytes (Burke, Thomas, Lewis, Steinberg, & Kjelleberg, 2011; Mancuso, D’hondt, Willems, Airoldi, & De Clerck, 2016), and phytoplankton (Sison-Mangus, Jiang, Kudela, & Mehic, 2016). An estimation of the total richness associated with coral reef animal surface microbiomes reached up to 2.5% of current estimates of Earth prokaryotic diversity, suggesting that coral reef animal surfaces should be recognized as a hotspot of marine microbial diversity (Chiarello et al., 2020). While most of the literature on marine holobionts focused on the beneficial services microbial communities can bring to their hosts or the surrounding ecosystems (Cheutin et al., 2021; HERNDL, GLASL, FRADE, et al., 2016; Peixoto, Rosado, Leite, Rosado, & Bourne, 2017), the importance of the host for microbe survival and dispersion has been greatly overlooked.

Recently, a new hypothesis coined as “sustaining the rare” (Troussellier, Escalas, Bouvier, & Mouillot, 2017) has been raised for describing the double role played by holobionts in the maintenance of microbial diversity through (i) their beneficial influence on rare community members and (ii) their role as dissemination vector for geographically restricted taxa. The first part of this framework refers to the fact that marine holobiont bodies (i.e.; skin, gut, mucus…) may constitute a nutrient-rich environment

favorable to the growth of opportunistic microorganisms present at low abundance (i.e. rare) in nutrient-poor seawater (Smriga, Sandin, & Azam, 2010). This enrichment of rare seawater amplicon sequence variants (ASVs) in marine holobionts has been documented for a wide variety of hosts (Burke et al., 2011; Chiarello et al., 2020; K.-H. Lee & Ruby, 1994; Webster et al., 2010). In addition, prokaryotic production (Fonte et al., 2011), diversity (Vlahos et al., 2013) and abundance have been shown to respond to organic matter excretion and exudation from marine organisms. The second part of the framework alludes to the role of holobionts in the direct dispersal of microbes in the marine realm through active or passive expulsion from their host (B. Jones, Maruyama, Ouverney, & Nishiguchi, 2007). Motile holobionts such as pelagic mollusks or vertebrates are particularly interesting for their high capacity of dispersion, including against currents and through stratified water layers. Taking into account the gut transit time of various marine holobionts and their sustainable swimming speed, Trousselier and colleagues showed that fishes and marine mammals may be efficient vectors of dispersal for their gut microbes, enhancing the original dispersion rate of one microbial cell by 200 to 200,000 times (Marc Troussellier, 2017). In addition, the "sustaining the rare" framework has important implications for ecosystems services and human populations when considering the particular case of potential human pathogenic bacteria (PHPB). These bacteria normally belong to the rare biosphere in the planktonic realm but recent findings using large-scale surveys showed that PHPB have a large distribution pointing the question of their persistence in marine waters (Troussellier et al., 2017). Results from this study indicated that the "sustaining the rare" framework could apply to PHPB since both occurrence and abundance of PHPB increased in marine holobionts comparing to the surrounding waters. This topic is only in its infancy and further experiments are needed to confirm that marine holobionts represent reservoirs and vectors of active PHPB and to evaluate the risks for human populations.

In this study, we sampled the surface and gut microbiomes of 265 coral reef animals belonging to 74 different taxa, including 32 and 18 genera of teleost fishes and Anthozoa (hard and soft corals, and anemones), respectively, and 12 taxa of crustaceans, echinoderms, mollusks and sponges, from a single coral reef ecosystem (Mayotte lagoon, Indian Ocean). In order to test the "sustaining the rare" framework, we in-

vestigated whether host-associated microbiota represented hotspots for rare microbial taxa found in surrounding planktonic and benthic communities. Then, we explored whether this framework may also be applied to PHPB. In other words, we test the hypothesis that marine macro organisms can be reservoirs and transmission vectors of PHPB.

2.2 Material and Methods

2.2.1 Sample collection and pre-treatment

Detailed description of the sampling campaign can be found in Chiarello et al. (Chiarello et al., 2018, 2020). Briefly, the campaign was conducted within one week in November 2015 around the Mayotte lagoon in the Western Indian Ocean. Two sites were chosen for the sampling including one on the fringing reef (S12°5417.46, E44°58) and the other on the inner slope of the barrier reef (S12°5733.72, E45°0449.38), which are located 15 km apart (Supplementary Material SM1-1).

The most common species of each main animal group (teleost fishes, crustaceans, echinoderms, mollusks, and sponges) were collected within a 50 m radius of each site (Supplementary Material SM1-1, 1-2 and 1-3). In order to take into account intraspecific variability of surface or gut microbiome, up to three replicates of each species were sampled in each site. To prevent contamination during capturing, fish were caught using a speargun or hook line and later handled following the European directive 2010/63/UE. The fish skin microbiome was collected by swabbing the entire untouched side of their bodies, excluding the head, using mouth swabs. Fish's gut microbiome was collected by squeezing digestive tracts (from below the stomach up to the rectum) to expel the gut content (Cheutin et al., 2021). For corals, individual colonies of 5-10 cm in diameter or a 15-20 cm2 part of the colony were sampled. We collected mucus that contains the microbiome directly after it was dripped from the coral (Leruste, Bouvier, & Bettarel, 2012). For the corals that didn't secrete mucus, we collected the surface microbiome by swabbing the entire untouched surface of the specimen using a mouth swab. Crustaceans, echinoderms, mollusks, and sponges were caught on the

sea floor using gloves and were fully swabbed on the boat for one minute. All invertebrates were released after their microbiomes were sampled. All samples were stored on board at -5°C in an electric cooler during the day and then at -80°C when returned to the lab until DNA extraction. For identification, fish species were classified at the species level, corals at the genus level, and other invertebrates at the species level when possible (Supplementary Material, SM1-3).

For environmental samples, six samples of seawater, each 200 mL, were gathered. Half of these samples were taken 50 cm below the sea surface, while the other half were collected 30 cm from the sea bottom. These samples were then kept in a dark cooler at 4°C and filtered within 6 hours using a 47 mm 0.2 µm polycarbonate membrane from Whatman, Clifton, USA. The filters were then frozen at -80°C. Sediment samples were collected in triplicates at each site, kept in sterile Falcon tubes, and stored at 0 ∘C in a portable icebox until arrival at the laboratory, and then stored at -80 ∘C until DNA extraction.

Overall, a comprehensive dataset comprising 462 samples, encompassing specimens from diverse ecological niches was collected. Specifically, this dataset included 265 samples derived from 44 fish species (comprising 144 skin and 121 gut samples), 84 coral samples from 16 species, 8 crustaceans samples from 2 species, 25 echinoderms samples from 7 species, 3 mollusks samples from 1 species, 7 sponges samples from 2 species, and 51 environmental samples (consisting of 15 sediments and 36 water samples) (with the remaining 19 samples with no information).

2.2.2 DNA extraction and sequencing

The DNA extraction process was carried out on all water filters, sediment, coral mucus, and swabs using the Maxwell® 16 Bench-top extraction system, with some adjustments to the manufacturer's instructions. The swabs and water membranes were treated with 570 µL lysis buffer from Maxwell® Buccal Swab LEV DNA kits (Promega Corporation, Madison, USA) and 2 µL of 37.5-KU.µL-1 Ready-Lyse lysozymeTM, then incubated for 30 minutes at 37°C. Following this, 30 µL proteinase K was added and

the tubes were left to incubate overnight at 56°C. The coral mucus was treated separately with lysis buffer (two 570 µL mucus samples with 570 µL lysis buffer each), and then extracted using two Maxwell® cartridges before being pooled. The whole solution was then used in the extraction kit. For sediment samples, a maximum of 0.25 grams was used. The DNA extraction was carried out as per the manufacturer's instructions, and the DNA was eluted in 50 µL elution buffer and then quantified using spectrophotometry (Nanodrop® 1000, Willmington, USA).

The V4 region of the 16S rDNA gene was amplified using modified prokaryotic primers for Illumina sequencing as 515F (5'- GTGYCAGCMGCCGCGGTAA - 3') and the modified version of 926R (5' – CCGYCAATTYMTTTRAGTTT - 3') (Caporaso et al., 2011; Parada, Needham, & Fuhrman, 2016). This was done using PuRe Taq Ready-To-Go PCR Beads, 1µL of the extracted DNA, and 0.4 µM of each primer. The process involved an initial denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. The DNA samples from each reef type were pooled and sequenced in two separate runs by an external laboratory on an Illumina platform using the 2×250 bp MiSeq chemistry (INRA GeT-PlaGE platform, Toulouse, France). Seven PCR blanks were included in each sequencing run to check for contaminants, which were removed in the subsequent sequence processing steps. For the environmental samples, one of each type showed no amplified DNA, and thus was removed from our dataset. Finally, a total of 460 samples including 49 environmental samples, 290 samples of surface microbiome, and 121 gut microbiome from animals were available for sequencing.

2.2.3 Amplicon sequence variants processing

The 'DADA2' R package v.1.2 (`https://benjjneb.github.io/dada2/index.html`) and R software v. 3.4.3 were used to process sequence reads, following a script provided in the electronic Supplementary Material SM1-4. Briefly, forward reads were trimmed at their 5' end extremity to remove forward and reverse primers, then trimmed according to a quality score of 2. Reads were later filtered to remove reads containing more than 2 expected errors, before dereplication. Both forward and reverse reads

were later merged, chimeric sequences were then removed and the remaining Amplicon Sequence Variants (ASVs) were taxonomically classified using the SILVA database v138.1, with the removal of non-bacterial or archaeal sequences in the end.

Rarefaction of 1000, 5000, and 10,000 reads per sample were tested in order to look at the impact of this process on the whole dataset. Coverage for each sample was calculated using Zhang & Huang's coverage estimator (Z. Zhang & Huang, 2007) with the 'entropart' R package v. 1.6-1 (Marcon & Hérault, 2015). Results of the 'coverage' function show 98.9%, 97.5% and 88.4% with 10,000, 5000 and 1000 reads per sample respectively. However, the number of raw samples removed during these rarefaction processes increases with a higher sample size. A sample size of 10,000 reads resulted in an 11.7% removal rate of samples. Therefore, analyses with a rarefied dataset at 5000 reads per sample are shown in the main document because it provided the best samples coverage without losing too many samples. Raw sequence data are available in the National Center for Biotechnology Information (NCBI) sequence reads archive (SRA) database under Bioproject accession number PRJNA895209.

The ASV data was filtered using two methods to eliminate contaminants. Initially, "decontam" R package (N. M. Davis, Proctor, Holmes, Relman, & Callahan, 2018) was employed to identify ASV contaminants in the dataset, using the package's "prevalence method". However, certain known extraction kit contaminants mentioned by Salter, such as ASVs from the *Bradyrhizobium* and *Cupriavidus* genera, were still present in the data (Salter et al., 2014). These were then manually eliminated from the final dataset. To detect potential human pathogenic bacteria (PHPB) in our data, we compared all ASVs to a full-length 16*S rRNA* gene dataset (Tran Son et al. in revision). This dataset was created with the main objective to curate a collection of 16*S rRNA* datasets that facilitate the tracking of potentially pathogenic bacteria (PPB) among different hosts and enable their swift identification using BLAST or classifier software. The compilation of the PHPB dataset was derived from a range of studies detailing pathogenic bacteria relevant to humans (Wardeh et al., 2015a) and utilized pathogen detection tools like 16SPIP (Miao et al., 2017), FAPROTAX (Louca et al., 2016), MPD (T. Zhang et al., 2018), and MBPD (X. Yang et al., 2023). In the end, our dataset included 196,770 full-length

16*S rRNA* from the 1757 human bacterial cargos. Only ASVs that matched a 16*S rRNA* sequence with 100% similarity, 100% coverage, and more than 239 base pairs were included in our pathobiome data (Tran Son et al. in revision).

2.2.4 Microbial biodiversity and statistics tests

Taxonomic diversity and composition of each PHPB community were measured using the relative abundance (number of PHPB ASVs/total ASVs), species richness ("Observed" index), and species diversity ("Shannon" index). Statistical tests were conducted on alpha diversity comparisons using a Kruskal-Wallis test (KW test), followed by a Dunn post-hoc test. The p-value was corrected using Bonferroni's method and a Winconxon test was also implemented for visualization. For beta diversity, we used Bray-Curtis distance with the vegan package in R, as suggested by Oksanen (Oksanen et al., 2013) and the LCBD (local contributions to beta diversity) index for the uniqueness of the ASVs. We performed statistical assessments using permutational analyses of variance (Permanova) and Bray-Curtis dissimilarity was depicted in a principal coordinate analysis (PCoA) plot. The identification of ASV biomarkers (i.e. differential abundance of ASV between coral holobionts and water and sediment, or among different animal types, etc.) was accomplished through an analysis of the bacteriome composition with bias correction. This was performed using ANCOM-BC (Cao et al., 2022) method.

2.3 Results

2.3.1 Marine Host-Associated Microbiotas Harbor Rich and Unique Prokaryotic Assemblages

A total of 67,976 bacterial ASVs were found from the 458 samples examined in this study, with 2 samples being discarded during prior processes. The analysis of these ASVs highlighted differences in the composition and diversity of the whole bacterial communities between marine holobionts (MH) and environmental samples. Individ-

ual fish gut samples contained significantly less ASV richness and diversity than MH surface, planktonic or sediment samples (Figure 1.5A&B) (Dunn post-hoc test, $p<0.05$, Supplementary Material SM1-5). Inversely, individual sediment samples presented the highest values of richness and Shannon diversity compared to the other type of samples. Within MH samples, crustacean microbiota showed higher diversity and richness compared to fish, coral, and echinoderm microbiotas (Dunn post-hoc test, $p<0.05$, Supplementary Material SM1-6). On the other hand, local contributions to beta diversity (LCBD) values indicated that microbiotas (gut & surface) and sediment microbial communities contained more original and unique ASVs than the planktonic communities (Figure 1.5C) (Dunn post-hoc test, $p<0.05$, Supplementary Material SM1-5). As a result, we observed a strong dissimilarity between the four community types (i.e.; surface, gut, planktonic and benthic) in terms of composition (Figure 1.5D), confirmed by the high percentage of variance explained by sample type (Permanova, $p<0.05$, $R^2=0.15$). The PCoA ordination also showed that the variability of MH samples was greater than the variability observed in sediment or water samples. As a consequence of the high turnover of ASVs within animal samples, prokaryotic richness hosted by MH microbiotas contributed up to 98.4% (91.6% and 45.3% for surface and gut microbiota, respectively) of the whole dataset genera richness (Figure 1.5F). Additionally, the number of genera unique to animal surfaces or gut (i.e., absent in plankton or sediment) represented over 47% of the total genera richness, with 6.4% and 30.4% of the total genera being exclusive to gut and surface microbiota, respectively (Figure 1.5F). The most abundant bacterial classes across all samples were *Gammaproteobacteria*, *Alphaproteobacteria*, and *Bacteroidia* (39%, 21.1%, and 11.1% of the total ASVs, respectively), as illustrated in Figure 1.5E.

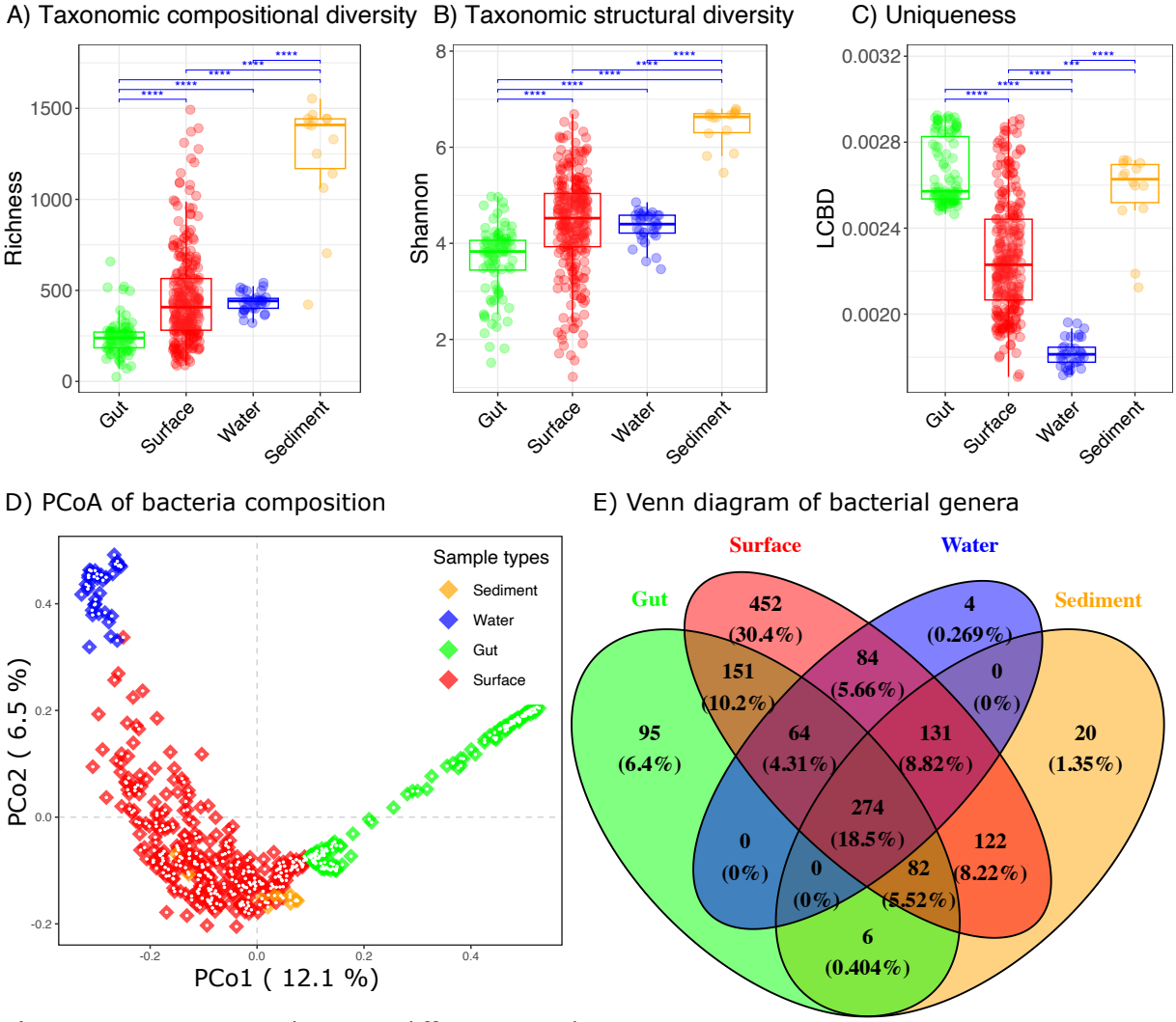
A) Taxonomic compositional diversity
B) Taxonomic structural diversity
C) Uniqueness
Richness
Shannon
LCBD
Gut
Surface
Water
Sediment
D) PCoA of bacteria composition
Sample types
Sediment
Water
Gut
Surface
PCo2 (6.5 %)
PCo1 (12.1 %)
E) Venn diagram of bacterial genera
Surface
Water
Gut
Sediment
452 (30.4%)
4 (0.269%)
84 (5.66%)
151 (10.2%)
0 (0%)
95 (6.4%)
64 (4.31%)
131 (8.82%)
20 (1.35%)
274 (18.5%)
0 (0%)
0 (0%)
82 (5.52%)
122 (8.22%)
6 (0.404%)
F) Bacteria composition between different sample types
Bacteria composition (%)
100%
75%
50%
25%
0%
Class
Gammaproteobacteria
Alphaproteobacteria
Bacteroidia
Oxyphotobacteria
Clostridia
Verrucomicrobiae
Acidimicrobiia
Erysipelotrichia
Planctomycetacia
Others

Figure 1.5: Composition, and alpha diversity of the whole bacterial community. A: Species richness (Observed index); B: Species diversity (Shannon index); C: Uniqueness of species (local contributor of beta-diversity index). A, B and C: Dunn post-hoc test between sample types (****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$). D: PCoA plot with Bray-Curtis dissimilarities. E: Venn diagram of unique and shared bacterial genera between each sample type. Percentage indicates the ratio of each group bacterial genera to the whole dataset bacterial genera. F: Bar plots representing the composition of the top 5 microbial classes in each sample type, remaining classes are listed as "Others" (ASV abundance >2%).

ANCOM-BC analyses revealed that 19 and 7 phyla (45.5% of the biomarkers) were significantly enriched in surface and gut samples, respectively, compared to environmental samples (Figure 1.6). *Tenericutes* and *Firmicutes* phyla were significantly systematically enriched in both gut and surface samples (Figure 1.6), with their respective ASVs making up to 0.6% and 7.5% of the total ASVs of MH samples, respectively, while representing only 0.1% and 2.1% of the total environmental ASVs. On the other hand, typical marine water and sediment phyla such as *Marinimicrobiota* and *Acidobacteria*, respectively, were relatively low from our MH surface samples, representing about 0.4% and 1.9% of the respective community, yet they accounted for up to 1.8% and 3.6% of the total water and sediment ASVs, respectively.

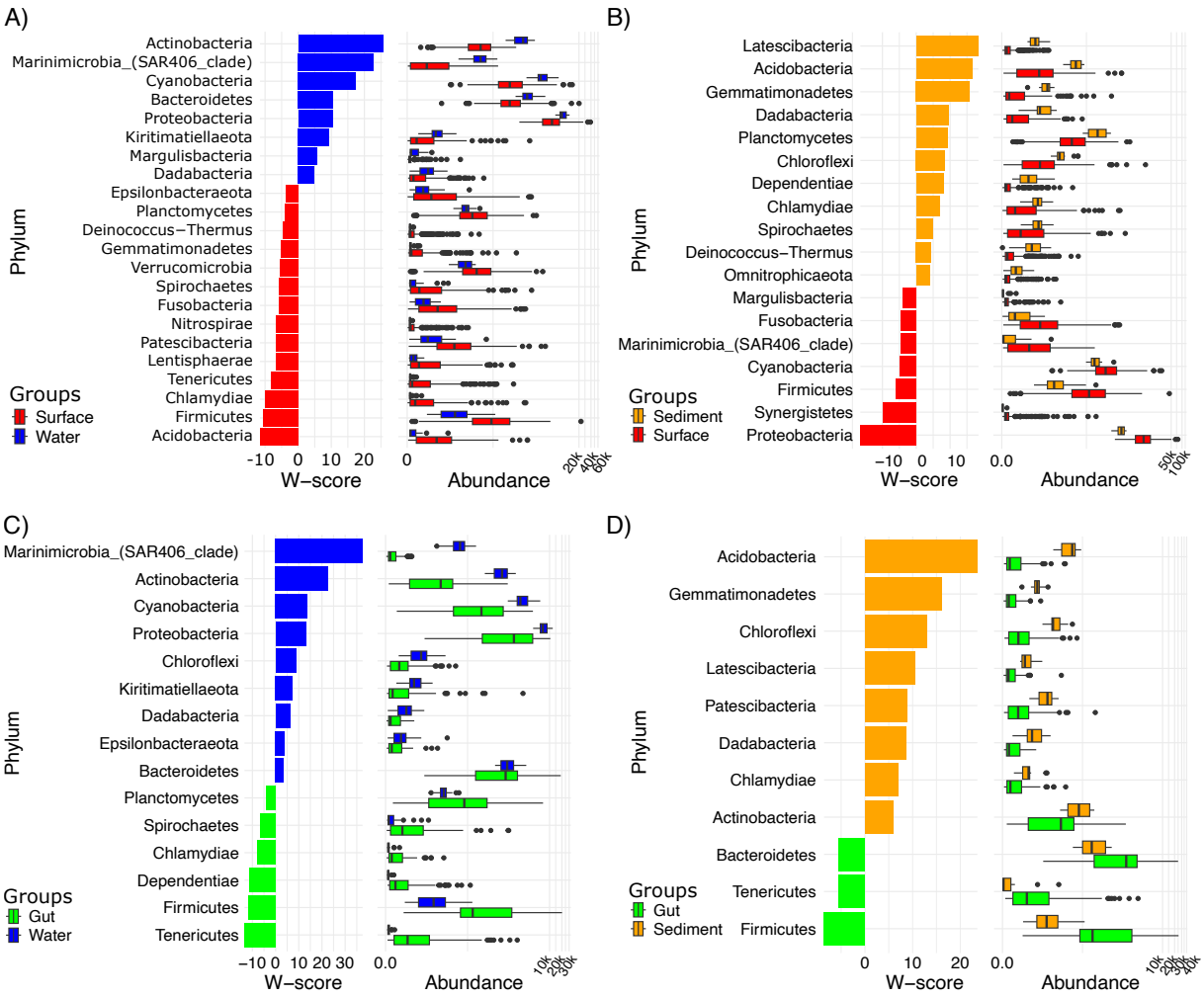

Figure 1.6: ANCOM-BC tests (W-score) and ASVs abundance according to the bacterial phylum in the microbial communities between. A: Animals surface and Water. B: Animals surface and Sediment. C: Gut and Water. D: Gut and Sediment.

2.3.2 Composition and Enrichment of Potentially Pathogenic Bacteria in Marine Holobionts

In the whole dataset, 697 ASVs (13.1% of the total reads) were identified as potential human pathogenic bacteria (PHPB) after being compared to the NEMESISdb pathogenic dataset. These 697 ASVs spanned 391 bacterial species, 169 genera and 85 families. The relative abundances of PHPB reads were significantly higher in MH samples compared to environment samples, as described in Figure 1.7A. While PHPB relative abundance accounted for 6.4 ± 7.3% in sediment and 2.4 ± 2.7% in water samples, they represented 13 ± 9.5% in fish guts and reached up to 14.7 ± 13.3% on the

surfaces of all animals. Significant differences in PHPB relative abundance were also observed among the surfaces of the different MH animals, with the highest numbers detected on fishes, corals and echinoderms (reads relative abundance of 15.6 ± 12.3%, 13.5 ± 12.2% and 25.4 ± 17.4%, respectively), while crustaceans, mollusks and sponges exhibited the lowest relative abundances (reads relative abundance of 2.7 ± 3.7%, 0.6 ± 1.0% and 0.4 ± 0.4%, respectively) (Figure 1.7B).

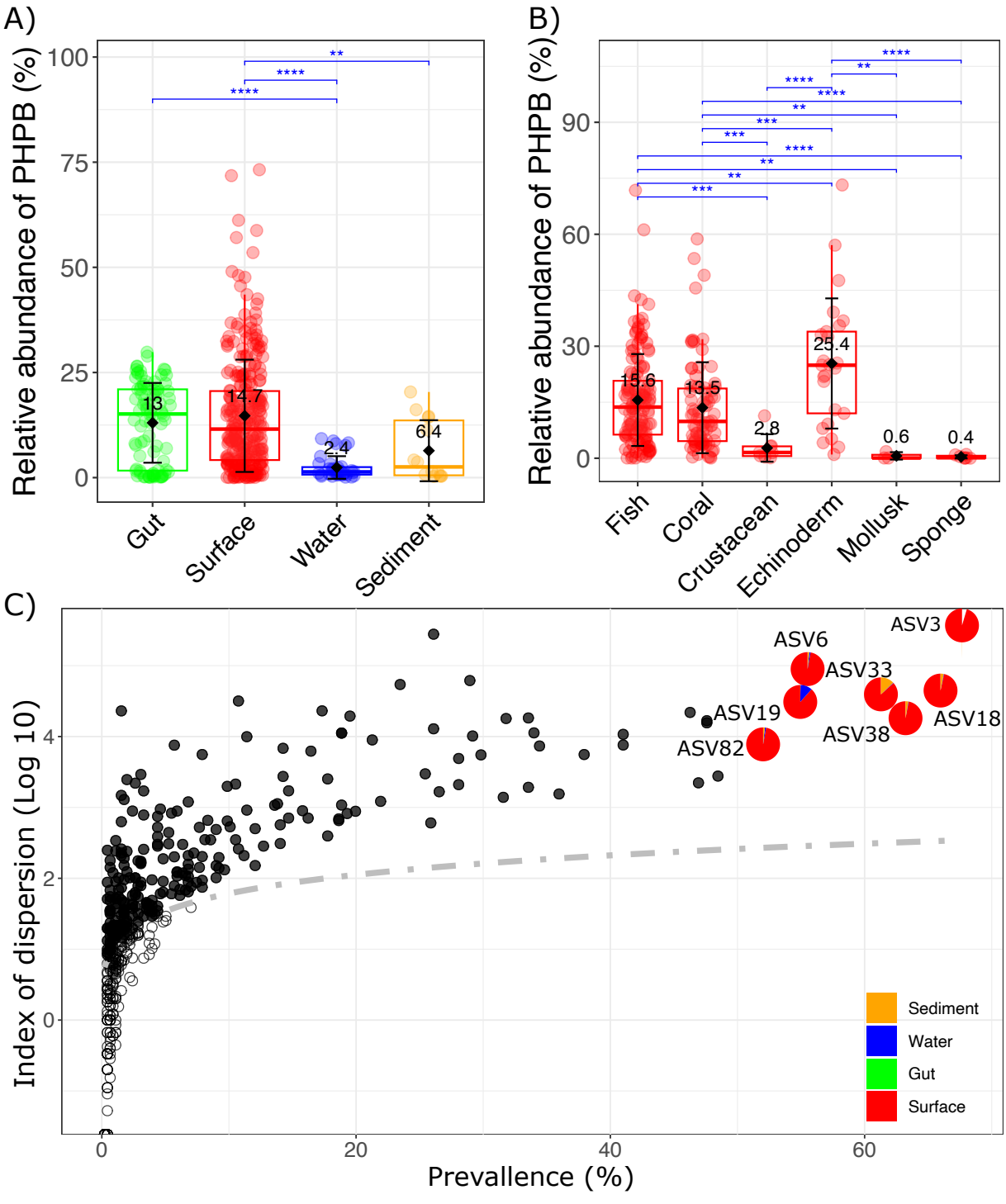

Figure 1.7: A: Relative abundance of PHPB ASVs to whole bacterial ASVs within each sample type; B: Relative abundance of PHPB ASVs to whole bacterial ASVs on surfaces of each MH. A&B: Jitter boxplots representing median values, numbers indicating mean values and error bar representing SD, Dunn post-hoc test between sample types (****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$) ; C: occurrence of core pathogenic ASVs among samples plotted against its dispersion index. The dotted line depicts the 5% confidence limit of the Chi^2 distribution: ASVs located above this line are non-randomly distributed among samples, whereas those below the line follow a random Poisson distribution. Pie charts indicate the reads proportion of each sample type. Taxonomic detail of core ASVs (occurrence > 50%): ASV3: *Vibrio vulnificus*; ASV18: *Methylobacterium radiotolerans*; ASV38: *Afipia broomeae*; ASV33: *Methylorubrum rhodesianum*; ASV6: *Vibrio tasmaniensis*; ASV19: *Vibrio parahaemolyticus*; ASV82: *Staphylococcus warneri*.

Overall, PHPB retrieved from gut samples were significantly less rich and diverse than in any other sample type (Figure 1.8A&B) (Dunn post-hoc test, $p<0.05$, Supplementary Material SM1-7). In contrast, surface samples displayed the highest PHPB values of richness and shannon diversity. Among surface samples from different MH, the PHPB richness of fish, coral, and echinoderm were significantly higher than crustaceans, mollusks, and sponges (Dunn post-hoc test, $p<0.05$; Supplementary Material SM1-6). However, local contributions to beta diversity (LCBD) values indicated that PHPB from the gut communities of fishes contained much more unique ASVs than other pathobiomes (Figure 1.8C) (Dunn post-hoc test, $p<0.05$, Supplementary Material SM1-7). This pattern is also reflected in the segregation of PHPB communities from gut samples to other sample types in the PCoA plot (Permanova, $R^2=0.20$, $p<0.05$) (Figure 1.8D). Additionally, host type between the MH and the sampling sites also had a significant effect on the PHPB compositions (Permanova pairwise test, Supplementary Material SM1-8). The most abundant PHPB families identified across all samples were *Vibrionaceae*, *Beijerinckiaceae*, and *Moraxellaceae* which accounted for 22.7%, 13.3%, and 9.4% of the total PHPB ASVs, respectively, as illustrated in Figure 1.8F. Examination of the species abundance distribution (SAD) of PHPB indicated that the core pathobiome was composed of 251 ASVs, with 7 PHPB found in more than 50% of the samples (*Afipia broomeae*, *Methylobacterium radiotolerans*, *Methylorubrum rhodesianum*, *Staphylococcus warneri*, *Vibrio parahaemolyticus*, *Vibrio tasmaniensis*, *Vibrio vulnificus*) (Figure 1.7C).

The number of exclusive PHPB genera to animal surfaces or gut (i.e., absent in plankton or sediment) accounted for over 48% of the total PHPB genera richness, with 1.8% and 36.7% of the total genera being exclusive to gut and surface microbiota, respectively (Figure 1.8E). Exclusive PHPB genera included *Anoxybacillus*, *Roseobacter*, *Helicobacter*, and *Variovorax* in the gut microbiota, and *Brucella* and *Kytococcus* in the surface microbiota. In contrast, environmental microbial communities did not harbor any unique PHPB genera. Although PHPB genera compositions varied between sample types, 47 PHPB genera including *Ralstonia*, *Vibrio*, *Acinetobacter*, *Pseudomonas* and *Methylobacterium* (3.9%, 3.9%, 3.6%, 3.4%, and 3.2% of the total PHPB ASVs, respectively), were ubiquist and shared between all MH and environmental samples (Figure 1.8E).

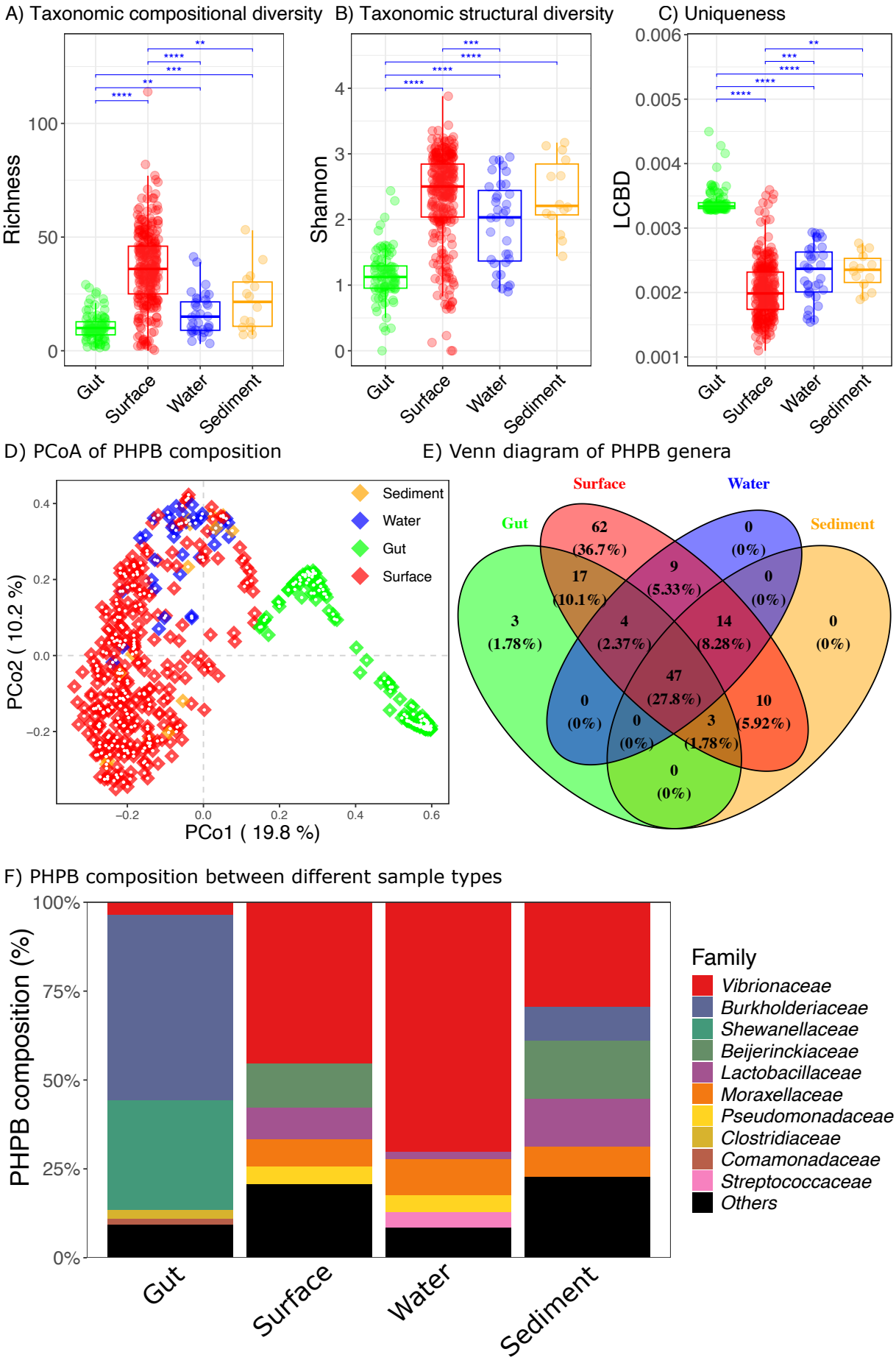
A) Taxonomic compositional diversity
B) Taxonomic structural diversity
C) Uniqueness
Richness
Shannon
LCBD
Gut
Surface
Water
Sediment
D) PCoA of PHPB composition
PCo2 (10.2 %)
PCo1 (19.8 %)
E) Venn diagram of PHPB genera
62 (36.7%)
0 (0%)
9 (5.33%)
17 (10.1%)
0 (0%)
3 (1.78%)
4 (2.37%)
14 (8.28%)
0 (0%)
47 (27.8%)
0 (0%)
0 (0%)
3 (1.78%)
10 (5.92%)
0 (0%)
F) PHPB composition between different sample types
PHPB composition (%)
Family
Vibrionaceae
Burkholderiaceae
Shewanellaceae
Beijerinckiaceae
Lactobacillaceae
Moraxellaceae
Pseudomonadaceae
Clostridiaceae
Comamonadaceae
Streptococcaceae
Others

Figure 1.8: Composition, and alpha diversity of the PHPB community. A: Species richness (Observed index); B: Species diversity (Shannon index); C: Uniqueness of species (local contributor of beta-diversity index). A, B and C: Dunn post-hoc test between sample types (****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$). D: PCoA plot with Bray-Curtis dissimilarities. E: Venn diagram of unique and shared PHPB genera between each sample type. Percentage indicates the ratio of each group PHPB genera to the whole dataset PHPB genera. F: Bar plots representing the composition of the top 5 PHPB families in each sample type, remaining families are listed as "Others" (ASV abundance >2%).

Using the ANCOM-BC approach, 62.1% (18/29) of the PHPB family biomarkers at the family level were found to be significantly enriched in surface (11 families representing 14 genera) and gut (11 families representing 13 genera) microbiotas compared to environmental microbial communities (Figure 1.9). Among these biomarkers, PHPB of the *Beijerinckiaceae* (*Methylobacterium radiotolerans* & *Methylorubrum rhodesianum*) and *Sphingomonadaceae* (*Sphingomonas rhizogenes*) families were the most enriched in surface-associated microbiotas relative to water communities (Figure 1.9A); and *Vibrionaceae* (*Vibrio tasmaniensis*, *Vibrio parahaemolyticus*, *Vibrio harveyi*) or *Oxalobacteraceae* (*Herminiimonas saxobsidens*, *Massilia timonae*) were also enriched in surface microbiotas compared to sediment microbiome (Figure 1.9B). On the other hand, in gut microbiotas, the most enriched PHPB biomarkers belonged predominantly to the *Shewanellaceae* family (*Shewanella* spp.) (Figure 1.9C&D)

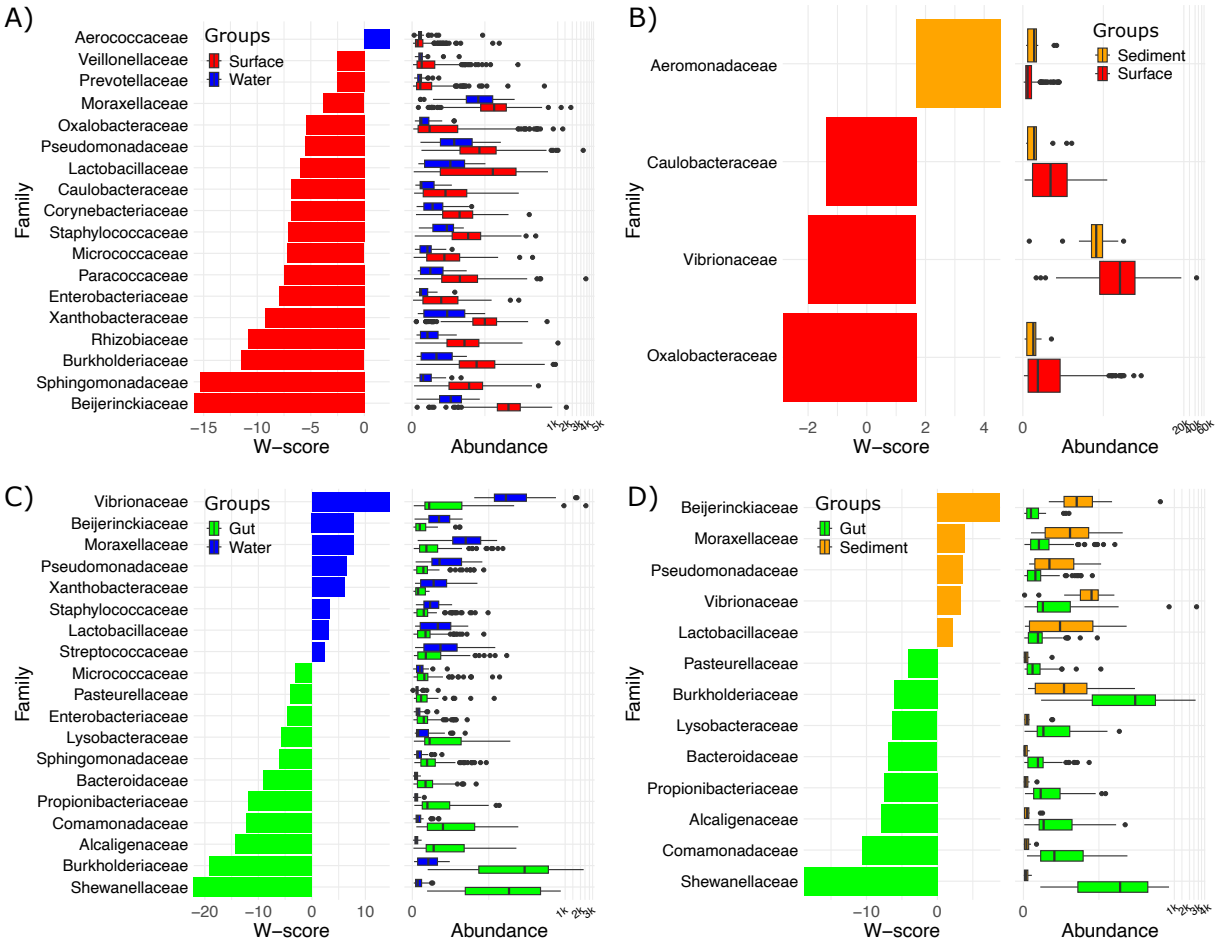

Figure 1.9: ANCOM-BC tests (W-score) and ASVs abundance according to the PHPB families in the PHPB communities between. A: Animals surface and Water. B: Animals surface and Sediment. C: Gut and Water. D: Gut and Sediment.

2.3.3 Testing the 'Sustaining the Rare' Hypothesis in Marine Holobiont Microbiotas

To explore the "sustaining the rare" hypothesis, we compared the frequency distribution of microbial genera in environmental and MH samples, specifically assessing variations in the relative abundance of rare microbial taxa (i.e.; taxa with a relative abundance < 0.1%, (Pascoal, Costa, & Magalhães, 2021). The richness of rare genera accounted for 91% and 70% in planktonic and benthic microbial communities, respectively. Surface microbiota also hosted 98.2% (337 genera) and 93.1% (351 genera) of these rare planktonic and benthic genera, respectively (Figure 1.10A). Of these shared genera, 21.7% (73 genera accounting for 23.2% of total surface microbiota reads Vs

0.82% in planktonic communities) and 15.4% (54 genera accounting for 10.2% of total surface microbiota reads Vs 1.01% in benthic communities) were significantly enriched in MH surface microbiotas compared to rare planktonic and benthic communities, respectively. 17.4% of the enriched genera exceeded the rarity threshold to become abundant in surface microbiotas. The five most enriched genera were *Methylobacterium* (ranging from 0.07% to 2.5%), *Pelomonas* (0.04% to 1.9%), *Rubritalea* (0.04% to 1.8%), *Halomonas* (0.01% to 1.4%), and *Bradyrhizobium* (0.03% to 1.4%) (Figure 1.10A). For their parts, fish guts hosted 57.1% and 49.6% of rare planktonic and benthic genera, respectively and 29.6% (accounting for 43.9% of total gut microbiota reads Vs 0.27% in planktonic communities) and 17.1% (accounting for 55.9% of total gut microbiota reads Vs 0.81% in benthic communities) were significantly enriched in fish gut microbiotas compared to planktonic and benthic communities, respectively (Figure 1.10B). 19.9% of these enriched genera exceeded the rarity threshold to become abundant in gut microbiotas. Among them, *Halomonas* (ranging from 0.01% to 21.1%), *Phaeodactylibacter* (0.08% to 10.5%), *Ralstonia* (0.01% to 9.8%), *Shewanella* (0.01% to 5.8%) and *Clostridium sensu stricto 1* (0.001% to 3.6%) were the most enriched.

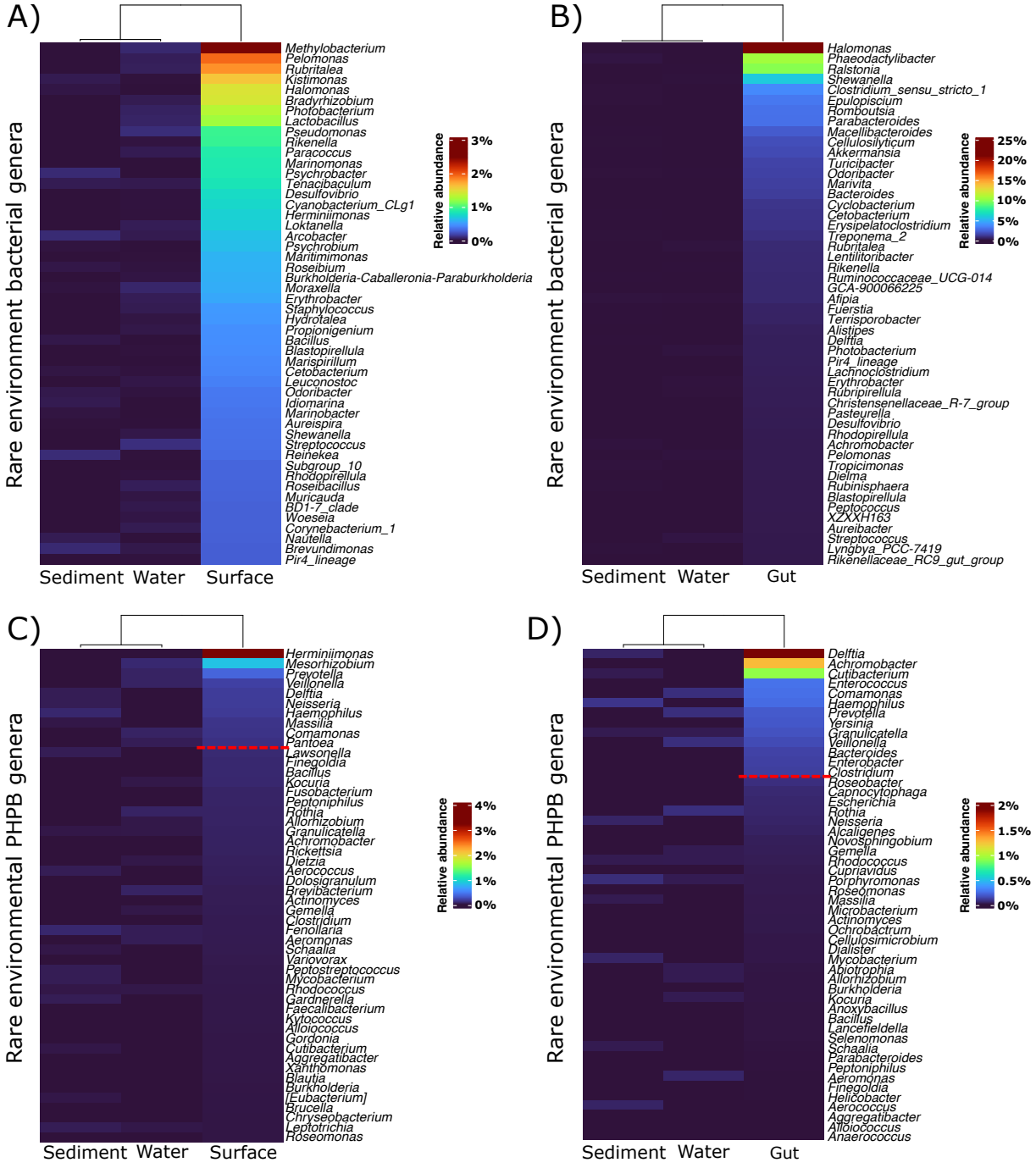

Figure 1.10: Heatmap of relative abundance distribution of: A&B: Rare bacterial genera within surrounding seawater microbial communities and the sediment microbial communities to the host-associated microbiotas (A: Surface, B: Gut). C&D: Rare PHPB genera within surrounding seawater microbial communities and the sediment microbial communities to the host-associated PHPB pathobiome (C: Surface, D: Gut). Data are shown by top 50 highest abundance genera in surface and gut pathobiome. A&B: All 50 genera from host-associated microbial communities have relative abundance >0.1%. C&D: Red dot-line represents the rare microbes threshold (0.1%) in host-associated pathobiome.

Of the 412 enriched genera (pathogens & non-pathogens) in MH, 16.9% (53/314) were identified as symbiotic in the surface microbiota and 14.4% (27/188) in the gut microbiota, while opportunistic genera accounted for 23.9% (75/314) and 14.9% (28/188) in the surface and gut microbiota, respectively. Beside the enrichment of pathogenic and non-pathogenic bacteria in host-associated microbiota, we also found that 13% (n=22) of the 169 PHPB genera are enriched in host microbiotas compared the rare environmental microbiome (8.4% and 17.6% of the bacterial richness in the surface and gut PHPB communities, respectively (Supplementary Material SM1-9). 7.5% and 28.3% of these PHPB genera switched from rare to abundant in surface and gut microbiotas, respectively (Figure 1.10C&D). PHPB genera *Herminiimonas* (*Herminiimonas saxobsidens*), *Mesorhizobium* (*Mesorhizobium loti*) and *Bacillus* (*Bacillus* spp) were the most enriched in surface microbiotas, whereas *Delftia* (*Delftia tsuruhatensis*), *Propionibacterium* (*Propionibacterium acnes*) and *Micrococcus* (*Micrococcus luteus*) showed the highest enrichment in gut microbiotas.

2.4 Discussion

2.4.1 Preserving the Invisible: The Hidden Role of Marine Holobionts in Sustaining Rare Microbes

Marine coral holobionts, which encompass not only the host organisms like corals, fish, and sponges, but also the intricate microbial communities living within them, are crucial to the structure and function of marine ecosystems (Wilkins et al., 2019). Several studies have shown that microbial communities in seawater and sediments differ markedly from those associated with coral holobionts, which offer distinct microhabitats, such as tissues, mucus, and guts, that shape unique microbial assemblages (sponges: (Dupont et al., 2013; Thomas et al., 2016); corals: (Sunagawa et al., 2015; Carlos, Torres, & Ottoboni, 2013); fish: (Balcázar, Lee, Pintado, & Planas, 2010; Schmidt et al., 2015; Parris et al., 2016; Chiarello et al., 2018, 2020; Cheutin et al., 2021); mollusks: (Pfister et al., 2014). Beyond differences in community composition, we recently showed that ASVs exclusive to coral holobiont surfaces (i.e., absent from plankton) accounted for over 85% of the total phylogenetic richness, indicating that the majority

of prokaryotic phylogenetic diversity was linked to animal-associated microbiotas in coral reefs (Chiarello et al., 2020). Here, we refined the estimates made in this previous studies by adding new compartments of the coral reef (i.e.; fish guts and sediments) and show that microbiotas associated with marine holobionts account for up to 98.4% of the total genera richness observed. However, gut microbiotas contributed significantly less (45.3%) than surface microbiotas (91.6%). While this lower contribution may partly reflect the fact that the guts of only fishes were sampled, it likely also stems from the greater exposure of holobiont surfaces to surrounding microbes and the more permissive settlement conditions they provide (e.g., lower anaerobic constraints, no digestive enzymes, no extreme pH, weaker immune regulation, weaker competition, concentration of environmental organic material creating more generalist-friendly conditions), making them a more favorable habitat for colonization by environmental microorganisms (Ley, Peterson, & Gordon, 2006). Nonetheless, the extreme contribution of marine coral holobionts to the microbial diversity confirm that marine coral holobionts may act as important reservoirs for environmental microbes (Chiarello et al., 2020; Frias-Lopez, Zerkle, Bonheyo, & Fouke, 2002; Hao, Gerdts, Peplies, & Wichels, 2015; Weiland-Bräuer et al., 2015) and particularly for rare ones, which, despite their low abundances, can have significant ecological roles due to their specialized and yet-to-be-understood interactions with other organisms (Litchman et al., 2024; Pedrós-Alió, 2012; Pascoal et al., 2021).

This hypothesis, recently termed the « sustaining the rare » concept (Troussellier et al., 2017), highlights the dual role of macroorganisms in preserving microbial diversity by (i) supporting rare microbial taxa and (ii) acting as vectors for the dispersal of geographically restricted species. The first component of this hypothesis posits that rare microbes are either maintained at their environmental levels within holobionts or become enriched due to favorable conditions for their growth. While Troussellier et al. (Troussellier et al., 2017) compellingly reviewed multiple lines of evidence supporting both processes, these mechanisms have never been rigorously tested at the ecosystem scale, nor specifically for rare microbes, using the conventional 0.1% relative abundance threshold. As a result, key questions remain unresolved, namely, what proportion of rare environmental microbes are hosted by marine holobionts, and what

fraction become enriched within them?

Our results show that surface and gut microbiotas serve as reservoirs for most rare environmental genera (95%) and foster the proliferation of a significant fraction (33.6%) of these taxa. This transition from rarity to abundance is characteristic of conditionally rare taxa (CRT) (Shade & Gilbert, 2015), which can be broadly categorized into two ecological types as outlined by Troussellier et al. (Troussellier et al., 2017). The first comprises host-associated specialists such as symbionts (mutualists or pathogens), which may dominate host-associated niches but persist at very low abundance in the environment, depending heavily on host presence (Pedrós-Alió, 2012; Bordenstein & Theis, 2015). The second group includes opportunistic copiotrophs, free-living seawater or sediment microbes that opportunistically colonize nutrient-rich host environments (e.g., guts, mucus, skin) but decline in abundance after leaving the host (Troussellier et al., 2017). We found that a substantial fraction (19.4% in surface and 21.8% in gut) of CRTs, particularly the most enriched, correspond to known marine animal symbionts. Except for *Phaeodactylibacter*, the ten most enriched genera in gut microbiotas (Figure 1.10B) are either generalist symbionts or part of the core marine fish gut microbiota known to be involved in pathogen defense (*Halomonas*, in cellulose/polysaccharide degradation (*Clostridium sensu stricto 1*, *Cellulosilyticum*, *Macellibacteroides*, *Epulopiscium*) and in protein degradation (*Ralstonia*, *Shewanella*, *Romboutsia*, *Parabacteroides*) (Jabari et al., 2012; Parris et al., 2016; Ngugi et al., 2017; Gerritsen et al., 2019; Huang et al., 2020; Y. Zhang et al., 2024; B. K. Singh et al., 2025). This trend appeared less pronounced in surface microbiotas, where a greater number of opportunistic bacteria (e.g., *Methylobacterium*, *Halomonas*, and *Bradyrhizobium*) were found among the most enriched genera (Figure 1.10A). The preferential enrichment of symbiotic bacteria in gut microbiotas may help explain why, despite representing a smaller reservoir of rare environmental diversity (52.3%) compared to surface microbiotas (94.5%), gut microbiotas enriched a higher proportion of genera (26.5% versus 21.5%).

These findings provide strong support for the "sustaining the rare" hypothesis, underscoring the pivotal role of marine macroorganisms in harboring and amplify-

ing rare microbial taxa that are otherwise scarce in the surrounding environment. In the current context of accelerating biodiversity erosion, particularly in marine ecosystems facing the combined pressures of climate change, pollution, and overexploitation (Hughes, Bellwood, Connolly, Cornell, & Karlson, 2014; Hughes et al., 2017), the extinction or decline of macroorganisms can therefore lead to the co-extinction of their associated microbial symbionts, many of which may have narrow ecological niches and limited dispersal capacities. This hidden dimension of biodiversity loss threatens not only microbial richness but also the functional resilience of ecosystems, as rare taxa often contribute disproportionately to ecosystem stability, adaptability, and long-term productivity (Shade & Gilbert, 2015).

2.4.2 The flip side of the coin: marine holobionts as a reservoir of potential pathogens

The "sustaining the rare" framework reveals a striking paradox with profound implications for both ecosystem services and public health. Traditionally viewed as a mechanism that supports microbial biodiversity by preserving low abundance taxa and the essential yet underexplored functions they may carry, our findings highlight that this ecological process can also serve as a double-edged sword. For the first time, we demonstrate that this same mechanism can inadvertently support the persistence, and even the proliferation, of potential human pathogenic bacteria (PHPB) in marine holobionts.

PHPB found in the ocean can be autochthonous such as some *Vibrio* and *Aeromonas* strains or others, such as *Mycobacterium marinum*, *Erysipelothrix rhusiopathiae*, and *Arcobacter* spp., that have only recently emerged as potential health threats (Doni, Martinez-Urtaza, & Vezzulli, 2023; Landrigan et al., 2020). Additionally, PHPB can originate from external, allochthonous sources, typically introduced into marine ecosystems through terrestrial runoff or human activities. These include well-known species such as *Helicobacter pylori*, *Escherichia coli*, *Enterococcus*, *Campylobacter*, *Clostridium*, *Shigella*, and *Salmonella* which are usually used as microbial indicators of fecal contamination or human activities (Wanjugi et al., 2016). Bacterial pathogens in the marine environment

are responsible for a wide range of acute and chronic diseases. These include diarrhea and gastroenteritis, ocular and respiratory infections, hepatitis, and wound infection (Landrigan et al., 2020). These bacteria typically belong to the rare biosphere in the marine environments, where their low abundance is thought to limit their ecological and epidemiological impact. However, recent large-scale surveys have shown that many PHPB are widely distributed across marine ecosystems, raising important questions about the mechanisms enabling their persistence (Troussellier et al., 2017).

Our study provides new insight into this question by identifying marine holobionts, through their surface and gut microbiotas, as potential biological sanctuaries for PHPB. These microbiotas appear to act not only as reservoirs but also as amplification systems. We show that 22 PHPB genera were significantly enriched in host-associated microbiotas compared to the surrounding environment. Even more strikingly, 7.5% and 28.3% of these enriched genera transitioned from rarity to dominance in surface and gut microbiotas, respectively, marking a clear shift in their ecological status (Figure 1.10C&D). When we broaden the lens to encompass the entire environmental pathobiome, not just the rare PHPB, the pattern becomes even more concerning: we detected a significant enrichment of 18 family biomarkers in host microbiotas only, including 11 families (14 genera) in surface samples and 11 families (13 genera) in gut samples (Figure 1.9). Furthermore, the number of exclusive PHPB genera to animal surfaces or gut (i.e., absent in plankton or sediment) accounted for over 48% of the total PHPB genera richness while environmental microbial communities did not harbor any unique PHPB genera (Supplementary Material SM1-10). Most of these enriched PHPB were autochthonous opportunistic pathogen, some of them considered as emergent such as Shewanella spp. which have been associated with several human disease cases (Fernandes, Sérvio, Silva, Tavares, & Rodrigues, 2023). Interestingly, many enriched PHPB are also well-established members of the marine holobiont microbiota such as members of the *Vibrionaceae* family. This may explain their enrichment in surface microbiotas. Although typically harmless, these microbes can shift to a pathogenic role when host or environmental conditions change. *Vibrio parahaemolyticus* and *Vibrio vulnificus* were one the most abundant and occurrent PHPB in our dataset (Figure 1.7). These two *Vibrio* species are known to pose serious threats to human health and

over the past decade, infections caused by *Vibrio* species have risen by 115%, with the sharpest increases observed along the US Gulf Coast, Northeast, and Pacific Northwest regions (Gilliss et al., 2013). Allochthonous PHPB belonging to the *Enterobacteriaceae* family (*Escherichia*, *Klebsiella* and *Enterobacter* genera) were also enriched but only in gut microbiotas suggesting that they could represent more sensitive sentinels than environmental samples for the detection of early contamination in surveillance systems.

As a consequence of this enrichment effect, the relative abundances of PHPB were approximately two times higher in MH samples compared to environmental samples (Figure 1.7), averaging 12% of total reads in holobiont microbiotas. Among the animal holobiont groups investigated in this study, fishes harbored significantly higher relative abundances of PHPB (15.6 ± 12.3%) along with corals and echinoderms. As marine animals, especially fish, can migrate and interact with various environments, they may act as vectors for the dissemination of bacterial communities, including PHPB. This dispersal mechanism, which is known to support microbial connectivity and diversity across ecosystems, may also carry health implications by promoting the spread of PHPB into new habitats (Troussellier et al., 2017; Khurana et al., 2020; Mills et al., 2022). In this context, not only the presence but also the abundance of these potential pathogens becomes critical. Emerging evidence suggests that pathogen load, the absolute or relative quantity of pathogens in a host or environment, plays a major role in determining infection risk, disease outcomes, and severity (Day et al., 2001). While it is important to note that the molecular detection of these PHPB does not prove their pathogenicity and that we did not investigate raw pathogen loads, their diversity, enrichment and transition from rare to dominant taxa within host-associated microbiotas underline a potential ecological shift that warrants further attention. The ability of marine holobionts to harbor, concentrate, amplify and and possibly disseminate both indigenous and externally derived pathogens suggests that they may play an underestimated role in the marine pathobiome dynamics, especially in increasingly anthropized coastal areas.

2.5 Conclusion

The dual role of holobionts, as protectors of rare, possibly beneficial taxa, and as reservoirs for potentially pathogen microbes, highlights the complexity of microbial interactions within marine ecosystems. In a context of global change, including biodiversity loss, climate warming, and escalating anthropogenic pressures, these dynamics may be further exacerbated. The disruption of host-microbiota relationships or the collapse of microbial diversity could not only reduce the buffering capacity of holobionts but also favor the emergence and transmission of pathogenic microbes.

2.6 Acknowledgments

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Chapter 2: Inventory of Potentially Pathogenic Bacteria and Antibiotic Resistance Genes in Different Environmental Compartments

Foreword

The previous Chapter has successfully displayed the establishment of NEMESISdb, a curated dataset of full-length 16*S rRNA* gene sequences for potentially pathogenic bacteria in human, fish and crustaceans. Especially, result from Chapter 1-2 which utilized NEMESISdb, described significant enrichment of several pathogens in marine holobionts compared to the planktonic and benthic microbiome in the Mayotte coral reef ecosystem, indicating the effectiveness of the dataset.

Among various marine ecosystems, coastal environment often acts as integrative hubs where human, animal, and environmental microbes can co-exist, and also proliferates the diversity of pathogenic bacteria community. However, most current studies only focus on inland systems, leaving these diverse marine pathobiome unexplored. Addressing this gap, our study provide insights on the influence of both intensive anthropogenic inputs and seasonality on the pathobiome and their respective resistome in Nha Trang Bay, Vietnam.

The second Chapter of this thesis investigates the presence of not only potentially pathogenic bacteria (PPB) but also antibiotic resistance genes (ARGs) in various environmental compartments of an anthropogenically impacted bay in Nha Trang. The first objective in this Chapter is to combine both phenotypic and genetic approaches to assess the occurrence of multidrug-resistant (MDR) bacteria from *Enterobacteriaceae* family and ARGs in the region (Chapter 2-1). To inherit and promote the advances of the NEMESISdb, the second objective examines the environmental drivers of phenotypic and genetic resistance within the different microbiomes in the same region (Chapter 2-2).

These analyses are detailed in two scientific articles, with one already published (Chapter 2-1), and the other currently under review (Chapter 2-2).

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Occurrence of multidrug-resistant *Enterobacteriaceae* and antibiotic-resistant genes in the anthropogenic impacted bay of Nha Trang, Viet Nam

Son Hoang Tran[1,2,6], Dinh Quang Vu[1], Dang Quang Nguyen[1], Duc Thinh Bui[1], Thu Uyen Do[1], Thi Thu Hang Le[1], Thi Thanh Tam Tran[1], Hoang Nam Pham[1], Kim Hanh Nguyen[3], Trung Du Hoang[3], Anne-Laure Banuls[4], Gwenn Pulliat[5], Sylvain Thery[5], Yvan Bettarel[2], Jean-Christophe Auguet[2], Quang Huy Nguyen[1]

[1]*MICH Group, LMI DRISA, University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology, Hanoi, Viet Nam;*

[2]*UMR MARBEC, Univ Montpellier, CNRS, Ifremer, IRD, Montpellier, France;*

[3]*Institute of Oceanography, Vietnam Academy of Science and Technology, Hanoi, Viet Nam;*

[4]*MIVEGEC, UMR-University of Montpellier-CNRS-IRD, LMI DRISA, Montpellier, France;*

[5]*ART-Dev, Univ Montpellier, CNRS, CIRAD, Univ Paul Valery Montpellier 3, Univ Perpignan Via Domitia, Montpellier, France;*

[6]*Institute of Genome Research, Vietnam Academy of Science and Technology, Hanoi, Viet Nam*

1 Occurrence of multidrug-resistant *Enterobacteriaceae* and antibiotic-resistant genes in the anthropogenic impacted bay of Nha Trang, Viet Nam

Antimicrobial resistance (AMR) has increased significantly due to the widespread transmission of multidrug-resistant (MDR) bacteria and antibiotic resistance genes within and between animals, environments, and humans. Unfortunately, the AMR situation in the coastal areas is little known. In this study, we investigated the occurrence and distribution of MDR *Enterobacteriaceae* from water and sediment samples collected in Nha Trang Bay, Viet Nam. Overall, 48/107 (45.8 %) identified and isolated bacteria belong to risk group 2 pathogens. More than 57% of *Enterobacteriaceae* isolates exhibited MDR phenotypes, in which *Escherichia coli*, *Klebsiella pneumoniae*, and *Citrobacter freundii* were the most prevalent species. In addition, eight ARGs including *sul*1, *sul*2, *sul*3, *tet*Q, *tet*B, *mec*A, bla_{VIM} and bla_{KPC} were detected with high abundance in most of water and sediment samples. Notably, class 1 integron-integrase gene *intI*1 was widely distributed (95.8%) in all samples with a total absolute abundance of 2.9 ×105 copies/ml water and 9.7 ×107 copies/g sediment. We also identified significant relationships between *sul*3, *tet*B, bla_{VIM}, bla_{KPC} and *intI*1 with bacterial density, chlorophyll A, phosphorus and temperature. These observations suggest that *intI*1 gene and nutrients could promote the emergence and propagation of antibiotic-resistance bacteria (ARB) and ARGs in the Nha Trang Bay.

1.1 Introduction

In 2019, the World Health Organization (WHO) declared antimicrobial resistance (AMR) as one of the top 10 global public health threats facing humanity (Organization et al., 2019). The emergence of antibiotic resistance genes (ARGs) in microorganisms precedes human use of antibiotics, nevertheless, it cannot be disputed that human activities dramatically accelerate the proliferation and transmission of ARGs (B. Chen et al., 2013; J. Perry, Waglechner, & Wright, 2016). To tackle AMR, the Food and Agriculture Organization of the United Nations (FAO), the UN Environment Programme (UNEP), the World Health Organization (WHO) and the World Organization for Animal Health (WOAH), known as the Quadripartite are joining forces to develop the One Health Priority Research Agenda for AMR (`https://www.who.int/publications/i/item/9789240075924`). Within the One Health context, the environment acts as the connection between humans and animals, or in other words they make up the dissemination route for resistance factors to moving between the different compartments (Bengtsson-Palme et al., 2018). Although AMR in human and veterinary health has been well investigated, environment is the most neglected part.

The occurrence and circulation of antibiotic resistant bacteria (ARB) and ARGs have been investigated in many studies from surface waters (Eckert, Di Cesare, Coci, & Corno, 2018; Proia et al., 2018; Reddy et al., 2022; Grenni, 2022), urban wastewater (Lorenzo et al., 2018; Nappier, Liguori, Ichida, Stewart, & Jones, 2020; Ibekwe et al., 2023), and wastewater treatment plants (Tehrani & Gilbride, 2018; Triggiano et al., 2020; Uluseker et al., 2021), identifying these areas as AMR reservoirs. However, antibiotic resistance in marine environments has not been studied as extensively despite recent efforts (Amarasiri, Sano, & Suzuki, 2020; H. Q. Anh et al., 2021; Alexander, Hembach, & Schwartz, 2022). Marine environments, particularly coastal areas can pose significant health risks to nearby people who have direct contact with the water through recreational activities or seafood consumption (Köck et al., 2018; Gabashvili et al., 2022). Coastal areas can function as reservoirs for potentially pathogenic bacteria (PPB) and/or AMRs because of the concentrated aggregations of bacteria from different sources, both marine and terrestrial, where environmental, human, and/or

animal-related bacteria can coexist, at least temporarily (Leonard et al., 2018; Siri et al., 2023). This mixing and the highly altered condition of coastal marine ecosystems i.e. anthropization, contaminants, and pharmaceutical residues, can affect species interactions, select antibiotic resistant bacteria (ARB), and trigger disease emergencies. Recent discoveries of potential human pathogenic bacteria in coastal area (Gyraite, Katarzyte, & Schernewski, 2019) emphasize the risk of infections by pathogenic antibiotic resistant bacteria associated with the marine habitat or marine products (Oliver, 2005; Park, Jung, Jung, Shin, & Hwang, 2009), posing a major world health challenge. In 2003, an estimated 120 million people contracted gastroenteritis and 50 million developed a severe respiratory infection after swimming or sea diving in marine waters, resulting in an estimated economic loss of some 12 billion dollars (Shuval, 2003). Similarly, of the 20% animal proteins provided to human population by aquatic livestock, approximately 10% are lost due to infectious diseases alone; this challenges the ability of aquaculture to feed the growing human population (Subasinghe et al., 2023). Furthermore, the overuse of antibiotics in aquaculture farming has led to high levels of AMR in aquatic environments and/or products, which have been reported in many intensive aquaculture farming countries. The aquaculture environment could be an important reservoir of resistance genes transferable to human or marine livestock pathogens and the emergence of pathogens with newly acquired antibiotic resistance (Reverter et al., 2020; Thornber et al., 2022; Suyamud et al., 2024).

With half a million inhabitants in Nha Trang city (southern central Viet Nam) and more than 2,000 aquaculture farms distributed along the coast, coastal waters are heavily impacted by anthropogenic inputs (of urban and/or aquaculture origins). A recent survey revealed that more than 5 kg of antibiotics were used per ton of lobster produced, and about 0.6 kg per ton of fish, which is considerably higher than findings from other studies. Notably, several antibiotics deemed "critical" and "highly important" by the WHO for human medicine such as tetracycline, trimethoprim, rifampicin, nifuroxazide, and colistin were administered routinely and preventively, with insufficient regulatory control and enforcement (Hedberg et al., 2018). Therefore, Nha Trang is a good model for studying the AMRs circulating in Viet Nam's coastal areas. This study aimed to investigate the distribution and abundance of potentially pathogenic

antibiotic resistant bacteria and ARGs in the marine environment of Nha Trang Bay, East Sea, Viet Nam. Our findings present the coastal environment as a significant reservoir of antibiotic resistant bacteria and ARGs and underline the urgent need to monitor the dissemination of AMR in these ecosystems.

1.2 Materials and Methods

1.2.1 Sample collection and water parameters measurement

Nha Trang Bay is a popular touristic destination for marine recreational activities located on the South coast of East Sea, Viet Nam (Figure 2.1). The total area of Nha Trang Bay is 249.65 km^2, which includes 211.85 km^2 of sea surface and 37,8 km^2 of islands (Figure 2.1). With half a million inhabitants in Nha Trang City and more than 200 aquaculture farms distributed along the coast of the Nha Trang Bay, the waters of the bay are heavily impacted by anthropogenic inputs (of urban and/or aquaculture origins), creating local gradients of anthropogenic influence. The origins and consequences of these contaminations remain however complex and unclear, with a variety of potential sources contributing, including urban inputs from the two rivers (Cai and Be) draining the whole Nha Trang urban area as well as aquaculture activities that produce eutrophication and release antibiotics into the water (H. N. K. Nguyen, Van, & Coloe, 2016). As a consequence, higher sedimentation fluxes, lower transparency and higher AMR have been recorded in zones closer to the coastline compared to off-shore zones (Latypov, 2015; Hedberg et al., 2018).

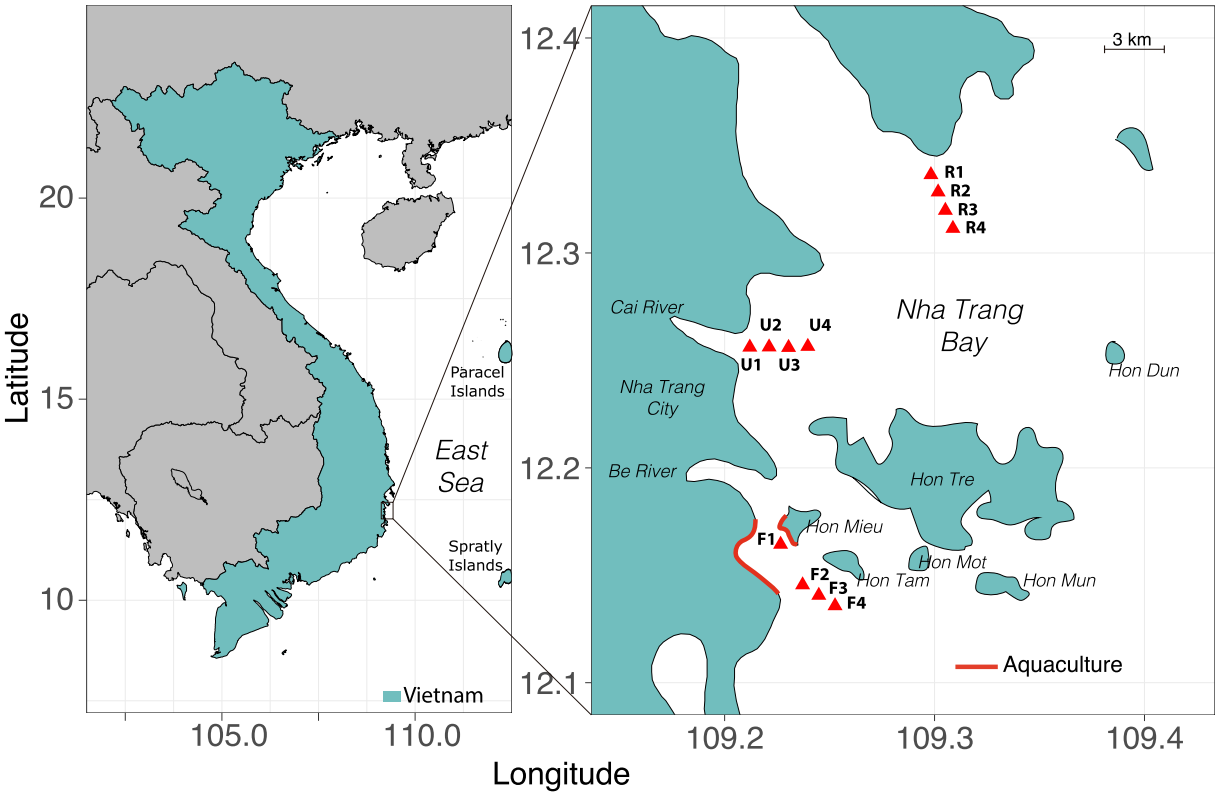

Figure 2.1: Sampling sites within the Nha Trang Bay. R : Recovery transect, U : Urban transect, F : Aquaculture farming transect.

Water column and sediment samples have been collected within two weeks in November 2022 along three transects representing these local differences in anthropogenic inputs (aquaculture versus urban) and a control area corresponding to an area of the bay where aquaculture activities have stopped for more than 5 years (Figure 2.1). In agreement with the coast to off-shore AMR gradient observed, four sampling stations (0, 1, 2 and 3 km from the coast) were sampled within each transect. The direction of the sample collection in each transect was chosen based on the direction of the currents and waves (`https://earth.nullschool.net`). At each station, water quality parameters including dissolved oxygen (DO - mg/L), salinity (‰), temperature (°C), and pH were immediately measured using a Multiparameter Water Quality Meter (Hanna Instrument, UK). Then, surface water (1L, 15 cm from the surface) and sediments (50g, 5 – 10 cm from the sediment surface) were collected at each station and stored at 4°C during transport to the laboratory for further analysis. Water and sediment samples were collected in triplicates and pooled together in order to integrate the variability of microbial and environmental parameters at each station. Other wa-

ter quality parameters including chlorophyll-a (Chl-a - µg/L), total dissolved nitrogen (NO_3 and NH_4 - µg/L), and total phosphorus (PO_4 – µg/L) were also measured. The coordinate and water quality parameters at each station are shown in Supplementary Material SM2-1.

1.2.2 Bacterial isolation and identification

In this study, we focused on some major waterborne and foodborne pathogens associated with human and aquatic animals, including *Enterobacteriaceae* family and *Aeromonas* genus. Bacteria were cultured on selective media (MacConkey Agar for *Enterobacteriaceae*, Aeromonas Agar for *Aeromonas* spp.) and Marine Agar (for total culturable bacteria). For water column samples, 100 milliliters of each sample were filtered through a 0.2 µm pore size cellulose acetate filter membrane (Sartorius Stedim Biotech, France) to capture the bacteria cells. Then, these filter membranes were put on the surface of the culture media (MacConkey Agar, Aeromonas Agar, Marine Agar) and incubated at 35°C for bacterial growth. For sediment samples, 200 mg of each sample was diluted in 5 ml of distilled water with 3.5% NaCl included (g/L) to mimic the sea water condition. Then, 150 µL of these diluted sediment sample were spread on the similar set of media and temperature. After 2-3 days of incubation, depend on growth time of different bacterial species, once the bacteria had become clearly visible on the plate, 107 pure colonies of the isolates were identified based on their morphological features using the MALDI Biotyper® Sirius One IVD System and the manufacturer's IVD kit (Bruker Daltonics, Germany). Bacterial isolates were then classified as potential pathogens according to the Risk Group Database (`https://my.absa.org/Riskgroups`).

1.2.3 Antibiotic susceptibility testing of *Enterobacteriaceae*

Only those bacterial species classified as potential enteric pathogens were subjected to antimicrobial susceptibility testing using the Kirby Bauer disc diffusion method on Mueller–Hinton Agar plates, according to the Clinical and Laboratory Standard Institute (CLSI) guideline. Antibiotics selected for the test included Amoxicillin + Clavu-

lanic acid (AUG-30µg); Ticarcillin (TIC-75µg); Cefoxitin (FOX-30µg); Cefotaxime (CTX-5µg); Ertapenem (ETP-10µg); Imipenem (IPM-10µg); Tobramycin (TOB-10µg); Gentamicin (CN-10µg); Nalidixic acid (NA-30µg); Ciprofloxacin (CIP-5µg); Levofloxacin (LEV-5µg); Trimethoprim + Sulfamethoxazole (SXT-25µg); Ticarcillin + Clavu-lanic acid (TCC-85µg); Aztreonam (ATM-30µg); Cefepime (FEP-30µg); Tigecycline (TGC-15µg); Fosfomycin (FF-200µg); Piperacillin (PIP-30µg); Ceftazidime (CAZ-10µg) and Piperacillin + Tazobactam (PTZ-36µg). A multidrug-resistant phenotype was defined as a bacterial isolate resistant to at least three antibiotics belonging to different antibiotic groups. A Multiple Antibiotic Resistance (MAR) index was calculated as the number of resisted antibiotics / total number of antibiotics used for the test. *Escherichia coli* ATCC25922 was included as a control in all the experiments.

1.2.4 Total environmental DNA extraction

For water column samples, 2 liters were also filtered through a 0.2 µm pore size cellulose acetate filter membrane (Sartorius Stedim Biotech, France) to trap the bacteria cells. Then, these filter membranes were cut into small pieces and used for DNA extraction using a DNeasy PowerSoil Pro kit (QIAGEN, Germany). For sediment samples, 200 mg of sample was used directly for DNA extraction with the same kit as above. The DNA extraction was carried out as per the manufacturer's instructions. The quality and quantity of DNA were verified by agarose gel electrophoresis, and measured using NanoDrop™ 2000/2000c Spectrophotometers (Thermo, USA).

1.2.5 Quantification of target genes

To evaluate the occurrence of ARGs within the marine environment in the Nha Trang Bay, 18 target ARGs associated with resistance to beta-lactams (bla_{VIM}, bla_{KPC}, bla_{NDM}, bla_{IMP}, $bla_{\text{OXA-48}}$), sulfonamides (*sul*1, *sul*2, and *sul*3), tetracyclines (*tet*B, *tet*X, *tet*M, and *tet*Q), methicillin (*mec*A), fluoroquinolone (*qnr*A), macrolide (*erm*B), multidrug efflux pump (*qep*A), aminoglycosides (*rmt*B) and colistin (*mcr*-1), were primarily screened in all water column and sediment samples using standard PCR assay (Detail of PCR primers for ARGs screening is listed in Supplementary Material SM2-5). The

abundance of ARGs with positive PCR results were then quantified using a realtime PCR assay with a FastGene 2x IC Green qPCR Universal Mix on the Quanstudio5 system (Thermo, USA). In addition, the concentration of the 16*S rRNA* gene (as a proxy for the bacterial abundance) and the Class 1 integron integrase gene (*intI*1) - a gene often associated with proliferation and transmission of ARGs and virulent genes was also determined (N. Ali et al., 2024; Heidari et al., 2024). The primer sequences of targeted ARGs and qPCR conditions are shown in Supplementary Material SM2-9. For each gene, the quality of the calibration curve was prepared with an efficiency ranging from 90% to 100% and reliable correlation coefficients (R^2) ranged from 0.97 – 0.99. The melting temperature (Tm) of the amplified product was also evaluated. The copy numbers of target genes were calculated based on the calibration curves, and expressed as copies/ml and copies/g in water column and sediment samples, respectively. Genes with number of copies per reaction lower than either the Limit of Detection (LOD) or Limit of Quantification (LOQ) were considered as under-detected (Supplementary Material SM2-10). Finally, the absolute abundance was calculated for ARGs, *intI*1 and 16*S rRNA* gene.

1.2.6 Statistical analysis

The significance test for the distribution of the isolated bacteria was performed using the Wilcoxon test. Comparisons between the relative and absolute abundances of ARGs in different substrates and transects were analyzed by the Kruskal-Wallis test, followed by a Dunn post-hoc test for non-parametric data and ANOVA's Levene test, followed by Tukey post-hoc test for parametric data. The p-value was corrected using Benjamini-Hochberg's method. For correlation analysis, we used the "rcorr" function in the Hmisc package to compute the correlation matrix and p-values are approximated by using the t or F distributions (Harrell, 2024). Permutational multivariate analysis of variance (Permanova) and Bray-Curtis distant matrix were used to calculate the differences in the total absolute abundance of ARGs between substrates and transects and Principal Coordinates Analysis (PCoA) was used to visualize these similarities and differences. The regression analyses of each ARG with the environmental variables were conducted using the "stepAIC" function from MASS library. The

function estimated the relationships between each ARG with multiple variables and assessed the significance of the relationship to find the best-explained variable. All statistical analyses and visualizations were performed using R version 4.2.0 and RStudio build 394.

1.3 Results

1.3.1 Distribution of putative pathogenic antibiotic resistant bacteria

Overall, 107 isolates were obtained from water column (15 cm from the surface) and sediment samples (10 cm from the surface) collected within one week along three transects (3 km long) representing local differences in anthropogenic inputs within the Nha Trang Bay (i.e.; active aquaculture area versus recovery aquaculture area versus urban impacted area, Supplementary Material SM2-1). As expected, most isolates belonged to *Enterobacteriaceae* (14/48, 36.8%) for water column samples and Bacillaceae (13/59, 22%) for sediment samples. The taxonomic affiliation of the 107 isolates is described in Supplementary Material SM2-2. The bacterial composition showed no difference between the origin of sample types (Wilcoxon test, $p>0.05$, Supplementary Material SM2-3) but obtained significant differences when compared with the origin of transects (Farm versus Urban and Farm versus Recovery, $p<0.05$, Supplementary Material SM2-3).

Among the isolates, 48 out of 107 (44.8%) were identified as PPB (risk group 2) by Maldi-Tof-MS (Table 2.1 and Supplementary Material SM2-2). These PPBs were found in high abundance in the urban transect (n=22, 45.8%), followed by the recovery transect (n=14, 29.2%) and the farm transect (n=12, 25%). The proportion and abundance of PPBs isolated was also higher in water column samples (n=27, 56.3%) than in the sediment samples (n=21, 43.7%). Species belonging to the *Enterobacteriaceae* family represented 45.8% (22/28) of PPB isolates. These species included *Klebsiella pneumoniae* (8/22, 36.4%), *Escherichia coli* (7/22, 31.8%), and *Citrobacter freundii* (4/22, 18.2%). Notably, *Klebsiella pneumoniae* and *Escherichia coli* were commonly shared among transects,

and between water and sediment environments. For the non-*Enterobacteriaceae* PPBs, *Pseudomonas aeruginosa* was in fact the most abundant PPB isolated from the water column samples (n=7, 26%) and was also identified in sediment samples (n=2, 4.7%). Within the genus *Vibrio*, *Vibrio algenolyticus* was the most frequent species isolated in both water column and sediment samples with quite similar proportions of 14.8% and 14.3%, respectively. The composition of isolated PPBs and the *Enterobacteriaceae* species showed no significant difference between both origins of substrates (water and sediment) and transects (Wilcoxon test, $p>0.05$, Supplementary Material SM2-3). These later indicate that the PPBs and especially the *Enterobacteriaceae* were widely abundant across all transects and substrates.

Table 2.1: Detail of the potentially pathogenic bacterial isolates*

Potentially pathogenic bacterial isolates	Number of isolates	
	Water column	Sediment
Citrobacter freundii	1	3
Enterobacter asburiae	1	0
Escherichia coli	3	4
Klebsiella pneumoniae	5	3
Kluyvera ascorbata	1	0
Acinetobacter baumannii	1	0
Clostridium baratii	1	0
Pseudomonas aeruginosa	7	2
Pseudomonas alcaligenes	1	0
Serratia marcescens	1	0
Vibrio alginolyticus	4	3
Vibrio fluvialis	1	0
Enterobacter cloacae	0	1
Acinetobacter haemolyticus	0	1
Bacillus cereus	0	1
Pseudomonas mendocina	0	1
Shewanella algae	0	1
Vibrio parahaemolyticus	0	1
Total	27	21

* Bacterial identification was conducted on MALDI Biotyper® Sirius One IVD System using the manufacturer's IVD kit (Bruker Daltonics, Germany). Bacterial isolates were classified as potential pathogens according to the Risk Group Database (`https://my.absa.org/Riskgroups`) (detail in Supplementary Material SM2-2).

Given that *Enterobacteriaceae* species, which are frequently considered indicators

of environmental pollution, are also significant foodborne pathogens with highly dynamic horizontal gene transfers among and within these species, 26 *Enterobacteriaceae* isolates were chosen for phenotypic-antimicrobial susceptibility testing. The results revealed that 24/26 (92.3%) *Enterobacteriaceae* bacteria were resistant to at least one antibiotic tested (Figure 2.2A). These isolates were multidrug resistant to amoxicillin & clavulanic acid (92.3%), followed by ticarcillin (52%), cefotaxime (48%), nalidixic acid (36%) and cefoxitin (28%) (Figure 2.2A). Nevertheless, they were completely susceptible to aztreonam, cefepime, and piperacillin (100%), and were highly susceptible to amikacin, ertapenem, and tigecycline (>90%). Of note, 15 isolates (57.7%) showed resistance to 3-7 antibiotics. The highly resistant bacteria were *K. variicola* and *Citrobacter freundii* which were resistant to seven antibiotics tested. Overall, multidrug-resistant (MDR) bacteria were measured in all transects with Multiple Antibiotic Resistance (MAR) indexes ranging from 0 to 0.33 (Figure 2.2B, Supplementary Material SM2-4).

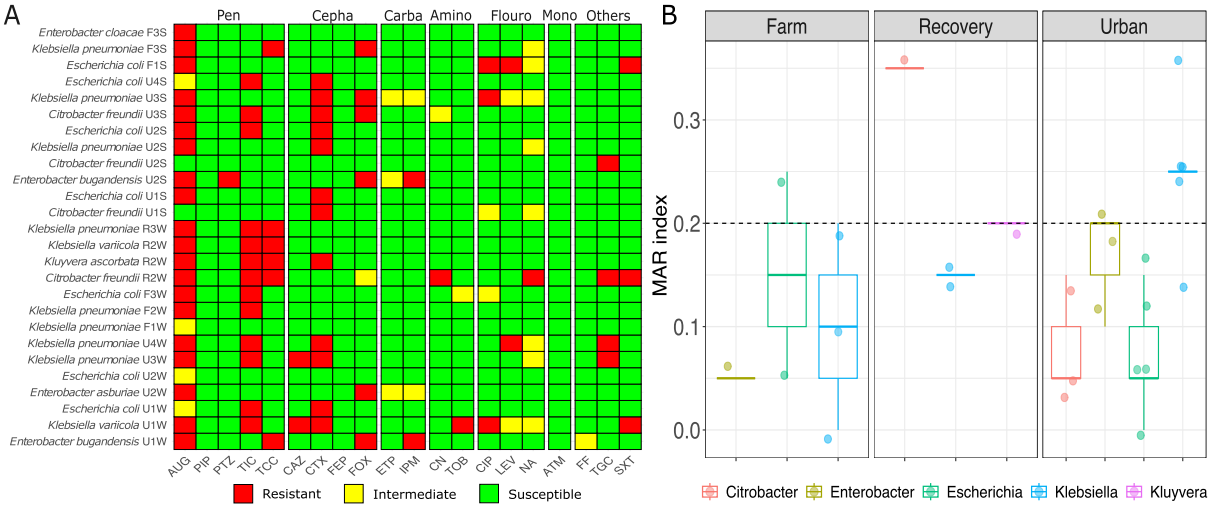

Figure 2.2: Phenotypic resistance profiles of bacterial isolates from *Enterobacteriaceae* species. (A) Antibiotic-resistant profile of *Enterobacteriaceae* species separated by antibiotic groups (Pen: Penicillins, Cepha: Cephalosporins, Carba: Carbapenems, Amino: Aminoglycosides, Fluoro: Fluoroquinolones, Mono: Monobactams; Others Antibiotics: Amoxicillin +Clavulanic acid (AUG-30µg); Ticarcillin (TIC-75µg); Cefoxitin (FOX-30µg); Cefotaxime (CTX-5µg); Ertapenem (ETP-10µg); Imipenem (IPM-10µg); Tobramycin (TOB-10µg); Gentamicin (CN-10µg); Nalidixic acid (NA-30µg); Ciprofloxacin (CIP-5µg); Levofloxacin (LEV-5µg); Trimethoprim + Sulfamethoxazole (SXT-25µg); Ticarcillin +Clavulanic acid (TCC-85µg); Az- treonam (ATM-30µg); Cefepime (FEP-30µg); Tigecycline (TGC-15µg); Fosfomycin (FF-200µg); Piperacillin (PIP-30µg); Ceftazidime (CAZ-10µg) and Piperacillin +Tazobactam (PTZ-36µg). (B) Multiple Antibiotic Resistance (MAR) index of *Enterobacteriaceae* isolates from different transects. Dot line at 0.2 represent the threshold for multidrug-resistant (MDR) bacteria.

1.3.2 Abundance and distribution of ARGs

Among the 18 ARGs screened by PCR in this study, only the *sul*1, *sul*2 and *sul*3 (sulfonamide resistance), *tet*Q and *tet*B (tetracycline resistance), *mec*A (methicillin resistance), and bla_{VIM} and bla_{KPC} (carbapenems resistance) genes exhibited positive results (Supplementary Material SM2-5). These 8 genes were all detected in water column samples (Supplementary Material SM2-6). *sul*1, *sul*3 and bla_{KPC} were found in all samples (12/12, 100%), followed by *sul*2 (11/12, 91.7%), *tet*Q (9/12, 75%), *mec*A (7/12, 58.3%), bla_{VIM} (5/12, 41.7%) and *tet*B (2/12, 16.7%). The average absolute abundance of *sul*1 (2.0 x 104 cop-ies/ml) was the highest among the 8 detected ARGs in water column samples, followed by *sul*3 (1.1 x 104 copies/ml), *tet*B (1.0 x 104 copies/ml), and *sul*2 (1.3 x 103 copies/ml). The average absolute abundances of *tet*Q, *mec*A, bla_{KPC} and bla_{VIM} was low, ranging from 3.0 x 101 copies/ml to 7.0 x 102 copies/ml.

For sediment samples, only 7 ARGs were detected as bla_{VIM} was under the detection limit of the assay in none of the sediment samples (Figure 2.3A). *sul*3 and bla_{KPC} were the most frequently detected ARGs (11/11, each, 100%), followed by *tet*Q (7/11, 63.6%), while *sul*1s, *sul*2, *mec*A, and *tet*B were found in only in 2 - 4 samples (18.2% - 36.4%). Average absolute abundance of *sul*1 (2.0 x 107 copies/g) was the highest among the 7 ARGs detected in sediment samples. Concentrations of other ARGs ranged from 5.4 x 104 to 2.1 x 106 copies/g. Class 1 integron integrase gene (*intI*1) was also detected frequently in both water and sediment habitats (100% and 90.1% of water and sediment samples, respectively), with abundance ranging from 6.01 x 103 to 7.04 x 104 copies/ml, and from 1.66 x 106 to 3.96 x 107 copies/g, respectively (Figure 2.3A&C and Supplementary Material SM2-6). Overall, ARGs were detected more frequently in the water habitat compared to the sediment habitat (Figure 2.3A) but the abundance of ARGs was higher in sediment (Figure 2.3C). Substrate type (water and sediment) was a major driver of the ARGs studied in this work (Permanova, R^2=0.55, $p<0.05$, Figure 2.3B and Supplementary Material SM2-3). In contrast, no significant effect of the transects on the ARGs on both sample types was observed (Permanova, $p>0.05$). However, when analyzing the ARGs only in the sediment habitats, a significant effect was obtained between transects (Permanova, R^2=0.54, $p<0.05$). In particular, the

ARGs abundances were higher in sediment samples compared to water column samples, especially for *intI*1 gene (Kruskal-Wallis, p<0.05) (Figure 2.3C). Besides the *intI*1 gene, *sul*1 and *sul*2 were the two ARGs displaying significantly different abundances between transects. The absolute abundance of *sul*1 was significantly higher in the recovery transect due to the fact that it could only be detected in this transect (Recovery vs Farm & Urban, p<0.05, Supplementary Material SM2-3& SM2-6). On the other hand, *sul*2 showed a significant higher abundance in urban transect compared to the two other transects (Urban vs Recovery and Farm, p<0.05, Supplementary Material SM2-3). The *tet*Q and *tet*B genes also showed a higher abundance in Urban and Farm transects to the Recovery transect based on the mean count but showed no significant differences when tested by Kruskal-Wallis (Figure 2.3A, Supplementary Material SM2-3).

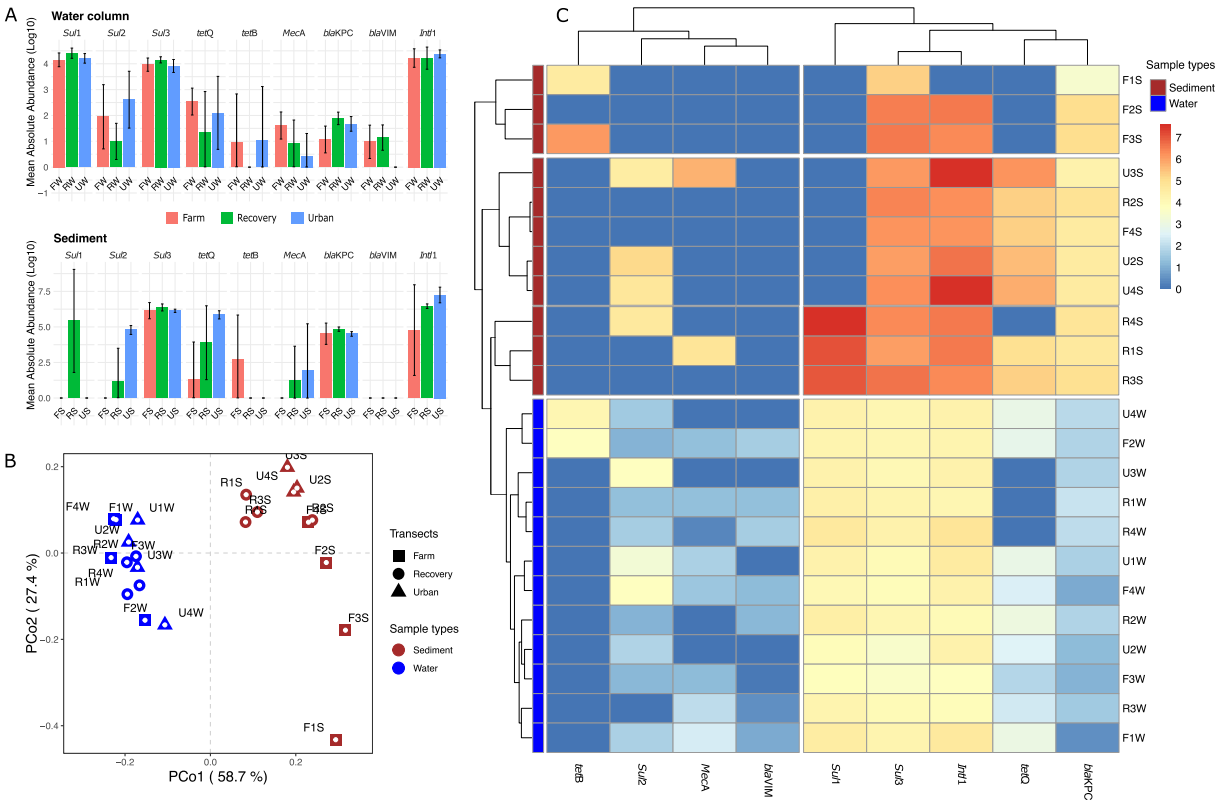

Figure 2.3: (A) Clustered bar plot of mean absolute abundance (log10) of 8 ARGs and intl1 gene; Standard Deviation (SD) of mean absolute abundance is calculated between the samples from 4 stations within each transect; (B) PCoA of the absolute abundance of ARGs between different substrates and transects; (C) Heatmap of absolute abundance (log10) of 8 ARGs and *intl*1 gene. Data used: absolute abundance was calculated by the number of copies/g for sediment samples and the number of copies/ml for water samples.

1.3.3 Environmental drivers of ARG abundances and ARG relationships

High positive correlations among the 8 quantified ARGs and the Class 1 integron integrase gene were found between *sul*3 and bla_{KPC} ($p<0.05$), *sul*3 and 16*S rRNA* gene ($p<0.05$), bla_{KPC} and 16*S rRNA* gene ($p<0.05$), bla_{KPC} and *intI*1 ($p<0.01$) (Supplementary Material SM2-8). Multiple linear regression models describing the relationships between each ARG and the environmental variables are shown in Figure 2.4 and Supplementary Material SM2-7. *Sul*3 abundance was strongly correlated with the bacterial abundances (16*S rRNA* gene) as shown in Figure 2.4, with nearly 70% of the gene's copies being explained by the number of bacteria. On the other hand, *sul*1 abundance was also explained by the relative abundance of pathogens (29.2%) but the major driver was salinity with 39.6% of variance explained ($p<0.05$, Supplementary Figure 6.3). In addition, most of the ARGs like bla_{KPC}, bla_{VIM}, *sul*3 and *tet*B, or *intI*1 gene were only explained by one variable each such as the 16*S rRNA* gene (bla_{KPC}, 39.8%), temperature (bla_{VIM}, 49.1%), 16*S rRNA* gene (*sul*3, 69%), Chlorophyll-a (*tet*B, 50.5%), or PO_4 (*intI*1, 39.5%).

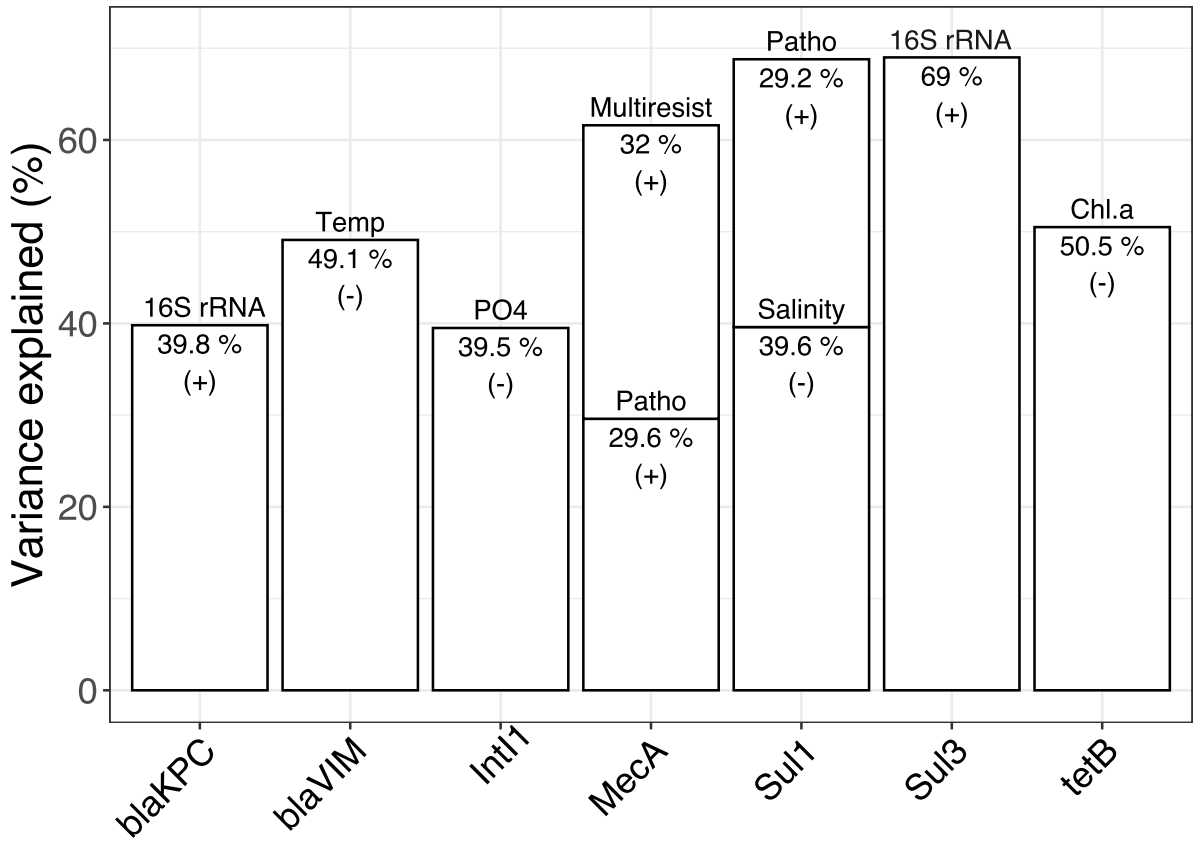

Figure 2.4: Multiple regression analysis between absolute abundance of ARGs or *intI*1 gene and the environmental variables or 16*S rRNA* gene (Positive relation: "+"; Negative relation: "-"). Data used: absolute abundance of ARGs, 16*S rRNA* and *intI*1 genes (number of copies/g for sediment samples and number of copies/ml for water samples); environmental variables (Supplementary Material SM2-1).

1.4 Discussion

Although antibiotic resistance is a natural phenomenon, it has been demonstrated that the use of antibiotics in human health, aquaculture, and veterinary and food-animal production has promoted the evolution and spread of ARB and ARGs (Prestinaci, Pezzotti, & Pantosti, 2015; Köck et al., 2018; Suyamud et al., 2024). ARB and ARGs prevalence have been extensively studied in humans and animals, compared to environmental settings. As bacteria and AMRs often cross environments and species boundaries, it is critical to study environment compartment to better understand the connections between the human, animal and environmental microbiomes (Köck et al., 2018; Reverter et al., 2020). Siri et al. 2023 conducted a literature review on AMRs in the

Southeast Asian water environments and emphasized the need for more investigation efforts on marine ecosystems as most of ARGs and ARB studies focused on freshwater and wastewater (Siri et al., 2023). In this context, our study provides extensive data on ARB composition, MAR values, and ARG abundances in the water column and sediments of a coastal bay impacted by both urban and aquaculture activities. Despite the various station locations selected in the methodology, we found that the lack of replicates for each sample type and the distances from the shore did not provide enough data to evaluate the impact of currents or waves on ARG abundance or antibiotic resistance. As a result, these stations would only serve as additional replicates within the transects.

We observed that 44.8% (48/107) of the total bacterial isolates belonged to pathogen risk group 2 which are potentially infectious agents for humans. In particular, potential human pathogenic bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Vibrio alginolyticus* and *Pseudomonas aeruginosa* distributed in all transects and substrate types (i.e.; water column and sediment). Comparison of the isolate's composition between different studies should be approached with caution due to biases associated with the different culture media used. However, our findings are consistent with those of Siri et al. (2023), who reported that *Escherichia coli* and *Vibrio* were frequently identified as predominant indicators in AMR detection, using both phenotypic and genotypic methods, in South East Asia water environments (Siri et al., 2023). In contrast, *Aeromonas spp.*, which is commonly found as antibiotic resistant bacteria in aquaculture systems worldwide (Caputo et al., 2023), was not detected in Nha Trang Bay. In addition to the potential bias related to culture media, this discrepancy could also be attributed to the fact that the majority of studies reviewed by Caputo and Siri have focused on freshwater habitats, where *Aeromonas spp.* thrives, although it has occasionally been isolated in marine aquaculture systems (Naudet et al., 2023). To a lesser extent, antibiotic-resistant *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* have also been retrieved from coastal waters and marine aquaculture waters (Ng et al., 2018; Suyamud et al., 2024).

Notably, 57.7% of the *Enterobacteriaceae* isolates (*Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*) showed MDR phenotypes with a MAR index ranging from

0.05 to 0.33. It was suggested that isolates with MAR index > 0.2 were recovered from sources with high risk of antibiotic resistant contamination (Krumperman, 1983). Nevertheless, in our study, such isolates with high MAR indexes were recovered in all transects (Figure 2.2B) but were in higher numbers in the urban area, indicating an influence of riverine inputs on ARGs of the coastal bay. It is noteworthy that the MAR indexes of *K. variicola* and *Klebsiella pneumoniae* isolates (0.33 and 0.24) were the highest among the *Enterobacteriaceae* species, emphasizing their dominant resistant abilities as ARB in the potentially pathogenic communities of the Nha Trang Bay. In their review, Suyamud et al. 2024 reported similar trends at the scale of South East Asia aquacultures with MAR values ranging from 8 to 79% for isolates and 10 to 40% for bacteria mixture (Suyamud et al., 2024). In another study, the mean MAR index of global aquaculture-related bacteria species was calculated to be 0.25 (Reverter et al., 2020). Hence, this study revealed lower level of antibiotic resistance in aquaculture isolates and samples compared to the level observed at the regional or global scale. The over-representation of freshwater aquaculture and pond aquaculture samples in Siri's review and Reverter's meta-analysis may explain in part this difference. Indeed, freshwater samples had a higher MDR percentage than seawater samples (Siri et al., 2023) and fish raised in pond systems were found to exhibit higher levels of ARB and ARGs compared to those raised in floating cage systems (P. T. P. Hoa et al., 2011).

ARGs, particularly conferring resistance to sulfonamides, tetracyclines, quinolines, macrolides and beta-lactams, have been reported to be highly abundant in marine sediments (J. Chen et al., 2019). In this study, antimicrobial resistance was most often against beta-lactams (i.e.; amoxicillin + clavulanic acid, ticarcillin, cefotaxim) consistent with the occurrence and abundance of bla_{KPC}, a gene associated with resistance to carbapenems, which are used as antibiotics of last resort (Figure 2.2A & Figure 2.3C). Overall, the 8 ARGs detected (i.e.; *sul*1, *sul*2, *sul*3, *tet*B, *tet*Q, bla_{KPC}, bla_{VIM} and *mec*A) in the three transect samplings of the Nha Trang bay showed high absolute abundances ranging between 3.0 x 101 - 2.0 x 105 copies/ml and 2.8 x 103 - 3.7 x 107 copies/g in water column and sediment samples, respectively. For *sul*1, *sul*2 and bla_{KPC}, these abundances are several orders of magnitude higher than those found in the sediment and water column of a previous study adopting the same study design as ours (Ng et

al., 2018). This pattern reflects the main antibiotics used in the surrounding clinical and aquaculture settings. For aquaculture in particular, Hedberg investigated the antibiotic practices in sea cage farms in Nha Trang region and reported that a large amount of critically important antimicrobials for human including rifampicin, sulfamethoxazole, trimethoprim, tetracycline, doxycycline, oxytetracycline, ampicillin, chloramphenicol, ciprofloxacin, enrofloxacin, colistin sulfate, and nifuroxazide are used in fish and lobster farming, and are therefore discharged directly into the marine environment every day (Hedberg et al., 2018). Even more remarkable, the amount of antibiotics used per ton of fish or lobster produced which was 100 and 1000 times higher, respectively, than in other aquaculture systems in Viet Nam or Thailand (Hedberg et al., 2018; Siri et al., 2023). Although we did not measure antibiotic concentrations in the present work, the overuse of antibiotics may in part have resulted in the high levels of ARG concentrations observed here. Moreover, a connection was observed between the antibiotic resistance profiles of the isolates and the prevalence of ARGs within the samples. The antibiogram results (Figure 2.2A) revealed that bacteria isolated from water column samples exhibited resistance to multiple antibiotics, in contrast to bacteria obtained from sediment samples. A similar pattern was also observed where ARGs being more frequently detected in the water column samples than in the sediment samples (Figure 2.3A). In particular, the *sul*1, *sul*2, *mec*A, and bla_{VIM} genes, associated with resistance to sulfonamides, penicillins, and carbapenem antibiotics, were predominantly detected in water column samples and the high resistance profiles to these antibiotics from water column's isolates summarized the strong correlation between the ARGs and ARBs. Sulfonamide and tetracycline resistance genes frequently occur in aquatic environments and are accompanied by the presence of integrons (Makowska, Koczura, & Mokracka, 2016; H. Shin, Kim, Han, & Hur, 2022; Suyamud et al., 2024). In our study, *intI*1 gene, an important indicator for the horizontal gene transfer (HGT) of ARGs, has been detected in 95.8% (23/24) of the samples with the average absolute abundance of 2.4 x 104 copies/ml and 8.8 x 106 copies/g in water column and sediment samples, respectively. In addition, we found a strong positive correlation between the abundance of *intI*1 and bla_{KPC} ($p<0.01$). *intI*1 gene is usually closely linked with various ARGs (Liao & Chen, 2018; H. Li et al., 2022) and is often found in the presence of disinfectants and heavy metals (Piergiacomo et al., 2020; Gupta, Sreekrishnan, & Ahammad,

2022) being detected in both pathogenic and non-pathogenic bacteria (Gabashvili et al., 2022). Furthermore, its abundance can fluctuate rapidly due to the short generation times of its host cells and the efficient horizontal gene transfer (HGT) between bacteria (Sidhu, Bae, Ogram, O'Connor, & Yu, 2021). A study reporting that 89.25% of multidrug-resistant (MDR) isolates carried *intI*1 suggests that this gene capture platform plays a critical role in the propagation and dissemination of MDR bacteria and ARGs (Chaturvedi et al., 2021). In agreement with these studies, the high ARG concentrations observed in our study suggests that *intI*1 facilitate the spread of resistance to beta-lactam by HGT within bacterial communities in the Nha Trang Bay. Furthermore, the *intI*1 gene is suggested to be a proxy for anthropogenic pollution and is allochthonous in water ecosystems (Gillings et al., 2015). This result should alert the stakeholders to the urgent need to implement measures to limit the spread of antibiotic resistance in coastal areas, notably by reducing the use of antibiotics and improving wastewater management practices, in order to protect the quality of marine ecosystems and public and veterinary health in the long term.

One important objective of our study was to investigate the potential environmental drivers of ARB composition and ARGs abundance in Nha Trang Bay. This bay has multiple input points for antibiotic and ARGs contaminations including the Cai and Be Rivers draining the 500,000-inhabitant urban area of Nha Trang and more than 200 aqua-culture farms distributed along the coast. While various factors may influence ARB and ARGs composition and abundance in the environment, the significant differences in the abundance of ARGs such as *sul*2 and *tet*Q in both sediment and water column samples (Figure 2.3A), among transects, support our hypothesis that urban and aquaculture inputs plays a significant role on the dynamics of ARB and ARGs in the Nha Trang Bay. However, our results showed that this is the substrate type (i.e.; sediment vs water column) that has the greater influence on the ARGs abundances (Figure 2.3B). In agreement with the study of Ng et al. 2018 who found the same patterns, ARG abundance was on average 260 times higher in sediments than in water samples in our study (Ng et al., 2018). Similarly, various studies have found that the variety and concentration of ARGs in sediment even surpass those in the water (Luo et al., 2010; J. Zhang et al., 2018). Sediments are therefore considered to be reservoirs

for the accumulation and spread of ARGs, unlike the water column (Jiang et al., 2018; Y. Liu et al., 2024). The accumulation of high levels of organic matter in sediments can reduce dissolved oxygen levels and promote the spread of ARGs through several mechanisms. First by stimulating bacterial (and among them ARBs) growth and activity, as nutrients or organic components (such as total nitrogen, total organic carbon, nitrate, and phosphate) are easily biodegraded, provide energy for their proliferation (Suyamud et al., 2024). These high microbial densities and the formation of biofilms facilitate close contact between bacteria, increasing the likelihood of horizontal gene transfer (HGT) and ARG dissemination in sediments. Second, anthropogenic pollutants such as antibiotics, heavy metals, and biocides tend to bind to organic matter, prolonging their presence in the sediment and continuously creating positive selective pressure on environmental microbiomes due to their attempt to resist anthropogenic stressors, thus prompting them to acquire ARGs and leading to antimicrobial resistance (AMR) (Pal et al., 2015). Finally, low-oxygen environments act as a stressor, triggering microbial adaptations such as an increase in plasmid transfer or plasmid number enhancing resistance due to higher plasmid copy numbers (McMahon, Xu, Moore, Blair, & McDowell, 2007). However, recent works show a complex landscape of changes in the performance of resistance genes in anaerobiosis (Deng et al., 2023; Pauzé-Foixet, Mathieu-Denoncourt, & Duperthuy, 2024; Bombaywala, Bajaj, & Dafale, 2024). Hence, along with the antibiotic residues, heavy metals and organic pollutants, environmental factors may play a crucial role in the dissemination of ARGs. Consistent with previous studies (Z. Lu et al., 2015; Pan, Lin, Zhang, Dong, & Yang, 2020), the total abundance of *sul*1 or *tet*B was significantly explained by environmental factors including salinity and chlorophyll A, indicating the potential impacts of these factors on the propagation of ARB and ARGs in the Nha Trang Bay. Similar to oxygen, salinity may act as an environmental stressor contributing to the complex evolution and prevalence of ARGs (Y.-J. Zhang et al., 2019). Besides, salinity is also recognized as a key environmental factor globally structuring prokaryotic communities (Lozupone & Knight., 2007; Auguet, Barberan, & Casamayor, 2010) and consequently their associated resistome. Recent studies indicate however that the effect of salinity on AMR or ARG abundance is contrasted with positive (M. Liu et al., 2018; Y.-J. Zhang et al., 2019) or no effect at all (Bergeron, Brown, Homer, Rehage, & Boopathy, 2016). Similarly, the

influence of soil salinity on the resistome may be indirect, exerted through its effect on the solubility of soil organic matter (Rath, Fierer, Murphy, & Rousk, 2019), the mobility of heavy metals (Acosta, Jansen, Kalbitz, Faz, & Martínez-Martínez, 2011), and soil pH. These factors are recognized as pivotal in shaping the resistome (McMahon et al., 2007; Hu, Gao, & Zhu, 2017; Q.-L. Chen, An, Zheng, Ma, & Su, 2018). Correlation between chlorophyll A concentrations and ARG abundances have been rarely socumented (Rajasekar, Qiu, Wang, Murava, & Norgbey, 2022). Another study found a negative correlation between chlorophyll A and *sul* genes in the water column of the Yangtze Estuary Estuary. So far, no explanation has been proposed, but since chlorophyll A is a proxy of primary productivity and indirectly of the load of organic matter and nutrients in the system, a relationship with the growth and activity of bacteria, and among them ARBs, seems evident. Taken together, these patterns confirm that both urban inputs and aquaculture activities are sources of ARB and ARGs contamination in the Nha Trang Bay. Even if we highlight the complexity of factors influencing the dynamics of ARB and ARGs in the Nha Trang Bay, sediment may play a dominant role as a reservoir for ARGs. The findings suggest that environmental factors such as nitrogen levels, chlorophyll A, and salinity may play a significant role in ARG propagation. These data emphasize the need for integrated management strategies that address not only direct anthropogenic inputs but also the broader environmental conditions that facilitate the persistence and dissemination of ARGs.

1.5 Conclusion

The occurrence and abundance of 8 ARGs and *intI*1 were widely detected in both water column and sediment samples in the Nha Trang Bay. Notably, the high abundance of *intI*1 particularly detected in the upper surface of sea water may facilitate the proliferation and spread of AMRs at a larger scale. Environmental factors such as pH, nitrogen, and phosphorus in the water were correlated with ARGs, indicating that they may play key roles for the formation or control of ARGs. Nha Trang's coastal habitat in Nha Trang is a hotspot for the accumulation of MDR, ARGs and MGEs, which can pose potential risks to human and animal health. Our study could provide a scientific basis for reservoir management to address the emergence of ARB and ARGs in aquatic

environments.

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CRediT authorship contribution statement

Anne-Laure Banuls: Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. Trung Du Hoang: Project administration, Methodology, Investigation, Funding acquisition, Formal analysis. Son Hoang Tran: Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis. Thi Thanh Tam Tran: Project administration, Funding acquisition, Formal analysis. Thi Thu Hang Le: Project administration, Funding acquisition, Formal analysis. Kim Hanh Nguyen: Project administration, Investigation, Funding acquisition, Formal analysis. Hoang Nam Pham: Project administration, Funding acquisition, Formal analysis. Yvan Bettarel: Project administration, Investigation. Dang Quang Nguyen: Methodology, Formal analysis. Sylvain Thery: Writing – original draft, Visualization, Project administration, Investigation. Dinh Quang Vu: Writing – original draft, Visualization, Methodology, Formal analysis. Quang Huy Nguyen: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. Thu Uyen Do: Formal analysis. Jean-Christophe Auguet:

Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. Duc Thinh Bui: Formal analysis. Gwenn Pulliat: Project administration, Investigation.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.rsma.2025.104084.

Data Availability Statement

Data will be made available on request.

This study is under review by the journal Environmental Pollution

Aquaculture and Human Activities Fuel Pathogen and Resistance Gene Dissemination in a Tropical Bay

Son-Hoang Tran[1,2], Trung Du Hoang[3], Kim Hanh Nguyen[3], Minh Hieu Nguyen[3], Hai Thi Vo[3], Thi Mien Pham[3], Marc Troussellier[1], Sébastien Villéger[1], Yvan Bettarel[1], Thierry Bouvier[1], Fabien Rieuvilleneuve[1], Claudia Ximena Restrepo Ortiz[1], Arthur Cousson[1], Anne-Laure Banuls[2,4], Constantin de Magny Guillaume[4], Ramsès Djidjou Demasse[4], Sylvain Goderuil[4,5], Gwenn Pulliat[6], Christian Culas[6], Bui Van Ngoc[7], Thi Kieu Oanh Nguyen[2], Quang Huy Nguyen[2], Jean-Christophe Auguet[1]

[1]UMR MARBEC, University of Montpellier, CNRS, Ifremer, IRD, Montpellier, France;

[2]LMI DRISA, Department of Life Sciences, University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology, Hanoi, 100000, Vietnam;

[3]Institute of Oceanography, Vietnam Academy of Science and Technology, Hanoi, Viet Nam;

[4]MIVEGEC, UMR-University of Montpellier-[5]Laboratoire de Bactériologie, Centre Hospitalier Universitaire de Montpellier Arnaud Villeneuve [6]ART-Dev, Univ Montpellier, CNRS, CIRAD, Univ Paul Valery Montpellier 3, Univ Perpignan Via Domitia, Montpellier, France;

[7]Institute of Biology, Vietnam Academy of Science and Technology (VAST), Hanoi 10072, Vietnam

2 Aquaculture and Human Activities Fuel Pathogen and Resistance Gene Dissemination in a Tropical Bay

Coastal ecosystems are increasingly threatened by microbial pollutants, including potential human pathogenic bacteria (PHPB) and antibiotic resistant bacteria (ARB), which pose major health risks at the human–ocean interface. In this study, we combined metabarcoding 16S rRNA gene and shotgun sequencing with culture-based approaches to characterize the diversity, composition, and resistance profiles of the pathobiome and resistome in Nha Trang Bay (Vietnam). Pathobiome diversity was high with 485 PHPB amplicon sequence variants spanning 49 genera and 98 species. PHPB communities were dominated by autochthonous opportunistic pathogens (*Staphylococcus*, *Bacillus*, *Acinetobacter*, *Pseudomonas* and *Aeromonas*) but also including enteric pathogens (e.g., *Prevotella* and *Arcobacter*) indicative of fecal contamination. Both phenotypic resistance and resistome data indicated high levels of multidrug resistance, with rifampicin resistance being particularly widespread, consistent with its massive use in aquaculture and tuberculosis treatment. Seasonal and spatial patterns revealed the strong influence of hydrological parameters (rainfall and riverine inputs) and anthropogenic pressures (aquaculture practices and tourism) on the structure of both the pathobiome and resistome. Together, these findings highlight that Nha Trang Bay represents a hotspot for pathogen and resistance gene dissemination, raising concerns for local populations and global health. Mitigation strategies should prioritize the reduction of fecal pollution, stricter regulation of antibiotic use in aquaculture, and the implementation of integrated One Health monitoring frameworks to manage microbial risks in coastal ecosystems.

2.1 Introduction

Coastal ecosystems, including estuaries, mangroves, coral reefs, seagrass beds, and sandy beaches, are among the most biodiverse and productive environments on Earth (Ray, 1991; Rashid, 2019). Although they cover only 17.4% of the land surface within 100 km of the sea, they are critical for global food supply, providing around 15% of agricultural crops and serving as nursery grounds for key fish species. However, these ecosystems face growing pressures from climate change (e.g., rising temperatures, sea levels, salinity shifts, acidification, and intensified storms) and human activities such as deforestation, shoreline development, industrialization, urbanization, aquaculture and tourism (Mellinger, Sachs, & Gallup, 1999; Doney et al., 2012; Santojanni et al., 2023; Crossland & Kremer, 2001; Culhane et al., 2024; A.-N. Zhang et al., 2021; Hamid et al., 2021; Newton et al., 2020). Beyond their ecological and economic importance, these pressures also have profound consequences on coastal microbiomes. Human-driven alterations not only reshape community composition but can also facilitate the emergence and spread of pathogenic bacteria and antibiotic resistance genes (Siri et al., 2023; Lin et al., 2022; D. Li et al., 2025; Booton et al., 2021; Powers, Wallgren, Marbach, & Turner, 2020; Landrigan et al., 2020).

The environmental resistome, the collection of all types of acquired and intrinsic resistance genes (Peng et al., 2024; Goh et al., 2024; Serrana, Nascimento, Dessirier, Broman, & Posselt, 2025; H. S. Tran et al., 2025) and the environmental pathobiome, collection of all potentially pathogenic bacteria (Jurelevicius et al., 2021; Cui, Fang, Huang, Dong, & Wang, 2017; Cui, Huang, Wang, & Fang, 2019; Y. Guo, Wu, & Sun, 2022; H.-J. Kim et al., 2023) in aquatic ecosystems is receiving growing interest and particularly coastal habitats. Potentially human pathogenic bacteria (PHPB) and antimicrobial resistance (AMR) in coastal ecosystems represents a critical concern for both environmental and human health, as these environments can serve as pathobiome and resistome reservoirs (Niegowska, Sanseverino, Navarro, & Lettieri, 2021; Cuadrat et al., 2020; Reverter et al., 2020; Zheng et al., 2021; Long et al., 2023). Among marine bacterial pathogens, species of *Vibrio* and *Aeromonas* are widespread across diverse aquatic habitats worldwide, causing diseases in both humans and animals (Naudet

et al., 2023). Other pathogens present in marine environments originate from external sources (allochthonous), such as indicators of fecal contaminations or enteric bacteria such as *Helicobacter pylori*, *Escherichia coli*, *Enterococcus*, *Campylobacter*, *Shigella*, and *Salmonella*. These PHPB are linked to numerous acute and chronic illnesses, including diarrhea, gastroenteritis, respiratory and ocular infections, hepatitis, and wound infections. These diseases are primarily transmitted through exposure to contaminated water or the consumption of contaminated seafood (Landrigan et al., 2020). However most studies have focused on FIB because pathogen diversity assessments in environmental waters are hampered by the absence of reliable approaches for simultaneously identifying large numbers of bacterial pathogens (Aw & Rose, 2012). The most reported AMR in coastal habitats are aminoglycosides, beta-lactams, (fluoro)quinolones, tetracycline and multi-drug (Suyamud et al., 2024; Goh et al., 2024; Serrana et al., 2025). Aquaculture effluents, sewage discharges, and riverine runoff represent major sources of PHPB and antibiotic resistance genes (ARGs) in coastal waters (Zheng et al., 2021; Y. Guo et al., 2022). These inputs typically vary with the seasons, implying that the coastal resistome may also undergo seasonal shifts. In addition, the distribution of PHPB and ARG was found to be related to various environmental factors, especially temperature, salinity and nutrient levels (Jacobs, Rhodes, Sturgis, & Wood, 2009; Y. Guo et al., 2022; Vikesland et al., 2019). Yet, there is still no agreement on the primary environmental driver of antimicrobial resistance in complex aquatic ecosystems (Y.-J. Zhang et al., 2019; L. Zhou et al., 2022). In summary, only limited information is available regarding how many bacterial pathogens and ARG, the dominant risk agents involved, and their relationships with influencing factors in coastal ecosystems that are impacted by diverse anthropogenic pressures. Understanding the dynamics and drivers of coastal pathobiome and resistome is critical to assess how these ecosystems may act as reservoirs and transmission hubs for microbial threats.

Situated along Vietnam's south-central coast, Nha Trang Bay represents both a crucial economic and cultural center and an ecologically rich hotspot with high biodiversity. Despite its significance, the area faces intense anthropogenic pressures that remain poorly characterized, largely driven by rapid population growth and accelerated economic development (Le, 2007; A. Nguyen et al., 2013). Concentrated human activities

in the region have raised concerns over untreated sewage, waste from aquaculture and coastal settlements, and the impacts of tourism (Lindsey & Holmes, 2000; Van Dung & Minh, 2023). Given the intensity and variety of these impacts, there is an urgent need to evaluate how they shape pathogenic bacteria and antimicrobial resistance in the region. Consequently, characterizing the diversity and prevalence of PHPB and antibiotic resistance in this heavily impacted coastal environment provides critical insights into the potential health threats arising from human impacts on tropical coastal environments In this study, we assessed the diversity and prevalence of PHPB and antibiotic resistance by collecting environmental samples along transects subject to varying degrees of anthropogenic influence during both dry and rainy seasons. We employed multiple approaches, including 16S rRNA gene metabarcoding, metagenomics and phenotypic resistance assays from bacterial isolates, to profile PHPB and antibiotic resistances. By combining high-resolution molecular and phenotypic approaches across spatial and seasonal gradients, this study offers a novel, comprehensive assessment of the coastal pathobiome and resistome, providing critical insights into the environmental and public health risks posed by human pressures in tropical marine ecosystems.

2.2 Material and Methods

2.2.1 Sample collection and pre-treatment

This study focuses on the seasonal diversity and distribution patterns of pathogens and antibiotic resistance genes profiles within the tropical bay of Nha Trang, Vietnam. Nha Trang covers a total area of approximately 249.65 km^2 and sits on the shores of Nha Trang Bay, which is a 500 km^2 embayment that opens to the western South China Sea. The population of the city in 2025 is now estimated at 353,846 people. In the 1990s, the local economy was primarily based on agriculture and small-scale fisheries, but by the 2000s, marine aquaculture became increasingly prominent with currently more than 200 floating farms implanted mainly on the shore of Hon Mieu Island. The intensification of marine aquaculture has led to localized environmental degradation, including organic pollution, habitat modification, coral community degradation, particularly around Hon Mieu (Du, Hieu, & Kunzmann, 2022; P. T. Anh, Kroeze, Bush,

& Mol, 2010; Tkachenko, 2015). In recent years, tourism has emerged as the dominant sector driving economic growth (A. Nguyen et al., 2013) and Nha Trang is currently the nation's largest tourist destination (i.e., one-third of Vietnam's inbound tourism). According to Khanh Hoa department of tourism (of Tourism, 2020), tourism in Nha Trang has expanded steadily since the 1990s, with a sharp increase in recent years, reaching 7,000,055 visitors in 2019 (H. V. Nguyen, Quang, Alang, Ngo, & Nguyen, 2022). This influx significantly boosts the city's population during the tourist season (dry season). This rapid expansion of tourism, particularly mass tourism, has fueled extensive shoreline development, leading to the destruction of natural habitats, increased waste discharge, and deterioration of water quality (Minh-Thu et al., 2025).

The climate in Nha Trang Bay is typically tropical with with a dry season lasting from January to August, and a rainy season lasting from September to December. The annual temperature ranges from 14–39°C and daily temperature changes in the bay are significant and pronounced, due to three main factors: tropical influences, monsoons, and local conditions. The average rainfall of Nha Trang Bay is 1,285 mm with strong seasonal variations. Precipitations were multiplied by 5 times between the dry (April 2019, 60 mm) and rainy season of our sampling (November 2022, 333.9 mm).

Two main rivers drain into Nha Trang Bay, the Cai River (catchment area of 1900 km^2) and Tac River (catchment area of 120 km^2), both of which originate in the mountains that exceed 2000 m to the west of Nha Trang. The Cai River, which flows through the center of Nha Trang, has an annual discharge of 916 km^3 (Fulfer, Nguyen-Ngoc, & Walsh, 2025). Because of seasonal precipitations, the discharge of the Cai river, which is the main source of freshwater flowing into the bay, increased dramatically during the rainy season (782 m^3/s) compared to the dry season (35 m^3/s). The sea-level of the area in rainy season is often 20-30cm higher than the dry season, and is increasing rapidly, driven by several process factors and climate changes (Van Chung, Long, Hoan, & Van Tuan, 2019).

For each season, water and sediment samples were collected within one week per season along three transects representing these local differences in anthropogenic in-

puts (aquaculture versus urban and a control area). The urban transect, located at the mouth of the Cai River (12.2608-109.2006), highlights the impact of anthropogenic inputs from the main city. In contrast, the fish farm transect, situated on Hon Mieu Island (12.1124-109.1321), is an aquaculture village with approximately 3,000 inhabitants. This ecosystem is rich in marine species and coral systems. Lastly, the recovery transect (12.3390-109.2920), located further north in the bay, was once an aquaculture hotspot. Intensive aquacultural practices in the past caused severe damage to the coral reefs in the area, which eventually led to a ban on all aquacultural activities, hence the name "Recovery". In agreement with the coast to off-shore AMR gradient observed in a study from Hedberg and colleagues, four sampling stations (0, 1, 2 and 3 km from the coast) were selected for sampling within each transect (Hedberg et al., 2018). The direction for the stations was chosen based on the direction of the currents and waves (`https://earth.nullschool.net`).

Water (1 litter, 15cm below the water surface) and sediment (50 grams, 5cm into the bottom) for microbial analyses were collected in triplicate at each station and immediately kept in a dark cooler at 4°C before frozen and further conserved at -80°C at the laboratory. Then, water samples were filtered within maximum of 6 hours after collected using a 47 mm 0.2 µm polycarbonate membrane (Whatman, Clifton, USA). Membrane were then frozen and kept at -80°C. Sediment samples were stored at -80°C until DNA extraction. Overall, a dataset comprising 144 samples, including both water and sediment samples from 12 different stations across 2 seasons was prepared for further analysis. At each station, in situ measurements of temperature (°C), salinity (‰) were taken using a multiparameter water quality meter (Hanna Instrument, UK). Additional water quality variables, including chlorophyll-a (Chl-a, µg/L), total dissolved nitrogen (NO_3^- and NH_4^+, µg/L), and total phosphorus (PO_4^{3-}, µg/L), were also determined. Geographic coordinates and all measured water quality parameters for each station are provided in Supplementary Material SM2-11.

2.2.2 DNA extraction and sequencing

The DNA extraction process was carried out on all water filters, and sediment samples (max 250 mg) using the MagAttract® PowerSoil® DNA EP extraction kit with automated processing and the liquid handling system KingFisher FlexTM (ThermoScientific®, Waltam, MA, USA). Nucleic acids were eluted in molecular water (Merck MilliporeTM, Burlington, MA, USA) and quantified on a NanoDrop 8000 TM spectrophotometer (ThermoScientific®, Wilmington, MA, USA). The DNA extracts were stored at -20°C until metabarcoding and metagenomic analyses.

For metabarcoding, the V4–V5 region of the 16S rRNA gene was amplified using the universal primers 515F–Y (5'-GTGYCAGCMGCCGCGGTAA-3') (Caporaso et al., 2011) and 926R (5'-CCGYCAATTYMTTTRAGTTT-3') (Parada et al., 2016), each tagged with Illumina-specific adaptor sequences at the 5' ends. PCR reactions (50 µL) were prepared with 25 µL of Phusion® High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs, Ipswich, MA, USA), 1 µL of each primer (10 µm), 2 µL of template DNA, 1.5 µL DMSO, and 19.5 µL molecular-grade water. Thermal cycling conditions consisted of an initial denaturation at 98 °C for 30 s, followed by 35 cycles of 98°C for 10 s, 60°C for 1 min, and 72°C for 1.5 min, with a final extension at 72 °C for 10 min. Amplicon size (450 bp) and primer specificity were confirmed by electrophoresis on a 2% agarose gel. Negative controls (blank extractions) and standard mock communities (ZymoBIOMICS Microbial Community DNA Standard II, Zymo Research) were processed in parallel to assess the quality of the DNA extraction and sequencing workflow. Amplicon sequencing was performed on an Illumina MiSeq platform by GeT-Biopuces (INSA, Toulouse, France). Raw sequence data are available in the National Center for Biotechnology Information (NCBI) sequence reads archive (SRA) database under Bioproject accession number PRJNA895209.

For metagenomics, 12 sediment samples from each station of the dry season were used for subsequent shotgun sequencing. Only ten of them (R1-4, U2-4, F1,3-4) were successfully sequenced and analyzed. Sequencing libraries were prepared using the Nextera XT DNA library preparation kit, and short-read sequencing was performed

using MiSeq 150 single-end sequencing (both from Illumina Inc., San Diego, USA) according to the manufacturer's instructions. Samples were sequenced at the licensed and certified sequencing service provider Microsynth (Balgach, Switzerland). Tagmentation library preparation and 150 bp sequencing (both from Illumina Inc., San Diego, USA) were utilized. Raw data obtained for each sediment sample under the NCBI SRA accession number: PRJNA981088.

2.2.3 Amplicon sequence variants (ASVs) processing and pathogen detection

The 'DADA2' R package v.1.2 (Callahan et al., 2016) and R software v. 3.4.3 were used to process sequence reads, following a script provided in the electronic Supplementary Material SM2-12. In brief, raw sequences were quality-filtered and trimmed according to read-quality profiles (maxN = 0; maxEE = [4,5]; truncQ = 2; truncLen = [250,250]). Amplicon sequence variants (ASVs) were then inferred using the DADA2 algorithm after pooling dereplicated reads from all samples. Forward and reverse reads were subsequently merged, and chimeric sequences were removed. Taxonomic assignment of ASVs was carried out with the naive Bayesian RDP classifier implemented in DADA2, using the SILVA nr V132 reference database. The resulting ASV count table, taxonomy, and representative sequences were integrated into a phyloseq object for downstream analyses using the phyloseq package v1.28.0 in R (McMurdie & Holmes, 2013).

ASVs filtration process utilized two methods to eliminate any contaminants. Initially, "decontam" R package (N. M. Davis et al., 2018) was employed to identify ASV contaminants in the dataset, using the package's "prevalence method". However, certain known extraction kit contaminants mentioned by Salter et al., such as ASVs from the *Bradyrhizobium* and *Cupriavidus* genera, were still presented in the data (Salter et al., 2014). These remaining contaminant ASVs were then manually removed from the final dataset. The dataset was then normalized by the implementation of the 'rarefy' function from the 'vegan' R package v. 2.5-5 (Dixon, 2003). To evaluate the effect of this process on the dataset, rarefaction was performed at 1,000, 5,000, and 10,000

reads per sample. Sample coverage was estimated using Zhang & Huang's coverage estimator (Z. Zhang & Huang, 2007) implemented in the entropart R package v.1.6-1 (Marcon & Hérault, 2015). The average coverage values obtained were 99%, 97%, and 88% for datasets rarefied at 10,000, 5,000, and 1,000 reads per sample, respectively. For downstream analyses, the dataset rarefied to 5000 reads per sample was selected as it provided high sample coverage while retaining the largest number of samples.

To detect potential human pathogenic bacteria (PHPB), we compared all ASVs of the dataset to NEMESISdb (Tran Son et al. in revision), a 16S rRNA full length sequence curated dataset designed to enable the identification and tracking of potentially pathogenic bacteria (PPB) for human. NEMESISdb contains 196,770 sequences spanning 1,757 species and 12 phyla of PHPB and is optimized for use with popular tools such as BLAST, enabling rapid and accurate detection of PPB in metabarcoding and metagenomic data. Only the ASVs in our data that matched a 16S rRNA sequence in the dataset with 100% similarity, 100% coverage, and more than 400 base pairs were considered as PHPB and included in further pathobiome analysis.

2.2.4 Metagenomics data processing and annotation of resistance genes

Raw reads were first assessed with FastQC (Babraham Bioinformatics) to identify and filter out low-quality sequencing data. After quality control, reads from each sample were assembled separately with MEGAHIT software (v1.1.3) using the preset parameters for the metalarge option (–k-min 27 –k-max 127 –k-step) (D. Li, Liu, Luo, Sadakane, & Lam, 2015) Assembled contigs longer than 500bp were then used for the prediction of open reading frames (ORFs) using ANGEL (v.2.4) (Pacific Biosciences) trained on the ARG protein variant model of the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2023). To characterize ARG profiles, we built a reference database by downloading sequences from ARGminer (version A), a manually curated and non-redusdant resource that integrates several widely used ARG databases, including CARD, DeepARG-DB, ARDB, MEGARes, UniProt, NDARO, SARG, ResFinder, and ARG-ANNOT (G. A. Arango-Argoty et al., 2020). Open reading frames

(ORF coding sequences) were then queried against this local ARG database using BlastX implemented in AC-DIAMOND (v1.0, -e 0.0001, -z 6, -k 1). Alignments were considered significant when they met the following thresholds: identity $\geq$ 50%, bit score $\geq$ 30, and E-value $\leq 10^{-4}$, following Gatica protocol (Gatica, Jurkevitch, & Cytryn, 2019). Identified ARG hits were classified into resistance types (antibiotic classes) and subtypes (gene level) based on ARGminer-A structural annotations. Predicted ARG abundances were quantified as reads per kilobase per million mapped reads (RPKM), a normalization method accounting for differences in sequencing depth and gene length, calculated as: RPKM = numReads / ((geneLength / 10^3) × (totalNumReads / 10^6)) (Mortazavi, Williams, McCue, Schaeffer, & Wold, 2008). For each sample, relative ARG abundances were determined by summing RPKM values for all ARGs belonging to a given resistance type (antibiotic class) or subtype (gene level). Raw sequencing data is available under the accession number PRJEB41834.

2.2.5 Bacterial isolation and identification

This study focused on major environmental pathogens linked to humans, including both gram-positive and gram-negative bacteria, across two seasons. Bacteria were isolated and grown on various selective media (MacConkey Agar for Enterobacteriaceae, TCBS Agar for *Vibrio* species, Aeromonas Agar for *Aeromonas* species, SS Agar for *Salmonella* and *Shigella* species, Staph Agar for *Staphylococcus* species) as well as Marine Agar for all culturable marine bacteria. For water samples, 100 milliliters of each sample were filtered using a 0.2 µm pore size cellulose acetate filter membrane (Sartorius Stedim Biotech, France) to collect bacterial cells. These membranes were then placed on the culture media and incubated at 37°C to allow bacterial growth. For sediment samples, 200 mg of each sample was mixed with 5 ml of distilled water containing 3.5% NaCl (g/L) to simulate seawater conditions. Then, 150 µL of the diluted sediment sample was spread on the same media and incubated under similar temperature. After 2-3 days, depending on the growth rate of different bacteria, visible colonies were identified based on their morphology using the MALDI-Tof Biotyper® Sirius One IVD System and the manufacturer's IVD kit (Bruker Daltonics, Germany).

2.2.6 Antibiotic susceptibility tests

All bacterial species isolated in the previous steps were submitted to antimicrobial susceptibility testing using the Kirby-Bauer disc diffusion method on Mueller–Hinton Agar plates, following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (on Antimicrobial Susceptibility Testing et al., 2018). The antibiotic susceptibility tests were categorized into two groups: gram-positive and gram-negative bacteria, each tested with a specific set of antibiotics. For gram-positive bacteria, eight antibiotics were selected: Cotrimoxazole (SXT-25µg), Erythromycin (ERY-15µg), Fosfomycin (FOS-200µg), Gentamycin (GEN-10µg), Kanamycin (KAN-30µg), Ofloxacin (OFX-5µg), Rifampicin (RIF-5µg), and Tetracycline (TET-30µg). For gram-negative bacteria, 17 antibiotics were tested: Amoxicillin & Clavulanic acid (AUG-30µg), Aztreonam (ATM-30µg), Cefepime (FEP-30µg), Cefoxitin (FOX-30µg), Ciprofloxacin (CIP-5µg), Ertapenem (ETP-10µg), Fosfomycin (FOS-200µg), Gentamycin (GEN-10µg), Imi- penem (IPM-10µg), Levofloxacin (LEV-5µg), Nalidixic acid (NAL-30µg), Piperacillin (PIP-30µg), Piperacillin/Tazobactam (PTZ-36µg), Ticarcillin (TIC-75µg), Ticarcillin & Clavulanic acid (TCC-85µg), Tobramycin (TOB-10µg), and Cotrimoxazole (SXT-25µg). A multidrug-resistant phenotype was defined as a bacterial isolate resistant to at least three antibiotics from different antibiotic groups. The multiple antibiotic resistance (MAR) index is defined as the proportion of resistance to a panel of tested antibiotics. It was calculated for each strain or group of strains, following the method described by Krumperman: the MAR index is equal to a/b, where “a” represents the number of antibiotics to which the strain was resistant, and “b” represents the number of antibiotics to which the strain was exposed (Krumperman, 1983).

2.2.7 Definition of the core pathobiome and resistome

As in any habitat, PHPB and ARGs can range from permanent residents to transient members. To identify the core pathobiome and resistome, we examined species abundance distributions (SADs) of both PHPB and ARG and partitioned them into core and satellite groups (Magurran & Henderson, 2003). For each PHPB genus or ARG subtype, an index of dispersion was calculated as the variance-to-mean abun-

dance ratio (VMR) multiplied by its occurrence. This index was then used to assess whether PHPB genus or ARG subtype followed a Poisson (i.e., stochastic) distribution, with values falling within the 2.5%–97.5% confidence interval of the X^2 distribution (C. Krebs, 1999). PHPB genus or ARG subtype with index values <1 were classified as under-dispersed, indicating a uniform distribution and assignment as core taxa, whereas those with index values >1 were considered over-dispersed, reflecting clustered distributions characteristic of satellite taxa.

2.2.8 Microbial biodiversity and statistics tests

Alpha diversity within PHPB and ARGs communities was quantified using richness (number of PHPB ASVs or ARG subtypes), the Shannon diversity index (H) and the local contribution to beta-diversity index (LCBD). Differences in alpha diversity among season, transect or subtrate type were tested with a Kruskal–Wallis test followed by Dunn's post hoc comparisons, with p-values adjusted using Bonferroni correction. Relationships between alpha diversity indices and the environmental parameter measured were tested by means of multiple linear regression. These analyses were conducted using the "stepAIC" function from MASS library.

Beta diversity was calculated using Bray–Curtis dissimilarities calculated with the 'vegan' package in R (Oksanen et al., 2013) and community dissimilarities were visualized using principal coordinate analysis (PCoA). Influence of season, transect, subtrate type and environmental parameters was assessed by permutational analysis of variance (Permanova). To identify PHPB or ARG biomarkers (differentially abundant PHPB or ARG between sample categories) across season, transects or subtrate type, we applied an Analysis of Composition of Microbiomes with Bias Correction (ANCOM-BC), with p-values adjusted using the Benjamini–Hochberg procedure, implemented in the microbiomeMarker package (Cao et al., 2022) All statistical tests and visualizations were carried out using R version 4.2.0 and RStudio build 394.

2.3 Results

2.3.1 Whole bacterial community diversity and composition

ASV richness and diversity were significantly lower in the rainy season compared to the dry season ($p < 0.05$; Supplementary Material SM2-13 & SM2-14). Sediment samples showed higher richness, Shannon diversity, and LCBD values than water samples ($p < 0.05$), while no seasonal effect was observed for LCBD (Supplementary Material SM2-14 & SM2-15). Although overall richness and diversity did not differ significantly among transects, LCBD values indicated higher bacterial uniqueness in the Urban and Fish farm transects compared to the Recovery transect ($p < 0.05$). Community composition differed strongly between seasons and substrates (Permanova, $p < 0.05$; $R^2 = 0.15$ and $R^2 = 0.05$, respectively; Supplementary Material SM2-16 & SM2-17). PCoA ordination further showed greater temporal stability in sediment communities compared to the higher variability in water communities. Across all samples, 49% of genera were shared among transects, with *Proteobacteria* (40.5%), *Bacteroidota* (19.9%), and *Firmicutes* (9.1%) as the dominant phyla (Supplementary Material SM2-17).

2.3.2 Diversity and Drivers of Potential Human Pathogenic Bacteria (PHPB)

Comparing the ASV sequences retrieved from our 143 samples to the PHPB sequences of the NEMESIS-db database allowed us to detect 485 PHPB ASVs distributed over 49 genera and 98 species (Supplementary Material SM2-18). Transects and season were the two main factors shaping the alpha diversity of PHPB. On average, PHPB richness (i.e., number of ASVs) was significantly higher in Urban transect samples (7 ± 6 ASVs) compared to the other two transects (3 ± 2 ASVs and 4 ± 3 ASVs for fish farm and recovery transects, respectively) (Figure 2.5A). A similar pattern was observed for Shannon diversity (Figure 2.5B). In contrast, PHPB uniqueness, as measured by the local contribution to beta diversity (LCBD) index, did not differ significantly among transects (Figure 2.5C). Pronounced seasonal differences were detected in PHPB tax-

onomic richness, diversity, and uniqueness. Samples from the dry season exhibited higher diversity and uniqueness, as well as greater species richness, compared to those from the rainy season (Figure 2.5D-F). In contrast, no effect of subtract type (i.e.; water Vs sediment) on PHPB alpha diversity was found (Figure 2.5G-I).

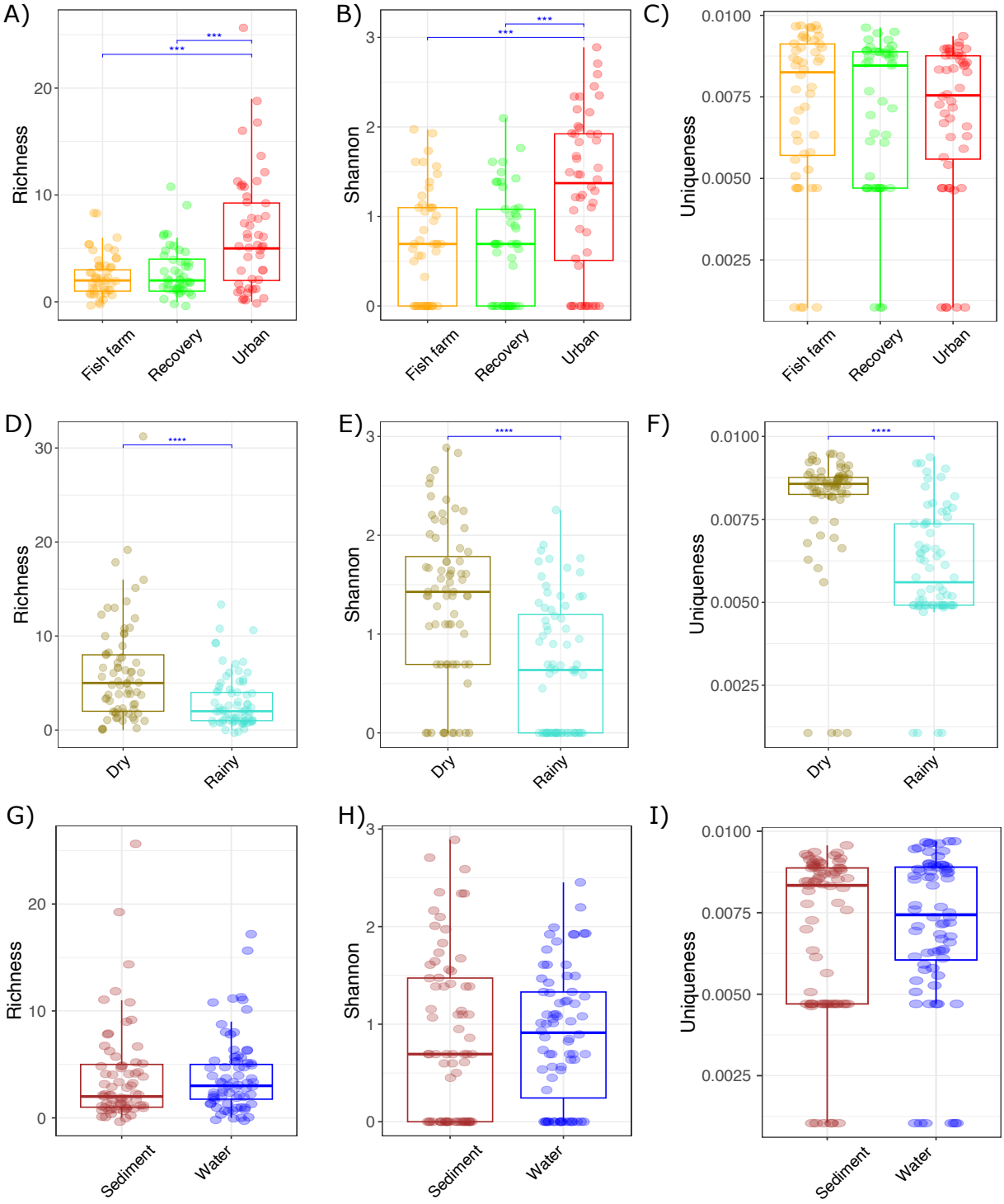

Figure 2.5: Alpha diversity indices of potential human pathogenic bacteria between transects, seasons and substrates. A&D&G: Species richness (Observed index); B&E&H: Species diversity (Shannon index); C&F&I: Uniqueness of species (local contributor of beta-diversity index). Significant differences were observed with Dunn post-hoc test between groups (****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$).

PHPB accounted for 0.28% of the total microbial community, with their relative

abundance being significantly higher in the urban transect (0.30 ± 0.29%) than in the fish farm (0.09 ± 0.09%) and recovery (0.15 ± 0.19%) transects (Supplementary Material SM2-15 & SM2-19). Overall, PHPB relative abundances exceeded the 0.1% rarity threshold in 74.4% of urban transect samples, compared to 33.3% and 43.4% in the fish farm and recovery transects, respectively (Figure 2.6). In the urban transect, distance from the shore was a significant factor, with relative abundance decreasing as distance increased during both seasons (Figure 2.6, Supplementary Material SM2-19). No seasonal variation in PHPB relative abundance was detected (p>0.05, Supplementary Material SM2-13).

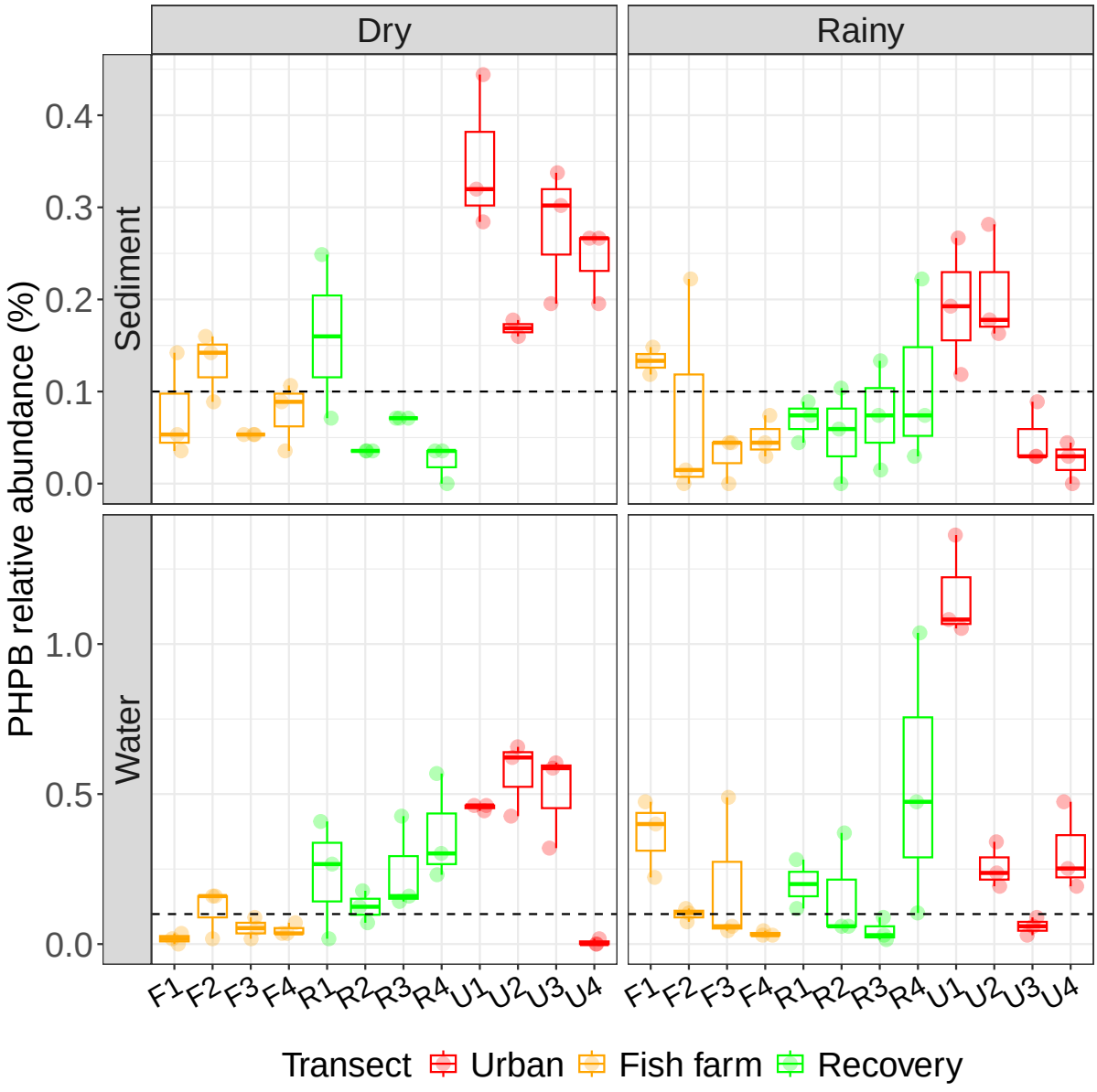

Figure 2.6: Relative abundance of PHPB to the whole bacteria between transects, seasons and substrates. Dot line represents the rare threshold (0.1%) for microbes.

Analyzing the PHPB composition, we detected that 18 PHPB genera were part of the core pathobiome of our samples, with *Staphylococcus* (*Staphylococcus epidermidis*), *Prevotella* (*Prevotella copri*), *Acinetobacter (Acinetobacter soli*), and *Vibrio* (*Vibrio harveyi*, *Vibrio parahaemolyticus*) as the dominant PHPB and representing 11.9%, 8.6%, 8.2%, and 8.2% of the total PHPB reads, respectively (Figure 2.7C). None of the PHPB species were present in more than 50% of the samples, indicating a strong turnover of species across the dataset. The potential infections associated with these species are listed in Supplementary Material SM2-20. The most abundant PHPB families were *Vibrionaceae* (14.9%), *Moraxellaceae* (10.5%), and *Bacillaceae* (9.8%), together representing more than one-third of the total reads. Community structure and composition of PHPB were strongly influenced by season (Permanova, R^2 = 15.2%, $p < 0.05$, Figure 2.7A), substrate type (R^2 = 5.3%) and transect (R^2 = 3.4%). Seasonal patterns showed that *Erysipe- lotrichaceae* (*Turicibacter sanguinis*), *Staphylococcaceae* (*Staphylococcus epidermidis*), and *Prevotellaceae* (*Prevotella copri*) were significantly enriched during the rainy season. Substrate type also shaped PHPB composition: *Xanthobacteraceae*, *Bacillaceae*, and *Mycobacteriaceae* were more abundant in sediments, whereas *Moraxellaceae* and *Staphylococcaceae* were enriched in the water column (Figure 2.7D, Supplementary Material SM2-21). Transects further revealed distinct patterns in family-level composition. For instance, *Lactobacillaceae* were significantly enriched in fish farm and recovery transects during the dry season, while *Pseudomonadaceae* and *Arcobacteraceae* were primarily associated with the urban transect (Figure 2.7D, Supplementary Material SM2-22). In terms of relative abundance, *Lactobacillaceae* accounted for 1.1% of PHPB reads in the urban transect, increasing to 1.7% in recovery sites and 4.7% in fish farm samples. Conversely, *Arcobacteraceae* represented 3.7% of PHPB reads in the urban transect, but remained below 1% in both fish farm and recovery transects (Figure 2.7D, Supplementary Material SM2-22).

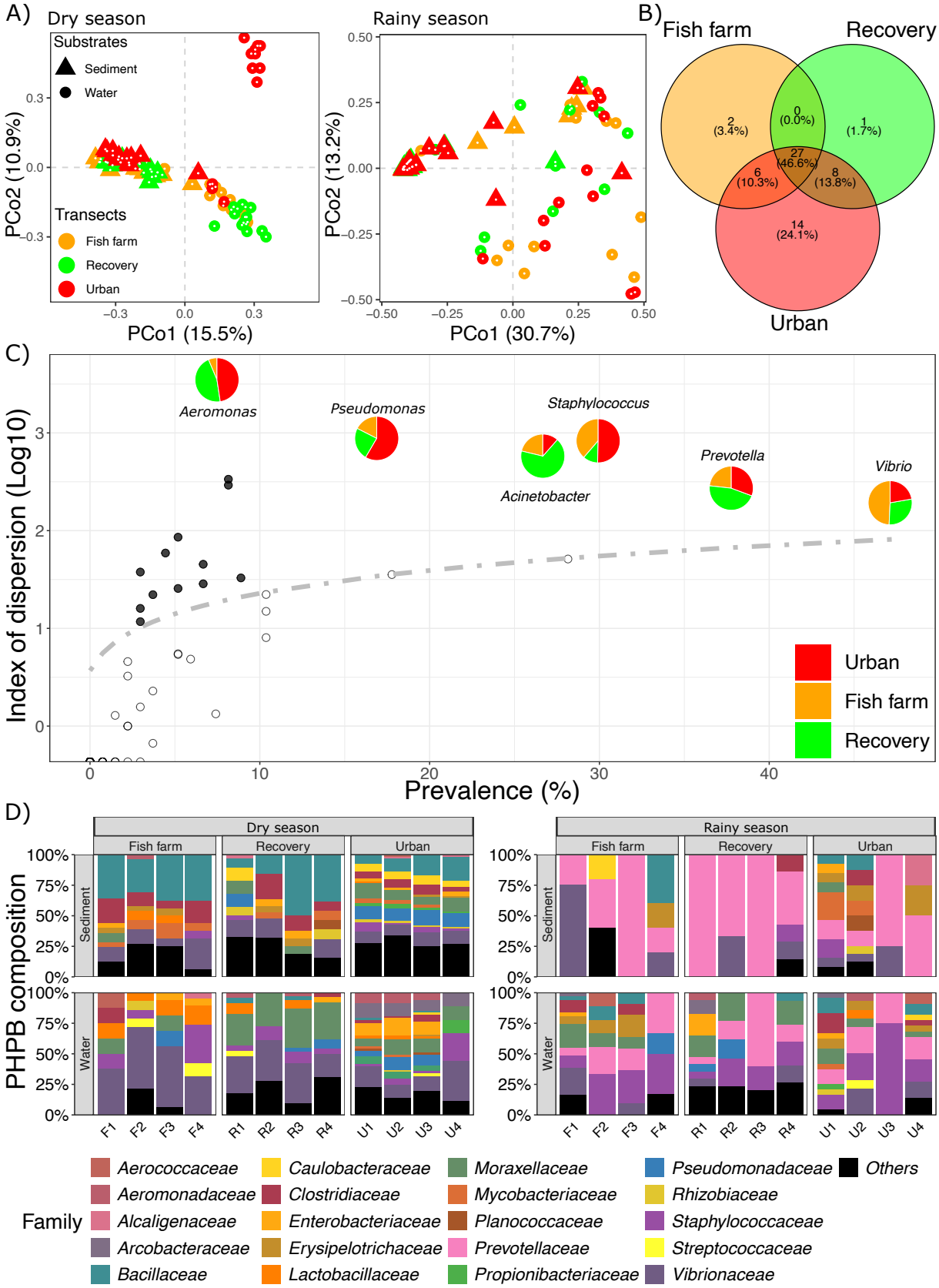
A)
Dry season
Rainy season
Substrates
Sediment
Water
Transects
Fish farm
Recovery
Urban
PCo2 (10.9%)
PCo1 (15.5%)
PCo2 (13.2%)
PCo1 (30.7%)
B)
Fish farm
Recovery
Urban
2 (3.4%)
0 (0.0%)
1 (1.7%)
27 (46.6%)
6 (10.3%)
8 (13.8%)
14 (24.1%)
C)
Index of dispersion (Log10)
Prevalence (%)
Aeromonas
Pseudomonas
Staphylococcus
Acinetobacter
Prevotella
Vibrio
Urban
Fish farm
Recovery
D)
PHPB composition
Dry season
Rainy season
Fish farm
Recovery
Urban
Sediment
Water
F1
F2
F3
F4
R1
R2
R3
R4
U1
U2
U3
U4
Family
Aerococcaceae
Aeromonadaceae
Alcaligenaceae
Arcobacteraceae
Bacillaceae
Caulobacteraceae
Clostridiaceae
Enterobacteriaceae
Erysipelotrichaceae
Lactobacillaceae
Moraxellaceae
Mycobacteriaceae
Planococcaceae
Prevotellaceae
Propionibacteriaceae
Pseudomonadaceae
Rhizobiaceae
Staphylococcaceae
Streptococcaceae
Vibrionaceae
Others

Figure 2.7: A: PCoA of PHPB composition between transects and substrates in dry season and rainy season. B: Venn diagram of PHPB genera between transects. C: occurrence of PHPB genera among samples plotted against its dispersion index. The dotted line depicts the 5% confidence limit of the Chi^2 distribution: PHPB genera located above this line are non-randomly distributed among samples, whereas those below the line follow a random Poisson distribution. Pie charts indicate the PHPB genera relative abundance in each transect. D: PHPB composition between transects and substrates in dry season and rainy season according to each sampling station.

Environmental parameters also had significant effects on planktonic PHPB alpha and beta-diversity. Water temperature (°C), salinity level (‰) and NO_3 concentration (µg/L) explained 10%, 6.7% and 6.2% of the dissimilarities in the water pathobiome (Permanova test, $p<0.05$, Supplementary Material SM2-16). Chlorophyll-A concentration in the environment were also positively correlated with PHPB richness and diversity, explaining 60.5% and 57.5% of the variance, respectively (p-value < 0.05, Supplementary Material SM2-23).

2.3.3 Resistome composition and diversity

After alignment of the metagenomic data set of Nha Trang bay marine sediments against the ARGminer-A database, a total of 565 ARGs encompassing 62 ARG classes were detected in the whole dataset (Figure 2.8A). Nha Trang bay sediment resistome was dominated by rifamycin, quinolone and multidrug ARG classes representing 79.8% of total ARGs. In total, 17 ARGs, accounting for 3% of the overall ARG richness, were identified as constituting the core resistome (Figure 2.8B), yet they represented 84% of the total ARG reads. Among these, the *rpo*B2 and *rph*B genes, which confer resistance to rifampicin, were both highly abundant and widespread. Other genes, including, *Nmc*R conferring resistance to beta lactam, *dfr*E (trimethoprim resistance), *mfd* (quinolone resistance), and *Cpx*R (multidrug resistance), were likewise present in high abundance and consistently detected across all sampling stations. ARG genes conferring resistance to multidrug resistance accounted for 41% (7/17) of the core ARG genes.

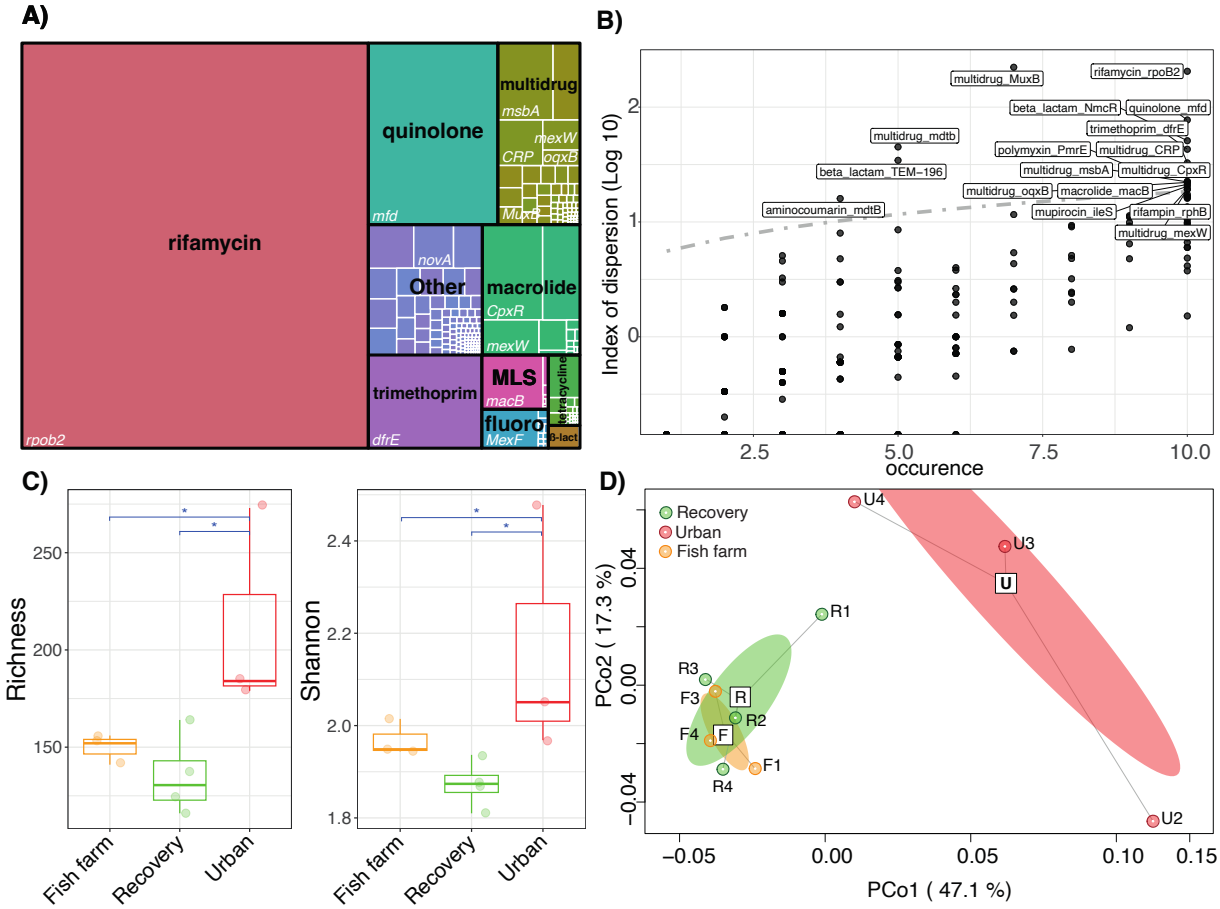

Figure 2.8: Resistome profiles in Nha Trang bay. A: Treemap of the total relative abundance of ARG classes. B: Occurrence of ARGs among samples plotted against its dispersion index. The dotted line depicts the 5% confidence limit of the Chi^2 distribution: ARGs located above this line are non-randomly distributed among samples, whereas those below the line follow a random Poisson distribution. C: Alpha diversity indices of ARGs between transects. D: PCoA of ARGs composition between transects.

Both alpha and beta diversity of ARGs showed significant variation across transects. The urban (U) transect stood out, exhibiting markedly higher ARG richness and Shannon diversity ($p < 0.05$, Kruskal–Wallis followed by Tukey post hoc test) (Figure 2.8C). On average, this transect contained 212 ± 53 different ARGs and harbored the greatest number of unique ARGs (Supplementary Material SM2-24). Similarly to pathobiome richness, ARG richness declined significantly ($p < 0.05$) with increasing distance from the coast both at urban and recovery transects ($P < 0.05$, Supplementary Material SM2-25). The role of environmental factors on the resistome alpha diversity was further explored through multiple linear regression analyses. Both richness and Shannon diversity were negatively correlated with PO_4 concentrations, which alone

explained 59% and 68% of their variance, respectively (Table 2.2). Other parameters, including NH_4 also explained a smaller yet significant portion of the variation.

Table 2.2: Multiple regressions between environmental variables and the richness and diversity of the pathobiome and resistome (Positive relation: "+"; Negative relation: "-"). Variance is explained in percentage.

Environmental variable	Richness		Diversity	
	Value	Correlation	Value	Correlation
Pathobiome				
Chlorophyll A	60.5%	(+)	57.5%	(+)
Resistome				
PO_4	59%	(-)	68%	(-)
NH_4	15%	(-)	-	-

Analysis of resistome composition revealed significant differences among transects (Permanova test, $R^2 = 0.15$, $p < 0.05$), with urban stations clearly separating from fish farming and recovery transects in the PCoA ordination space (Figure 2.8D). Differential abundance analyses showed that the differences observed in resistome composition between the urban and the two other transects were mainly due to 9 ARGs (Supplementary Material SM2-26). Although the ARG differed, the resistome of both fish farm / recovery and urban transects were significantly enriched in ARGs conferring multidrug resistances: *mac*B (MLS resistance) and *sme*R (multidrug resistance) for the fish farm / recovery transects and *olec*, *Mux*C, *ceo*B, *mex*f (multidrug resistance) for the urban transect. This later was also enriched in ARG biomarkers conferring resistance to beta-lactams (TEM-197) and macrolides (*ole*B) while the fish farm / recovery transect was enriched in *mup*B (mupirocin resistance).

2.3.4 Isolated strains and phenotypic resistance

Across the two sampling seasons, a total of 430 bacterial isolates were obtained, with 226 (52.5%) from the dry season and 204 (47.5%) from the rainy season (Supplementary Material SM2-27). The number of isolates did not differ significantly between seasons (Kruskal-Wallis test, $p > 0.05$). In contrast, transects strongly influenced culturable bacterial abundance: the urban transect yielded significantly more isolates (n

= 192, 44.7%) than the fish farm (n = 153, 35.6%) and recovery (n = 85, 19.7%) transects (Kruskal-Wallis test, $p < 0.05$).

MALDI-ToF identification successfully classified 139 of the 430 isolates (57 from the dry season and 82 from the rainy season) into 58 bacterial species (Supplementary Material SM2-28). PHPB strains accounted for 42 species and represented 67.6% of the identified isolates. In line with the most abundant genera found in metabarcoding results, *Vibrio*, *Staphylococcus*, *Acinetobacter* and *Pseudomas* were isolated from the Nha Trang Bay. Members of the *Bacillaceae* family dominated culturable isolates (32.4%), followed by *Enterobacteriaceae*, *Pseudomonadaceae*, and, to a lesser extent, *Moraxellaceae*. Urban samples harbored the greatest family-level diversity (13 families), compared to only 6 in fish farm and 9 in recovery transects (Supplementary Material SM2-28). Several PHPB families, including *Aeromonadaceae*, *Yersiniaceae*, *Gordoniaceae*, *Erwiniaceae*, and *Streptococcaceae*, were exclusively detected in urban samples. This pattern was consistent with genus-level analyses, which revealed a disproportionately high number of PHPB genera unique to the urban transect (Figure 2.7B).

The 139 identified isolates (dry season: 36 Gram-positive and 21 Gram-negative; rainy season: 25 Gram-positive and 57 Gram-negative) were further tested for antibiotic susceptibility. The overall mean MAR index was 0.29 ± 0.17, with 45.9% of isolates exceeding a MAR value of 0.2, indicative of a high prevalence of multidrug resistance (Figure 2.9). A MAR index above 0.2 is typically considered a marker of high antibiotic contamination risk (Krumperman, 1983; Reverter et al., 2020). Seasonality significantly affected MAR values, with higher indices observed in Gram-negative isolates from fish farm and recovery transects during the dry season (Kruskal-Wallis test, $p < 0.05$, Figure 2.9). In contrast, no significant seasonal or transect-related variation was detected for Gram-positive isolates (Kruskal-Wallis test, $p > 0.05$, Figure 2.9).

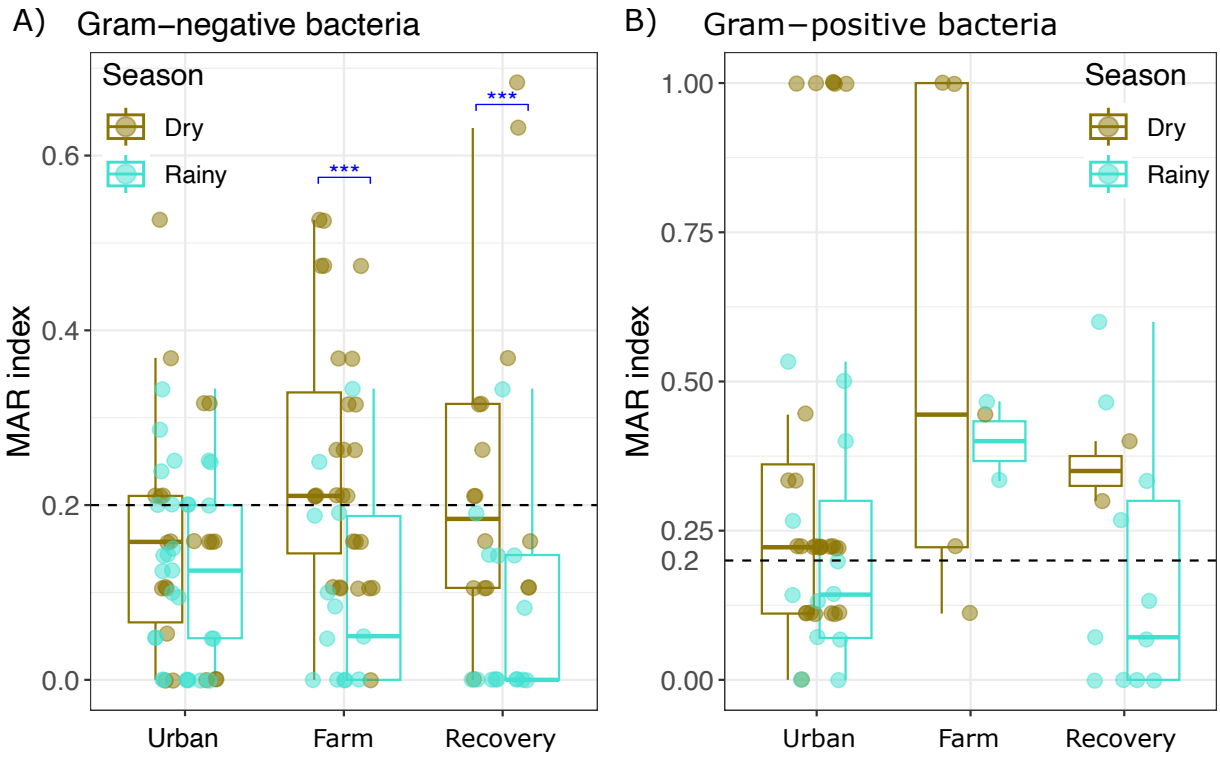

Figure 2.9: MAR index of gram-negative bacterial isolates (A) and gram-positive bacterial isolates (B) from phenotypic resistance in different transects and seasons. Dot line indicates 0.2 threshold of Multidrug resistance. Significant differences were observed with Kruskal-Wallis test between season (****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$).

Phenotypic resistance tests revealed that multidrug-resistant (MDR) bacteria accounted for 48.4% of the isolates (Figure 2.10). Notably, certain strains, including *Stenotrophomonas maltophilia* and *Enterococcus casseliflavus* among Gram-negative bacteria, and *Bacillus amyloliquefaciens* among Gram-positive bacteria, exhibited resistance to more than eight different antibiotics (Figure 2.10). As observed for PHPB beta-diversity, season significantly influenced the phenotypic resistance patterns of the 45 Gram-negative isolates (Permanova test, $R^2 = 0.41$, $p < 0.05$; Figure 2.10). Isolates collected during the rainy season exhibited high resistance to the penicillin group, particularly amoxicillin–clavulanic acid (77% of isolates, n = 20) and ticarcillin (50% of isolates, n = 13). In contrast, Gram-negative bacteria from the dry season displayed elevated resistance to multiple antibiotic classes, including penicillins (piperacillin (79%, n = 15), piperacillin–tazobactam (63%, n = 12), and ticarcillin (42%, n = 8)), carbapenems (Ertapenem, 37%, n = 7), cephalosporins (Cefoxitin, 58%, n = 11), and aztreonam (53%, n = 10). On the other hand, both seasonality and transect significantly affected

the phenotypic resistance of Gram-positive bacteria (Permanova test, $R^2 = 0.13$, $p < 0.05$ and $R^2 = 0.12$, $p < 0.05$, respectively; Figure 2.10). These bacteria exhibited particularly high resistance to rifampicin (60% of isolates, n = 29), a widely used antibiotic in aquaculture, as well as to erythromycin (31% of isolates, n = 15) and fosfomycin (31% of isolates, n = 15). In addition, substrate (water & sediment) has no effect on the phenotypic resistance of both gram-negative and gram-positive bacteria (Permanova test, $p > 0.05$).

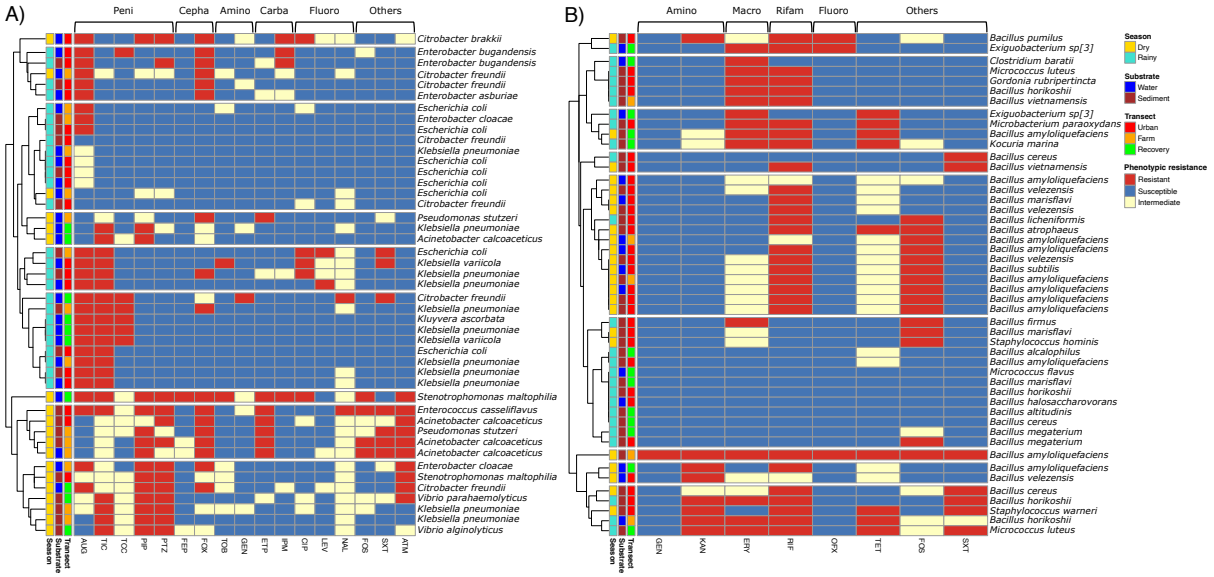

Figure 2.10: Antibiotic susceptibility test on gram-negative bacteria (A) and gram-positive bacteria (B). The breakpoints of each antibiotic were determined according to EUCAST's guidance. Samples are clustered by resistant distance matrix. Antibiotic class abbreviations are: Peni: Penicillins, Cepha: Cephalosporins, Amino: Aminoglycosides, Carba: Carbapenems, Fluoro: Fluoroquinolones. Antibiotic abbreviations are: AUG - Amoxicillin with Clavulanic acid; PIP - Piperacillin; PTZ - Piperacillin with Tazobactam; TCC - Ticarcillin with Clavulanic acid; TIC - Ticarcillin; GEN - Gentamicin; TOB - Tobramycin; ETP - Ertapenem; IPM - Imipenem; FEP - Cefepime; FOX - Cefoxitin; CIP - Ciprofloxacin; LEV - Levofloxacin; NAL - Nalidixic acid; ATM - Aztreonam; FOS - Fosfomycin; SXT - Cotrimoxazole; KAN - Kanamycin; OFX - Ofloxacin; ERY - Erythromycin; RIF - Rifampicin; TET - Tetracycline.

2.4 Discussion

2.4.1 Unveiling the Hidden Diversity of the Pathobiome and Resistome in the Nha Trang Bay

Human pathogenic bacteria in aquatic systems are often monitored through fecal indicator bacteria (FIB) such as total and fecal coliforms, *Escherichia coli*, *Clostridium perfringen*s, and *Enterococci* (Griffin, Lipp, Mclaughlin, & Rose, 2001; Boehm & Sassoubre, 2014). Other well-known enteric pathogens such as *Helicobacter pylori*, *Campylobacter*, *Shigella*, and *Salmonella* are widely used as indicators of fecal pollution and anthropogenic activity (Powers et al., 2020). These PHPB are allochthonous to marine habitats, entering coastal waters via runoff and human inputs. Yet, most potential human pathogenic bacteria (PHPB) occurring in the marine realm are not of fecal in origin, but rather autochthonous members of marine ecosystems, including opportunistic strains of *Vibrio* and *Aeromonas* (Landrigan et al., 2020; Doni et al., 2023)(Tran Son et al., submitted). Recent studies based on next-generation sequencing (NGS) revealed that wastewater, urban waters and coastal waters can harbor between 16 and 50 PHPB genera (Cui et al., 2017, 2019; T. Fang et al., 2018; H. Sun et al., 2017; Ye & Zhang, 2011; Y. Guo et al., 2022), suggesting that their diversity and their role in aquatic systems has been largely underestimated. To address this gap, we developed a new reference database of full-length 16S rRNA genes from 1,757 PHPB species (Tran Son et al., minor revision) and applied it to Nha Trang Bay. Using a conservative approach (100% similarity across the entire 450 bp amplicon), we identified 485 PHPB ASVs spanning 49 genera and 98 species, representing 0.28% of the whole microbial community. This diversity is nearly twice as high as reported in a recent semi-enclosed bay study (Y. Guo et al., 2022) although the contribution to the whole bacterial community they found was one or two order of magnitude higher, ranging from 0.13% to 24.65% (average 3%). Importantly, our estimates are based on stringent detection criteria (i.e.; taking into account only ASV that have blasted within a genus, not the whole genus) whereas studies relying on taxonomic nomenclature at the genus level may tend to inflate PHPB contributions. This may explain the discrepancy in PHPB relative abundance with the

study of Guo et al. (Y. Guo et al., 2022). These differences highlight the lack of methodological standardization across studies, including sequencing depth, database choice, and taxonomic thresholds, which complicates direct comparisons. While the accuracy of 16S rRNA amplicon sequencing for pathogen detection, particularly at the species level, remains debated (Aw & Rose, 2012; Tan & Tatsumura, 2015), our work demonstrates that with conservative thresholds, NGS remains a powerful tool to assess PHPB diversity and distribution at the genus level (T. Fang et al., 2018).

Consistent with previous reports (Cui et al., 2017; T. Fang et al., 2018; Naudet et al., 2025; Y. Guo et al., 2022; Leight, Crump, & Hood, 2018; A.-L. Xu, Niu, Song, Lang, & Guo, 2018)(Tran Son et al., submitted), PHPB assemblages in Nha Trang Bay were dominated by autochthonous opportunists such as *Vibrio*, *Staphylococcus*, *Bacillus*, *Acinetobacter*, *Pseudomonas* and *Aeromonas* (Figure 2.7C). The ubiquity of *Vibrio* and *Bacillus* in marine systems is well established (Y. Wang et al., 2020). However, we found that enteric opportunistic genera, notably *Prevotella*, *Arcobacter*, and *Enterobacter*, were part of the core PHPB microbiome and reached substantial relative abundance, with *Prevotella* dominating the PHPB community during the rainy season. Both *Prevotella* and *Arcobacter* are recognized markers of human fecal contamination (C. Lee, Agidi, Marion, & Lee, 2012; Koskey et al., 2014), pointing to direct anthropogenic inputs into Nha Trang Bay.

Beyond PHPB diversity, we also uncovered a worrisome prevalence of antibiotic resistance. Multidrug-resistant (MDR) strains accounted for 48.4% of isolates, and PHPB represented 67.6% of these. This high prevalence of MDR bacteria is in line with the high prevalence (i.e.; 41%) of ARGs coding for efflux pump and conferring multidrug resistance within the core resistome (Figure 2.8B). Coastal ecosystems expose microorganisms to a diverse array of antibiotics and other environmental pollutants that may help explain the high prevalence of multidrug resistance genes observed in these environments (Kunhikannan et al., 2021). The mean multiple antibiotic resistance (MAR) index was 0.29 ± 0.31, with more than half of isolates (51.7%) exceeding the 0.2 threshold, indicative of a high level of multiple antibiotic resistance. This level is comparable to values reported for marine bacteria in Vietnam (0.39), Indonesia (0.35)

and India/Sri Lanka (0.36), and considerably higher than those in African coastal waters (0.19 in South Africa; 0.15 in Mauritius) (Suyamud et al., 2024; Naudet et al., 2023; Reverter et al., 2020). Such patterns point to strong selective pressures in the bay, likely linked to intense use of antibiotics and other co-selectors (e.g. metals, pharmaceuticals, pesticides).

Rifampicin resistance emerged as a striking feature of our dataset: 60% of Gram-positive isolates were resistant, consistent with the dominance of rifamycin ARGs (rpoB2, rphB) in sediment resistomes (Figure 2.8A&B). Rifampicin is heavily prescribed for tuberculosis, a highly prevalent disease in Vietnam (Mason, Lyttleton, Marks, & Fox, 2020), and its ARGs may reach the bay through riverine inputs and horizontal gene transfer. In parallel, rifampicin is massively applied in local aquaculture, particularly in lobster cage farming, at an average of 2,114 g per ton of lobster, and is also used prophylactically in fish farming (Hedberg et al., 2018). Our findings show that this combined medical and aquaculture use has generalized rifampicin resistance across marine bacterial communities. Given that rifampicin belongs to the WHO list of essential medicines and is a last-resort treatment for tuberculosis and leprosy, its uncontrolled use in aquaculture is of particular concern and should be urgently reconsidered. Finally, the high abundance of beta-lactam and multidrug resistance genes in Nha Trang Bay mirrors patterns observed in other estuarine and coastal ecosystems (F. Guo et al., 2016; Zhu et al., 2017; L. Zhou et al., 2022; Peng et al., 2024; H. S. Tran et al., 2025). Their likely introduction through river discharge and human activities (Su et al., 2023) further confirms the heavy anthropogenic imprint on the bay.

2.4.2 Dry-season intensification of multidrug-resistant pathobiomes in tropical coastal waters

Similarly to the whole bacterial community, our findings demonstrate that the coastal pathobiome and resistome are shaped by a dynamic interplay between hydrological regimes, nutrient availability, and anthropogenic pressures. Seasonal shifts were particularly striking: during the dry season, pathobiome diversity, species richness, and uniqueness all peaked, while dissimilarities between transects increased,

pointing to a more heterogeneous and diversified pool of potentially pathogenic bacteria (Figures 2.5 & 2.7A). In parallel, phenotypic resistance displayed a marked seasonal signal, with dry-season isolates exhibiting significantly higher MAR indices (Figures 2.9 & 2.10).

These patterns can be understood in light of the hydrological context of the Nha Trang Bay. Rainfall is multiplied by 3 during the rainy season compared to the dry season (van Kessel, Kockelkorn, Speelman, & Wierikx, 2021), driving a drastic increase in Cai River discharge (600 m^3/s vs. 30 m^3/s), which produces a massive freshwater influx. This pulse likely exerts a dilution effect, lowering PHPB diversity and resistance potential while homogenizing communities across the bay. However, consistent with previous studies showing that rainfall and runoff can enrich coastal waters with enteric pathogens and ARGs (Leonard, Morris, Schmitt, & Gaze, 2022), the dominance of *Prevotella* within the PHPB community during the rainy season suggests that anthropogenic inputs extend into areas of Nha Trang Bay where no fecal contamination biomarkers are detected during the dry season. Our results reveal a complementary process: dry-season conditions, characterized by reduced flushing, higher temperatures, and elevated salinity, instead appear to favor the diversification of PHPB (Powers et al., 2020) and resistant bacteria (Serrana et al., 2025; Peng et al., 2024; Schages, Wichern, Kalscheuer, & Bockmühl, 2020; Harnisz et al., 2020; Y.-J. Zhang et al., 2019). Consistent with this, we found that mean temperature and salinity increased by 3°C and 2 PSU in the dry season (Supplementary Material SM2-11), both of which were significantly associated with PHPB community structure. Higher temperature is known to enhance the growth and virulence of PHPB (Maurelli, 1989; Vojvodic, Jensen, James, Boomsma, & Eilenberg, 2011) and to elevate MAR indices (Reverter et al., 2020), while salinity stress can directly induce resistance through plasmid transfer, ARG expression, and activation of multidrug resistance operons (McMahon et al., 2007).

Beyond environmental forcing, human activities likely amplify these seasonal differences. Nha Trang is Vietnam's largest tourist hub, concentrating one-third of the country's international tourism, with over 7 million visitors in 2019, mostly during the dry season. In the absence of a comprehensive sewage system (only 31% of wastewa-

ter treated), this massive seasonal influx likely fuels direct discharges of PHPB, ARGs, antibiotics, and nutrients into the bay (T. U. K. Hoa, 2024). Tourism-driven inputs, together with aquaculture effluents, are consistent with previous findings that recreational and industrial activities enhance the release of clinically relevant pathogens and ARGs in coastal waters (Ahammad, Sreekrishnan, Hands, Knapp, & Graham, 2014; Jani, Bandal, Rale, Shouche, & Sharma, 2019; Jurelevicius et al., 2021). Nutrient enrichment in particular is a key driver of microbial growth and ARG abundance (Jacobs et al., 2009; Pianetti, Bruscolini, Rocchi, Sabatini, & Citterio, 2006; Cui et al., 2019; Y. Guo et al., 2022), and our data confirm strong relationships between Chla, phosphate, ammonium, nitrate concentrations and the alpha and beta diversity of the pathobiome and the resistome.

Finally, phenotypic assays revealed that Gram-negative isolates from the dry season harbored broader resistance spectra spanning multiple beta-lactams (penicillins, carbapenems, cephalosporins) and aztreonam, suggesting cumulative selective pressures under reduced hydrological flushing. This broadening of resistance profiles during the dry season underscores the capacity of tropical coastal waters to act as reservoirs and amplifiers of multidrug-resistant pathogens when subjected to combined stressors of tourism, aquaculture, and environmental forcing.

Taken together, our results provide one of the clearest demonstrations to date that tropical dry seasons, rather than rainy seasons, may constitute periods of heightened risk for the proliferation of multidrug-resistant PHPB. This has immediate implications for public health, given the high exposure of recreational bathers, aquaculture workers, and marine livestock to resistant PHPB in coastal waters under dry-season conditions.

2.4.3 Spatial variation in the resistome and pathobiome

Our results revealed also pronounced spatial heterogeneity in both the pathobiome and resistome during the dry season, with the strongest contrasts observed between the fish farm / recovery transects and the urban transect. This contrast is particularly striking because limited rainfall and reduced river discharge minimize hydrolog-

ical mixing and dilution. Under these conditions, inputs from aquaculture facilities and urban sewage may exert more localized and persistent impacts on microbial communities. This spatial compartmentalization leads to the co-existence of aquaculture-associated pathobiomes and resistomes (Figures A 2.7 & 2.8D), dominated by livestock influence and veterinary antibiotic selection pressures, and urban-associated pathobiomes and resistomes, reflecting the complexity of human-derived sewage inputs.

In the fish farm transect, the domination (17.8% of the PHPB community) and enrichment by autochthonous opportunistic PHPB belonging to the Vibrionaceae family likely reflect the concentrated presence of marine livestock (fish and lobsters) from surrounding floating farms settings. Although ubiquitous and abundant in marine ecosystems (Chatterjee & Haldar, 2012; Ina-Salwany et al., 2019), Vibrionaceae are also well-established members of the marine holobiont microbiota where there are enriched compared to the surrounding environments (Tran son et al., submitted). This family has received considerable attention, particularly in relation to aquaculture environments (Naudet et al., 2023, 2025; X. Sun et al., 2020; J. Lu, Zhang, Wu, & Luo, 2019) since many species are pathogenic, either causing human diseases (Rivas, Lemos, & Osorio, 2013; Mustapha, Mustapha, & Nozha, 2013; Schröttner, Tille, Lück, & Bunk, 2020; Letchumanan et al., 2019) or contributing to mass mortalities in aquaculture (Austin & Austin, 2016; Stentiford et al., 2017; Ina-Salwany et al., 2019). *Vibrio alginolyticus* and *Vibrio parahaemolyticus*, both known human and fish pathogens, were the most abundant and occurrent PHPB in fish and recovery transects (Figure 2.7D). For technical reasons, the vast majority of the *Vibrio* isolates were lost and only three isolates corresponding to *Vibrio alginolyticus* and *Vibrio parahaemolyticus* could be tested for phenotypic resistance. Consistent with previous reports (Naudet et al., 2023; Y.-J. Zhang et al., 2019; R. E. Moore, Millar, & Moore, 2020), these *Vibrionaceae* isolates displayed high resistance to beta-lactam antibiotics of the penicillin class (66% resistant to piperacillin with tazobactam; 100% resistant to ticarcillin and piperacillin).

Similarly to the pathobiome, the higher MAR index found in the fish and recovery transects (Figure 2.9) and the composition of the resistome (Figure 2.8A), likely reflect the intensive use of diverse antibiotics in floating farm aquaculture. Hedberg et al.

showed that the amount of antibiotic per ton of fish or lobster used in floating farm in Vietnam is 100 to 1000 fold higher than in any other country (Hedberg et al., 2018). A total of 13 different antibiotics, particularly sulfamethoxazole, tetracylin, thimethoprim and rifampicine, were used with 11 of them classified by WHO as "Highly important" to "Critical for human use". These antibiotics are often used not only for therapeutic purposes but also prophylactically and as growth promoters (P. T. P. Hoa et al., 2011; Pham et al., 2015; Rico et al., 2013). Such practices are widely documented in Vietnam and other Asian countries, where residues of tetracyclines, sulfonamides, and beta-lactams have been repeatedly detected in aquaculture ponds and effluents, where they exert strong selective pressure on microbial communities (Cabello et al., 2013; Henriksson et al., 2018). This selective environment not only enriches multi-resistant bacterial strains but also favors horizontal gene transfer (HGT), as aquaculture environments are hotspots of plasmid-mediated ARG dissemination (Shah et al., 2014; Watts et al., 2017). The high MAR index of dry season isolates from the fish and recovery transect (Figure 2.9), the enrichment in mutltidrugs ARGs in the resistome, as well as the high abundance of ARGs related to rifamycin and thrimethoprim resistances in the fish and recovery transect resistomes are all consistent with these mechanisms, including resistance to several aquaculture-related antibiotics, suggesting that aquaculture operations may act as reservoirs for ARGs with potential transfer to human pathogens. Notably, the strong similarities between the resistome and pathobiome of the fish farm and recovery transects suggested that the impacts of aquaculture activities can persist long after farming has ceased.

Conversely, the urban transect harbored the highest prevalence and diversity of pathogenic taxa, alongside the greatest ARG richness (mean 2985 ± 108 ARG hits), Shannon diversity, and number of unique ARGs. suggesting that human activities contribute substantially to both the expansion and diversification of the local pathobiome and resistome. Notably, the resistome showed a clear enrichment in beta-lactamase ARGs (Supplementary Material SM2-26), paralleled by increased abundances of *Arcobacteraceae* (*Arcobacter butzleri*, *Arcobacter cryaerophilus*), and *Moraxellaceae* (*Acinetobacter soli*). These formers are recognized as emerging fecal-associated pathogens (C. Lee et al., 2012). The predominance of beta-lactamase resistance genes is particularly re-

vealing, since beta-lactams are among the most widely used antibiotics in clinical settings, and their detection in coastal resistomes is a strong signal of human-derived pollution (Su et al., 2023). These findings are best explained by the direct discharge of untreated or insufficiently treated urban sewage into the Cai river, which flows into Nha Trang Bay. This interpretation is reinforced by the distance-to-shore dilution pattern observed for PHPB relative abundance and diversity in the urban transect (Figure 2.7D). Our results are consistent with previous studies showing that urban effluents elevate pathogen loads (Cui et al., 2019; Powers et al., 2020; Y. Guo et al., 2022; Jurelevicius et al., 2021) and ARG levels (Peng et al., 2024), largely driven by the continuous influx of wastewater-derived bacteria and residual antibiotics. In Nha Trang, where only a small fraction of wastewater undergoes treatment prior to discharge, urban effluents likely represent a major point source of fecal bacteria, human pathogens, ARGs, and residual antibiotics. In line with our results, such inputs have been shown to introduce clinically relevant pathogens (e.g., *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus* spp., *Acinetobacter* spp.) carrying extended-spectrum beta-lactamases (ESBLs) or carbapenemases into coastal waters (Rizzo et al., 2013; Hendriksen et al., 2019). Even more concerning is their coexistence, as seen with multidrug-resistant (MDR) *Acinetobacter* spp. and *Klebsiella pneumoniae*, which can engage in synergistic interactions that enhance both their resistance and virulence, placing them among the most dangerous priority multidrug-resistant pathogens (Semenec et al., 2023). In addition to pathogens, wastewater discharges increase nutrient concentrations, promoting bacterial growth and interactions, which in turn foster horizontal gene transfer (HGT) and the mobilization of ARGs (Jacobs et al., 2009; Y. Guo et al., 2022).

Taken together, these findings underscore that in addition to seasonal dynamics, localized human activities strongly modulate the distribution and structure of coastal pathobiomes and resistomes. The dry season, with its reduced hydrological flushing, exacerbates these differences and may increase risks of exposure for humans and livestock. Addressing these risks will require improved management of both antibiotic use in aquaculture and urban wastewater treatment, as both contribute significantly to the coastal burden of potentially pathogenic and resistant bacteria.

2.4.4 Conclusions

Our results demonstrate that Nha Trang Bay is a hotspot of both fecal contamination and antimicrobial resistance, hosting a diverse pathobiome dominated by opportunistic and enteric pathogens, as well as multidrug-resistant strains. Seasonal shifts and spatial gradients further shaped the composition of the resistome and pathobiome, highlighting the influence of rainfall, riverine inputs, and aquaculture activities on microbial risks. The pervasive rifampicin resistance, likely driven by its extensive use in aquaculture and human medicine, illustrates how local practices can amplify global health threats. These findings underscore the urgent need for stricter regulation of antibiotic use in aquaculture, improved wastewater treatment to reduce fecal inputs, and systematic monitoring of coastal microbial communities. Such measures are essential to safeguard marine ecosystems and to mitigate the growing threat of pathogen and resistance gene transmission to human populations. More broadly, Vietnam and other Southeast Asian countries could serve as model regions for implementing integrated One Health strategies, aligning environmental, agricultural, and public health policies to curb the spread of antimicrobial resistance at the human–ocean interface.

2.5 Acknowledgments

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Chapter 3: Circulation of Potentially Pathogenic Bacteria and Antibiotic Resistance Gene between Marine Environments and Human or Aquaculture Hosts

Foreword

The first two Chapters have explored the dynamics of both the pathobiome and resistome across different sample types and transects of various marine environments. Results from the studies in Chapter 1 indicates the higher abundance of potentially pathogenic bacteria (PPB) from marine holobionts compared to the environment. The composition and abundance of PPB, ARGs and phenotypic resistance were also driven by anthropogenic inputs as illustrated in Chapter 2. These results are consistent with many other studies of pathobiome and resistome in aquatic environment. However, the circulation pathway of the PPB and ARGs, potentially between different sample types remain unknown.

Nowadays, aquaculture is one of the fastest growing areas in global food production and floating cage farms play an important role in the aquaculture production of many countries including Vietnam. However, the close interactions between human, animals, and surrounding water in such systems can lead to increased circulation of pathogenic bacteria and antibiotic resistance genes (ARGs). Hence, the risk of pathogens and antimicrobial resistance dissemination in aquaculture environment increases.

Therefore, our last Chapter aims to provide insights on the potential circulation of pathobiome and resistome between compartments under seasonality effects, with a focus on aquaculture practices in a floating cage farms system in Nha Trang, Vietnam (Chapter 3). We combined both phenotypic and genomic methods to compare the microbiota of farmers with that of aquatic livestock and water samples in order to inspect the connectivity of the pathobiome and resistome between compartments. Results from this Chapter is presented in an article format, the genomic context part of the resistome will be finalized shortly and the manuscript will be submitted in the coming months.

This study manuscript is currently in preparation

Longitudinal dynamics of resistome and pathobiome in farmers, livestock and environment in aquaculture floating farms in Nha Trang, Vietnam

Son-Hoang Tran[1,2], Trung Du Hoang[3], Hai Thi Vo[3], Marc Troussellier[1], Sébastien Villéger[1], Yvan Bettarel[1], Thierry Bouvier[1], Fabien Rieuvilleneuve[1], Claudia Ximena Restrepo Ortiz[1], Anne-Laure Bañuls[2,4], Juliette Hayer[4], Gwenn Pulliat[6], Christian Culas[6], Quang Huy Nguyen[2], Dinh Quang Vu[2], Jean-Christophe Auguet[1,2]

[1]UMR MARBEC, Univ Montpellier, CNRS, Ifremer, IRD, Montpellier, France;

[2]LMI DRISA, Department of Life Sciences, University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology, Hanoi, 100000, Vietnam;

[3]Institute of Oceanography, Vietnam Academy of Science and Technology, Nha Trang, Viet Nam;

[4]UMR MIVEGEC, University of Montpellier-CNRS-IRD, Montpellier, France;

[5]Laboratoire de Bactériologie, Centre Hospitalier Universitaire de Montpellier Arnaud Villeneuve;

[6]ART-Dev, Univ Montpellier, CNRS, CIRAD, Univ Paul Valéry Montpellier 3, Univ Perpignan Via Domitia, Montpellier, France

1 Longitudinal dynamics of resistome and pathobiome in farmers, livestock and environment in aquaculture floating farms in Nha Trang, Vietnam

Aquaculture is among the fastest-growing branches of animal agriculture, where advances intended to enhance human well-being often lead to unintended consequences for humans, animals, and the environment, notably through the spread of potential pathogens and antibiotic resistance. To investigate this issue, we carried out an 18-month longitudinal survey (covering both the dry and rainy seasons) examining the fecal and skin microbiota of aquaculture farmers within the Nha Trang Bay, Vietnam. To examine the connectivity between compartments, we compared the microbiota of farmers with those of fish livestock and water samples using a combination of phenotypic and genomic approaches. Results from amplicon sequence variants (ASVs) analysis revealed the most dominant PPB lineages belonging to the *Prevotellaceae* and *Vibrionaceae* families across all samples. Both results from metabarcoding and bacterial isolation showed a potential circulation of PPB with 14.4% of the total PPB ASVs and 15.3% of isolated PPB shared between the three compartments. These shared PPB mainly belong to the *Prevotella*, *Escherichia*, *Klebsiella* and *Vibrio* genera. On the other hand, metagenomic analysis detected a total of 538 ARGs from 26 classes with 108 ARGs shared between the compartments. Notably, the gene *bla*OXA-347 exhibited 100% similarity across contigs from various compartments, suggesting an active circulation of ARGs. The relative abundance of the shared resistome reflected the main classes of antibiotic used on farms with a dominance of ARGs related to tetracycline, beta-lactams, macrolide-lincosamide-streptogramin, and multidrug resistance, which together accounted for 85.6% of the total ARGs abundance. In agreement, high phenotypic resistance to beta-lactams (penicillins, cephalosporins, monobactams) were also observed. Overall, floating farms in Nha Trang aquaculture facilitate the spread of both pathobiome and resistome between aquaculture compartments, with farmers both facing higher risk and acting as reservoirs for transmitting pathogens and antibiotic resistance genes to other compartments.

1.1 Introduction

Aquaculture has emerged as one of the fastest-growing sectors in global food production, serving as a vital source of animal protein to meet the needs of a growing human population (Food & Organization, 2022). With a production valued at over US$250 billion and a sector predicted to rise by 62% by 2030, aquaculture provides about half of the world's total fish population (Thornber et al., 2020; Nafiqoh et al., 2020). Currently, developing countries accounted for over 80% of the world's aquaculture production, with China plays the role of the world's largest contributor, producing more than 52.24 million metric tons (35% of the global aquaculture production) in 2020, primarily from inland aquaculture (X. Liu et al., 2022). To a lesser extent, 15% of the total aquaculture production also come from Association of Southeast Asian Nations (ASEAN) (Phillips et al., 2016; Chan et al., 2017). Notably, Indonesia and Vietnam, both members of ASEAN, are significant players in global aquaculture production. Indonesia ranks second worldwide, contributing 10% of the total aquaculture production, while Vietnam closely follows in fourth place with 5% of the world's aquaculture production (Food & Organization, 2022). Among many various forms of marine aquaculture, the use of floating cages has gained widespread adoption worldwide to rear fish and crustaceans stands out for its efficiency and scalability by optimizing marine space in open (Food & Organization, 2022). Vietnam, known for its long coastline of more than 3260km, has established itself as a significant contributor to global aquaculture production, ranking as the third-largest seafood exporter in the world in 2022 (exports exceeding 11 billion USD) (VASEP, 2023), with floating cages playing an important part. These systems are often utilized in open marine environments of Vietnam to cultivate species like fish and lobster (De Silva & Phillips, 2007; Kongkeo, Wayne, Murdjani, Bunliptanon, & Chien, 2010). As a result, floating cages farming in Vietnam has gone under a significant rise in number of lobster cage and fish cage units, which has increased from 10% to 500% in just 5 years (2010-2015) (of Agriculture & Development, 2015).

Aquaculture is the main livelihoods of 22 million individuals worldwide, mostly (95%) in Asia (Food & Organization, 2022). These workers, routinely comes into con-

tact with livestock and are directly exposed to marine animal and aquaculture associated microbial communities. Among these bacterial communities, aquaculture exposes humans to potential pathogens originating either from livestock or from the surrounding environment. Numerous human vibriosis cases have been documented and linked to ingestion of raw/undercooked seafood or exposure to contaminated water (Wachsmuth, Olsvik, Evins, & Popovic, 1994; Finkelstein, Edelstein, & Mahamid, 2002; Arab, Nalbone, Giarratana, & Berbar, 2020; Håkonsholm et al., 2020), and the continued spread of *Vibrio parahaemolyticus* underscores tight connections between aquatic ecosystems and human health (Tian et al., 2024). Pathogens such as *Escherichia coli*, *Aeromonas hydrophila*, and *Salmonella enterica* have also been detected in the aquaponic systems (Fox et al., 2012; Weller, Saylor, & Turkon, 2020; Y.-J. Wang, Deering, & Kim, 2020; Thaotumpitak, Sripradite, Atwill, Tepaamorndech, & Jeamsripong, 2022). Additionally, aquaculture accounts for approximately 5.7% of global antimicrobial consumption, largely for prophylactic and growth-promoting purposes (Schar et al., 2020). However, prolonged or excessive use of antibiotics causes the creation of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) in addition to residues (Watts et al., 2017). According to Lulijwa and colleagues, erythromycin, amoxicillin, sulphadimethoxine, and enrofloxacin were used in 55% of the major aquaculture producing countries, while oxytetracycline, florfenicol, and sulphadiazine were used in roughly 73% of them (Lulijwa, Rupia, & Alfaro, 2020). Antibiotic classes such as quinolone, bacitracin, and fosmidomycin are suggested to be under selective pressure from the influence of aquacultural practices (Cuadrat et al., 2020). Through horizontal gene transfer, antibiotic resistance genes in aquaculture can also spread to natural environment bacterial strains, hence impacting the entire ecosystem (Rodgers & Furones, 2009; Preena, Swaminathan, Kumar, & Singh, 2020). Mobile genetic elements also increase the chance of antimicrobial resistance to transfer to human and animal by facilitating the spread of ARGs among bacterial populations (Jian et al., 2021; Tao, Chen, Li, Wang, & Liang, 2022). Previous studies have documented the presence of diverse pathogens and antibiotic resistance genes (ARGs) in freshwater aquaculture settings, highlighting substantial shifts influenced by antimicrobial usage, environmental variables, and temporal changes (Downes et al., 2015; Delphino et al., 2019; Patil et al., 2020; Mao et al., 2025). Increasing evidence has shown that aquaculture systems act as

reservoirs for pathogenic bacteria and antibiotic-resistance genes (ARGs) (Naudet et al., 2023, 2025; Ferri, Lauteri, & Vergara, 2022; X. Yuan et al., 2023; Gundi et al., 2025). Hence, similarly to inland livestock farming, aquaculture farming may expose workers to occupational hazards that directly affect their health and may play a critical role in the dissemination of antimicrobial resistance. Despite its global relevance for public health, the impact of aquaculture activities on the circulation of both PPB and ARGs between farmers, livestock and environment microbiomes remains poorly understood.

Although recent studies have begun to investigate the temporal dynamics of the pathobiome and resistome, findings remain short and inconsistent concerning whether there are active circulation of microbes and ARGs across compartments (Mahmud et al., 2024) or just limited circulation under specific environmental conditions (Herrero et al., 2022). Given the limited understanding of the circulation of the pathobiome and resistome across human, animal, and environmental compartments, and how these dynamics may vary with seasonal conditions (e.g., dry versus rainy season), this study aims to: (1) characterize the composition of the pathobiome and resistome within aquaculture floating cage systems, and (2) assess their potential circulation between compartments over time, with a focus on aquaculture practices in Nha Trang, Vietnam. This research seeks to address critical knowledge gaps and inform sustainable aquaculture management and antimicrobial resistance (AMR) surveillance strategies.

1.2 Material and Methods

1.2.1 Experimental design

Briefly, the study was conducted in a farming village located in Hon Mieu island, Nha Trang Bay, South Central Vietnam. This community represents one of the largest portion of aquaculture activities in the region, with over half of the total 200 farms concentrated on the close water of the island. The climate in Nha Trang Bay is generally tropical, featuring a dry season from January through August and a rainy season from September to December. The yearly temperature fluctuates between 14–39°C, with notable daily variations in temperature influenced by three primary factors: tropical

elements, monsoons, and local conditions. Detail of the location for the sampling sites is presented in Supplementary Material SM3-1.

The aquacultural practices in the village mostly focus on different species of fish and other invertebrates like clams or lobsters. In the village, most of the small-scale aquaculture farms utilize traditional wooden cages, whereas industrial-scale farms often deploy high-density polyethylene (HDPE) net cages that are floating and circular in shape. The design of wooden cages is typically featuring standard rectangular dimensions of 4 by 4 meters. These cages are often assembled into "rafts" that comprise four or more units. Farmers can construct temporary shelters on these rafts to assist with their daily activities. These arrangements are well-suited for family-operated ventures, and the cages can only be situated in the sheltered bays on the calmer sides of the islands. The farmers utilized the floating cage technique, where each system was designed to nurture various animals, with some even using a two-level structure. In these specific systems, fish are often raised in the upper cages, while lobsters and clams are kept in the lower ones. Here, water flows directly between the cages and to neighboring farms. The high population density and intensive aquaculture practices have led to significant human-induced impacts on the waters, resulting from both daily routines and aquaculture practices, creating a localized gradient of anthropogenic influence.

A pre-campaign was carried out before the main sampling process to identify potential farmer participants based on selective criteria. Residents of these farms were contacted by telephone, followed by a face-to-face interview to receive the study description. For farmers, the participating criteria included: (1) being actively involved or residing on the farm or within the village, and (2) spent at least 6 hours daily on farm activities. Additionally, two groups of people were included in the study as controls for comparison. The first control group were limited 7 healthy female individuals aged from 24 to 70 with body mass indices (BMIs) ranging from 18.06 to 22.43 kg/m^2 (Hoang et al., 2021). These samples were included as non-farmers to compare the gut microbial communities of our participants, also representing variability in sex. The second control group included 10 participants without any known infections, no record of diarrheal episodes in the six months before enrollment, and no use of antimicrobials

in the three months before recruitment. These samples were added to our study to have the resistome profiles of healthy non-farmers. More details about the criteria for this control group can be found in the study by Pereira-Dias on the gut microbiome of healthy Vietnamese adults (Pereira-Dias et al., 2021). For farmers, all participants were informed with a consent form, and a questionnaire was also provided during recruitment and screening to gain knowledge about the farming practices.

In total, we recruited 38 aquaculture farmers from 13 floating farms. For aquatic livestock, a variety of species were selected in the same participant's farms, including fish such as longfin batfish (*Platax teira*), golden pomfret (*Trachinotus blochii*), carp (*Cyprinus carpio*), cobia (*Rachycentron canadum*), white-spotted spinefoot (*Siganus canaliculatus*), grouper (*Epinephelus lanceolatus*), and blue/red lobsters (*Panulirus ornatus* and *Panulirus longipes*). Detail of the main participants and selected animal species are included in Supplementary Material SM3-2.

1.2.2 Sample collection and preparation

The sampling process targeted various aquaculture compartments including humans, animals, and the environment, following a One-Health approach and accounting for seasonal variations that reflect the different environmental climates in the Nha Trang region of Vietnam. The first and third campaigns, conducted in November 2022 and December 2023, respectively, represented rainy seasons. The second campaign, carried out in July 2023, corresponded to the dry season. Sample types included water column (environment), animal gut, human (skin and fecal) samples.

Water column samples representing the surrounding environment in the floating cage farms ecosystems were collected around the village using sterile bottles (1.5 liters, taken 15 cm below the water surface). All water column samples were collected in triplicate and immediately stored in a dark cooler, at 4°C. They were later frozen and preserved at -80°C in the laboratory. Water samples were then filtered within a maximum of 6 hours after collection using a 47 mm, 0.2 µm polycarbonate membrane from Whatman, Clifton, USA. The filtered membranes containing bacteria were then frozen

and stored at -80°C until DNA extraction and further analysis.

For animal gut samples, the microbiota of fish and lobster guts were collected by squeezing the digestive tracts (from below the stomach to the rectum) to extract the gut content (Cheutin et al., 2021). These samples were kept in sterile 2ml Eppendorf tubes at -80°C before DNA extraction.

For human fecal samples, each participant received a collection kit containing multiple items such as gloves, tissues, and a specific sterile swab-tube three days before enrollment. Participants were instructed to collect fecal samples themselves, following the guidelines provided along with the consent form. Briefly, the fecal sample was collected by dipping the swab into the stool to ensure the tip made contact with the fecal matter (avoiding contact with the toilet). The swab was then placed back into its original tube and stored in a freezer at -20°C (or in a refrigerator at -4°C if freezing was not possible) to maintain the sample's condition. Human skin samples were collected on the same day the fecal samples were retrieved. Skin samples were taken directly after participants had finished their daily activities, without showering or washing their hands. A member of the research team collected skin microbiota by swabbing each participant's palms and fingers using sterile foam-tipped swabs for 20 seconds on each hand. The swabs were then carefully placed back into their plastic containers and sealed securely. Skin swabs were stored at 4°C until they were returned to the lab, where both fecal and skin swab samples were later preserved at -80°C.

1.2.3 DNA extraction and amplification

The DNA extraction process was performed on all samples using the MagAttract® PowerSoil® DNA EP extraction kit, with slight modifications to the manufacturer's instructions. Water filters were cut into the smallest possible pieces and placed into the 96 Power Bead Pro Well. For human skin and gut samples, swabs were trimmed into small pieces weighing no more than 0.25 grams before being placed in the 96 Power Bead Pro Well. Animal gut samples were similarly prepared with a maximum weight of 0.25 grams. The DNA extraction was then carried out following the default protocol,

with the DNA eluted in 100 µL of elution buffer and quantified using spectrophotometry (Nanodrop® 8000, Wilmington, USA).

DNA amplification was performed on the DNA products of all samples by the PCR technique using the V4-V5 region of the 16*S rRNA* gene. The DNA template was amplified using modified prokaryotic primers for Illumina sequencing with forward 515F (5'-GTGYCAGCMGCCGCGGTAA- 3') (Caporaso et al., 2011) and the modified version of reverse 926R by Parada (Parada et al., 2016) (5' – CCGYCAATTYMTTTRAGTTT - 3'). PCR protocol of the samples were ran using Taq Phusion, 3µL of the extracted DNA, and 1 µL of each primer to a total volume of 50µL. The process involved an initial denaturation at 98°C for 30 seconds, followed by 35 cycles of 98°C for 10 seconds, 60°C for 1 min, and 72°C for 1 min 30 sec, and a final extension at 72°C for 10 min.

1.2.4 16*S rRNA* sequencing and processing

After the DNA extraction process, a total of 149 samples collected across three seasons were prepared for sequencing. These included 76 human samples (38 gut and 38 skin samples), 9 water column samples, and 64 animal gut samples (42 fish and 22 lobster samples). The DNA was sequenced by an external laboratory using the Illumina platform with 2x250 bp MiSeq chemistry (INRA GeT-PlaGE platform, Toulouse, France). Raw sequence reads from our samples and the reads of the first control group were processed using the 'DADA2' R package v.1.2 (`https://benjjneb.github.io/dada2/index.html`). Briefly, reads were trimmed at the 5' end to remove forward and reverse primers based on the provided primer sequences. For control samples, the V3-V4 regions of the 16*S rRNA* gene were amplified using a different set of specific primers: 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'). After primer removal, reads were trimmed based on a quality score of 2 and filtered to exclude reads with more than two expected errors before dereplication. The forward and reverse reads were then merged, and chimeric sequences were removed. The remaining Amplicon Sequence Variants (ASVs) were taxonomically classified using the SILVA database v138.1, with non-bacterial or archaeal sequences excluded at the end. The ASVs were filtered using a two-step method to remove contam-

inants. First, the "decontam" R package (N. M. Davis et al., 2018) was used to detect ASV contaminants, then known extraction kit contaminants reported by Salter, such as ASVs from the *Bradyrhizobium* and *Cupriavidus* genera, were manually removed from the final (Salter et al., 2014). To normalize data in our samples, we implemented the 'rarefy' function from the 'vegan' R package v. 2.5-5 based on the lowest sample read (Dixon, 2003).

1.2.5 Potentially pathogenic bacteria identification

Potential human pathogenic bacteria (PHPB) were identified by blasting all acquired ASVs to NEMESISdb, a custom-made pathogenic bacteria dataset (Tran et al., in revision). NEMESISdb is a curated dataset of full-length 16*S rRNA* sequences, designed to improve identify and track potentially pathogenic bacteria (PPB) for humans, fish, and crustaceans. This dataset was built by extracting sequences from the SILVA database (v138.2 SSU Ref) and refining them through a strict curation process to ensure taxonomic accuracy and remove misclassifications. In this study, we focused only on PHPB from the dataset to analyze bacterial communities linked to human diseases in high-risk cohorts across compartments. NEMESISdb contains 196,770 full-length 16*S rRNA* sequences from 1,757 human bacterial strains. For pathobiome profiling, only ASVs matching a 16*S rRNA* sequence with 100% similarity, 100% coverage, and a length of more than 400 base pairs were included. The richness and diversity of the pathobiome were assessed using functions from the R package vegan (Dixon, 2003). Bacterial originality was evaluated using the local contribution to beta-diversity index (LCBD) within R package adespatial with the "hellinger" method (Dray et al., 2018). Bray-Curtis dissimilarity was calculated using the 'vegdist' function (method = 'bray'). Principal Coordinate Analysis (PCoA) of ASV relative abundance was performed and visualized by R packages ape (v5.0) and ggplot2 (Paradis, Claude, & Strimmer, 2004; Wickham, 2011).

1.2.6 Metagenomic sequencing and Metagenome-assembled genomes assembly

Among the 149 samples collected, 13 were chosen for the first batch of metagenomic sequencing using the sequencing platform of Montpellier MGX GenomiX with a sequencing depth of 15 Gb per samples. Due to their relatively low DNA concentration, most human skin samples were barely suitable for metagenomic sequencing, as the platform required a minimum DNA concentration of 2 ng/µl. Furthermore, contamination from host DNA limited the sequencing capacity for metagenomic content and reduced the accuracy of metagenomic profiling. Based on these initial results, metagenomic sequencing was performed on a total of 51 samples, including 9 water column samples, 14 animal gut samples (10 fish and 4 lobsters), and 28 human samples (24 gut and 4 skin samples).

The 'nf-core' framework was implemented for the assembly and binning of metagenomes through the 'mag' pipeline (v3.3.0) on our samples and the second control group (Ewels et al., 2020) (`https://zenodo.org/records/14526076`). The 'mag' pipeline was executed mostly with default parameters, starting with processes like read trimming and adapter removal using Fastp, quality control with FastQC, merging multiple sequencing runs, and read taxonomy assignment using Kraken2, followed by visualization with Krona (Bioinformatics, 2011; Ondov, Bergman, & Phillippy, 2011; S. Chen, Zhou, Chen, & Gu, 2018; Wood, Lu, & Langmead, 2019). Host removal was performed using Bowtie2, with host genomes corresponding to species such as *Homo sapiens* (GCA_000001405.15), *Platax teira* (NC_024580.1), *Trachinotus blochii* (NC_024026.1), *Cyprinus carpio* (GCA_905221575.1), *Rachycentron canadum* (GCA_038496675.1), *Siganus canaliculatus* (GCA_041146955.1), *Epinephelus lanceolatus* (GCA_005281545.1), *Panulirus ornatus* (GCA_036320965.1), and *Panulirus longipes* (GCA_032273845.1). After the quality control step, filtered reads were assembled using MEGAHIT (–min-contig-len 1000) and assessed for quality with Quast (Gurevich, Saveliev, Vyahhi, & Tesler, 2013; D. Li et al., 2015). Metagenomic binning was carried out using MetaBAT2 and MaxBin2, followed by quality checks with CheckM2 and refinement using DAS Tool (Y.-W. Wu, Simmons, & Singer, 2016; Sieber et al., 2018; D. D. Kang et al., 2019; Chklovski, Parks,

Woodcroft, & Tyson, 2023). Protein-coding genes were predicted using Prodigal for the assemblies and Prokka for the Metagenome-assembled genomes (MAGs) (Hyatt et al., 2010; Seemann, 2014). Finally, each MAG was taxonomically annotated by GTDB-Tk, with an option for virus identification with Tiara (Chaumeil, Mussig, Hugenholtz, & Parks, 2020; Karlicki, Antonowicz, & Karnkowska, 2022).

1.2.7 Resistome and mobilome profiling

The relative abundances of ARGs in our metagenomic datasets were calculated using ShortBRED (v.0.9.4) (Kaminski et al., 2015). This software first created a new reference set of ARGs from the CARD database (v.4.0.0). Using this reference, we generated ARG markers with the 'shortbred_identify.py' function (–clustid 0.95) based on our assemblies. These markers were then used to quantify ARG relative abundances, expressed as reads per kilobase per million (RPKM), using the 'shortbred_quantify.py' function with default settings. The richness, diversity, and originality indices of the resistome were calculated using the same package as the pathobiome. Spearman correlation coefficients between the relative abundances of ARG classes were determined using the rcorr function from the R package Hmisc (v.5.1.3) (Harrell Jr & Harrell Jr, 2019).

We also utilized the MetaCompare2 pipeline to identify antibiotic resistance genes (ARGs), mobile genetic elements (MGEs), and pathogens in our assembled contigs (Rumi et al., 2024). This pipeline assesses the ecological resistome risk of our samples by analyzing a wide range of both known and potential ARGs, their co-occurrence with MGEs, and various human bacterial pathogens annotated in the metagenome. MetaCompare2 employs DeepARG for ARG annotation, MobileOG for MGE annotation, and multiple datasets for pathogen annotation (M. Woolhouse, Gowtage-Sequeria, & Evans, 2007; G. Arango-Argoty et al., 2018; Brown et al., 2022; Parks et al., 2022).

Genome binning resulted in a total of 1,507 MAGs from our samples, including 477 high-quality MAGs (completeness > 90%, contamination < 5%) and 1,120 medium-quality MAGs (completeness > 50%, contamination < 10%), as evaluated using CheckM2

(Chklovski et al., 2023). To explore the genomic context of ARGs, we identified the IDs of assembled contigs carrying ARGs and/or MGEs, as previously detected by MetaCompare2. The sequences of these contigs from the corresponding MAGs were then retrieved and processed with Prokka to predict the genomic details of antibiotic resistance protein-coding genes based on the CARD database (v4.0.0) and MobileOG database (v1.6). Finally, Clinker (v0.0.31) was used to compare and visualize gene clusters (Gilchrist & Chooi, 2021).

1.2.8 Bacterial isolation and antibiotic susceptibility tests

This study concentrated on key pathogens associated with humans, which included both gram-positive and gram-negative bacteria, over the course of three seasons. Bacteria were isolated and cultivated on different selective media, such as MacConkey Agar for *Enterobacteriaceae*, TCBS Agar for *Vibrio* species, Aeromonas Agar for *Aeromonas* species, and Marine Agar for all culturable marine bacteria. For the water samples, each 100 milliliters was filtered using a cellulose acetate filter membrane (0.2 m pore size) from Sartorius Stedim Biotech, France, to capture bacterial cells. The membranes were subsequently placed onto the culture media and incubated at 37°C to promote bacterial growth. In the case of sediment samples, 200 mg of each was combined with 5 ml of distilled water containing 3.5% NaCl (g/L) to mimic seawater conditions. Then, 150 l of the diluted sediment sample was spread across the same media and incubated at the same temperature. For animal and human samples, 100 – 150 l of fluids contained in the sterile swab-tube was used to spread on same set of media and incubated for the same conditions. After a period of 2-3 days, depending on the varying growth rates of the bacteria, visible colonies were recognized based on their morphology using the MALDI-Tof Biotyper® Sirius One IVD System along with the manufacturer's IVD kit from Bruker Daltonics, Germany.

All bacterial species identified in the earlier stages were subjected to antimicrobial susceptibility testing utilizing the Kirby-Bauer disc diffusion technique on Mueller Hinton Agar plates, in accordance with the EUCAST guidelines (EUCAST, 2020). The tests for antibiotic susceptibility were divided into two categories: gram-positive and

gram-negative bacteria, with each group evaluated using a specific array of antibiotics.. For gram-positive bacteria, seventeen different antibiotics were selected: Amoxicillin & Clavulanic acid (AUG-30µg), Aztreonam (ATM-30µg), Clindamycin (DA-2µg), Cotrimoxazole (SXT-25µg), Ticarcillin (TIC-75µg), Erythromycin (ERY-15µg), Fosfomycin (FOS-200µg), Linezolid (LZD-10µg), Imipenem (IPM-10µg), Gentamycin (GEN-10µg), Kanamycin (KAN-30µg), Ciprofloxacin (CIP-5µg), Levofloxacin (LEV-5µg), Ofloxacin (OFX-5µg), Vancomycin (VAN-5µg), Rifampicin (RIF-5µg), and Tetracycline (TET-30µg). For gram-negative bacteria, twenty antibiotics were tested: Amoxicillin & Clavulanic acid (AUG-30µg), Amikacin (AK-30µg), Aztreonam (ATM-30µg), Cefepime (FEP-30µg), Cefoxitin (FOX-30µg), Cefotaxime (CTX-5µg), Ceftazidime (CAZ-10µg), Ciprofloxacin (CIP-5µg), Ertapenem (ETP-10µg), Fosfomycin (FOS-200µg), Gentamycin (GEN-10µg), Imipenem (IPM-10µg), Levofloxacin (LEV-5µg), Piperacillin (PIP-30µg), Piperacillin/ Tazobactam (PTZ-36µg), Ticarcillin (TIC-75µg), Ticarcillin & Clavulanic acid (TCC-85µg), Tigecycline (TGC-15µg), Tobramycin (TOB-10µg), and Cotrimoxazole (SXT-25µg). A multidrug-resistant phenotype was defined as a bacterial isolate resistant to at least three antibiotics from different antibiotic groups. The multiple antibiotic resistance (MAR) index is defined as the proportion of resistance to a panel of tested antibiotics. It was calculated for each strain or group of strains, following the method described by Krumperman: the MAR index is equal to a/b, where "a" represents the number of antibiotics to which the strain was resistant, and "b" represents the number of antibiotics to which the strain was exposed (Krumperman, 1983).

1.2.9 Statistical analysis

The Kruskal-Wallis test was applied to assess richness, diversity, and uniqueness in both pathobiome and resistome profiles across compartments and seasons, followed by Dunn's post-hoc test for non-parametric data. For parametric data, ANOVA's Levene test was used, followed by Tukey's post-hoc test. The p-value adjustments were made using Benjamini-Hochberg's method. Differences in bacterial taxonomy and ARG community compositions were analyzed using Permanova with Bray-Curtis distance matrices, utilizing the adonis2 function from the vegan package, while Principal Coordinates Analysis (PCoA) was used for visualization. ASV biomarkers (i.e., differ-

ences in ASV abundance between compartments) were identified through bacteriome composition analysis with bias correction, performed using the LEfSe (Linear discriminant analysis Effect Size) method (Segata et al., 2011). Differentially abundant ARGs and antibiotic classes were identified using MaAsLin2, considering biosample groups as the fixed effect (e.g., Farmers vs Animals) (Mallick et al., 2021). All statistical analyses and visualizations were conducted using R version 4.2.0 and RStudio build 394.

1.3 Results

1.3.1 Whole bacteria communities between compartments

In total, 18291 bacterial ASVs were found among 142 sequenced samples from animals, humans, and the environment, covering 49 phyla, 103 classes, 450 families, and 1328 genera. Overall bacterial richness and diversity of the environmental water microbiome were significantly higher than those from human and animal communities (Dunn post-hoc test, $p < 0.05$) (Supplementary Material SM3-3). However, local contribution to beta diversity (LCBD) index indicated that animals microbiota were significantly more unique than the other compartments (Dunn post-hoc test, $p < 0.05$) (Supplementary Material SM3-3). Differences in sample types and seasons were observed in the dissimilarity of bacterial composition, as demonstrated by principal coordinates analysis (PCoA) with Permanova results (Permanova, $p < 0.05$, $R^2 = 0.16$ and $R^2 = 0.03$, respectively). The analysis also revealed a distinct discrepancy in bacterial communities from human and animal samples (Supplementary Material SM3-3).

Generally, *Firmicutes* represented the most abundant phylum across all samples (34.8% of total ASV reads), followed closely by *Bacteroidota* and *Proteobacteria* (33.7% and 19.2%, respectively). In particular, *Bacteroidota* and *Firmicutes* dominated the total ASVs abundant of the bacterial communities in human and animals at 51.4% and 53.1%, respectively, whereas environmental communities were dominated by *Proteobacteria* (35.5%) (Supplementary Material SM3-3). At the genus level, *Prevotella* (20.5%) dominated the communities, followed by *Candidatus Bacilloplasma* (5.5%), *Photobacterium* (3.1%), *Clostridium* (2.7%), *Bacteroides* (2.5%), and *Faecalibacterium* (2.4%). More-

over, 14.4% (n = 192) of the richness of bacterial genera was found to be commonly shared across all types of compartments (human, animals, and environment) (Supplementary Material SM3-3). Within farmer samples, a total of 85 classes, 384 families and 1087 genera were found fecal and skin microbiota. Notably, *Bacteroidota* (51.4%), *Firmicutes* (25.6%), and *Proteobacteria* (16.7%) also dominated the microbiota, mimicked the whole bacterial ASV composition of the entire dataset. On the other hand, only 16 different classes, 32 families, and 100 genera were found in non-farmer samples. The total bacterial ASV reads within these samples was mainly dominated *Firmicutes* (94.8%), followed by *Proteobacteria* (2.4%) and *Cyanobacteria* (0.9%). Detail of the alpha diversity and composition of non-farmer whole bacterial communities is presented in Supplementary Material SM3-3 & SM3-4.

1.3.2 Identification and characterization of the PHPB

Blasting bacterial ASVs to the NEMESISdb revealed 532 ASVs (2.89%) as potential human pathogenic bacteria (PHPB). These pathogenic PHPB ASVs belong to 87 families, 150 genera, and 362 species. *Prevotellaceae* was the most abundant bacterial family among PHPB species, accounting for 36.5% of the total PHPB reads, followed by *Vibrionaceae* (13.7%), *Ruminococcaceae* (9.3%), and *Streptococcaceae* (8.5%). PHPB species from *Prevotellaceae* were also presented in all compartments, accounted for 61%, 48%, and 5.3% of total PHPB reads in water, farmers, and animals, respectively (Figure 3.1A). The core PHPB was composed by 246 ASVs dominated by 2 species present in more than 50% of the total samples: *Prevotella copri* and *Faecalibacterium prausnitzii*. Most of the core ASVs mainly belonged to families *Moraxellaceae* (7.7%, n = 19), *Bacteroidaceae* (5.7%, n = 14), and *Streptococcaceae* (5.7%, n = 14) (Figure 3.1B).

In terms of PHPB alpha diversity, farmers exhibited significantly higher values compared to environmental and animal samples (Figure 3.1C). The shared PHPB species between different compartments (farmers, animals, and environment) accounted for 11.54% (n = 45) of the total PHPB species richness (Figure 3.1D). Interestingly, within the floating cage system, the most abundant PHPB species of *Prevotella copri* (34%), *Faecalibacterium prausnitzii* (9.3%), *Photobacterium damselae* (8%), were also

shared between human, animals and water, along with *Staphylococcus epidermidis* (3%) and *Vibrio harveyi* (3%). Moreover, between farmers and animals, the hosts microbiotas also shared together a total of 120 exclusive species richness (in absent of water microbiome), accounted for 30.8% of the total PHPB species richness. In particular, the most abundant species that presented within all types of samples based on total number of ASV reads were *Prevotella copri* (34%), following by *Faecalibacterium prausnitzii* (9.3%), *Photobacterium damselae* (8%), *Streptococcus iniae* (4.8%). The relative abundance of the PHPB to the whole bacteria communities were also higher in farmers (0.32 ± 0.11%) than the other two sample types (0.16 ± 0.21% and 0.02 ± 0.04% in animals and environment, respectively) (Figure 3.1E). However, compared to human samples, the LCBD index was significantly higher in animal samples, explaining the unique in origin of PHPB from animals microbiota (Figure 3.1C) (Supplementary Material SM3-5). Principle coordinate analysis (PCoA) also illustrated that differences in PHPB composition was mostly based on the sample types (Permanova test, $R^2 = 0.17$, $p < 0.05$) and seasonal influence (Permanova test, $R^2 = 0.02$ $p < 0.05$) (Figure 3.1F) (Supplementary Material SM3-5). The most abundant PHPB species in the farmers' pathobiome were *Prevotella copri* (45%), *Faecalibacterium prausnitzii* (12.7%), *Prevotella stercorea* (3.3%), *Bacteroides vulgatus* (2.9%). In the animal's pathobiome, *Photobacterium damselae* (28.4%), *Streptococcus iniae* (17.4%), and *Prevotella copri* (5%) were the dominant species. Meanwhile, in the environmental pathobiome, *Prevotella copri* (61.1%), *Vibrio parahaemolyticus* (7%) *Faecalibacterium prausnitzii* (5.1%) were predominant (Figure 3.1A). Using LEfSe analysis, we also detected 12 biomarkers with the highest prevalence from *Faecalibacterium* (25%, n = 3), and *Prevotella* (16%, n = 2) genera, both enriched in farmer microbiota (Figure 3.1G). Notably, all of these biomarkers taxonomy were also part of the core ASVs except *Holdermanella bioformis*, with the majority are enriched in human samples, mainly from *Prevotella copri* and *Faecalibacterium prausnizii*. In contrary, only *Photobacterium damsele* was enriched in animals microbiota and while *Bacteroides vulgatus*, *Enterobacter cloacae* and *Vibrio aestuarianus* were enriched in the water (Figure 3.1B&G).

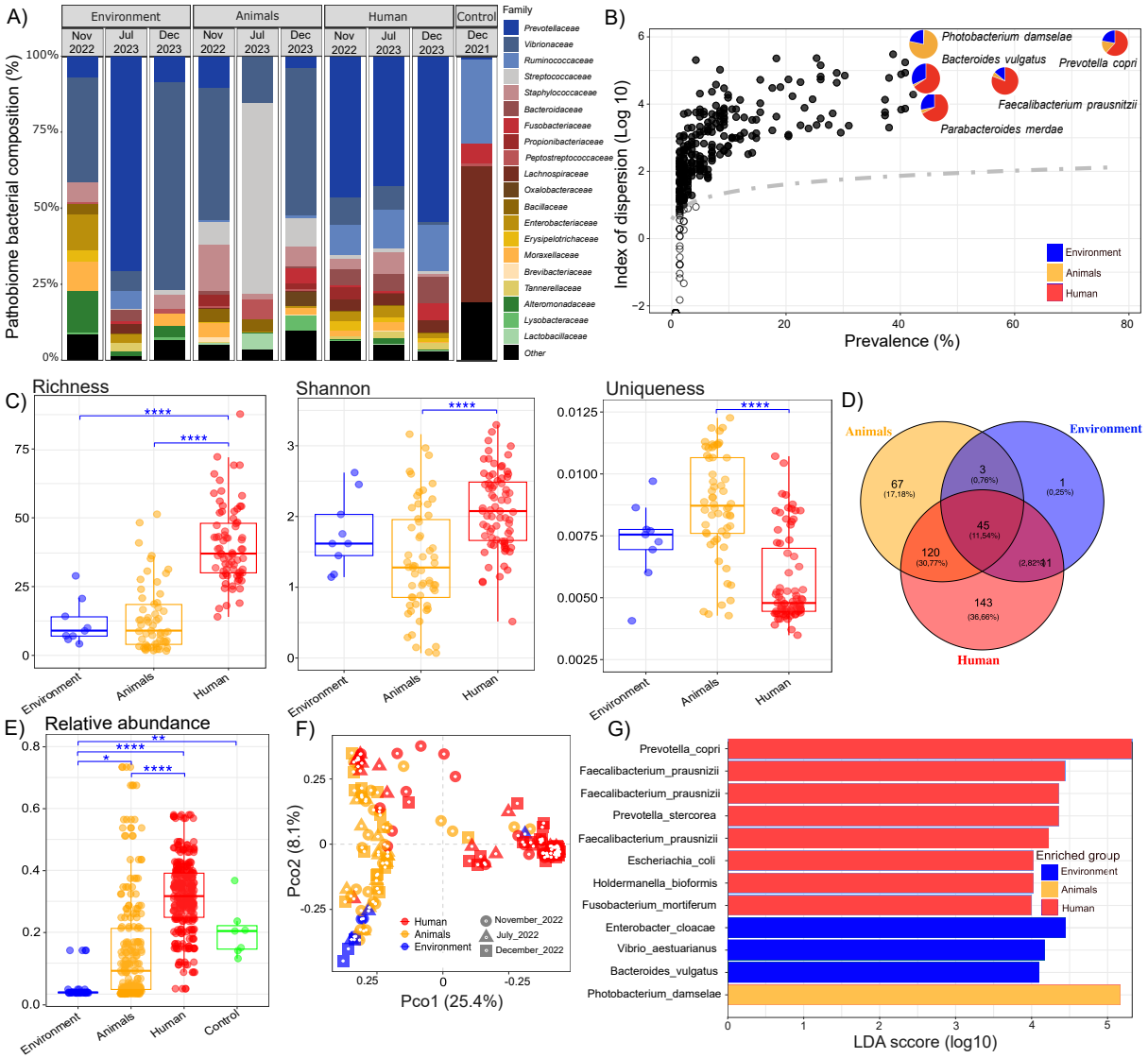


Figure 3.1: Pathobiome analyses between compartments. A: PHPB composition of each compartments across seasons. Top 20 families in each sample types are presented with colors. B: Occurrence of pathogenic ASVs among samples plotted against its dispersion index. The dotted line depicts the 5% confidence limit of the Chi^2 distribution: ASVs located above this line are non-randomly distributed among samples, whereas those bellow the line follow a random Poisson distribution. Pie-charts display the relative abundance among sample types of the most occurrent ASVs. C: Alpha indices of PHPB richness, diversity, and originality across compartments. D: Venn diagram showing PHPB richness and the shared PHPB composition between compartments in the floating farms setting. E: Relative abundances of the pathogenic bacteria to the whole bacteria in water column (environment), animals, farmers, and non-farmer individuals. F: Principal coordinate analysis of Bray–Curtis dissimilarities in the PHPB compositions. G: Pathogenic biomarkers identified in each sample type by LEfSe. The x-axis represents log fold changes of Linear Discriminant Analysis (LDA) score for these differentially abundant species.

1.3.3 Bacterial isolates and antibiotic susceptibility test

During the whole longitudinal survey, a total of 308 bacterial isolates were obtained, with 101 (32.8%), 84 (27.3%), and 123 (39.9%) isolates from November 2022, July 2023 and December 2023, respectively. Unfortunately, due to technical problems, no human isolates were obtained in July 2023. Animal samples yielded more culturable isolates (n = 168, 54.5%) than the farmers (n = 98, 31.8%) and water (n = 42, 13.7%).

Results from MALDI-ToF successfully identified 124 (40.2%) of the 308 isolates. These bacteria are classified into 12 genera and 29 species. *Proteus*, *Escherichia*, *Klebsiella*, and *Bacillus* species were the most abundant bacterial isolates in our data, representing 22.6%, 15.3%, 14.5% and 11.3% of the total identified taxa, respectively. Among the bacterial isolates, 90.3% (n = 112) were identified as potential human pathogenic bacteria (PHPB). Additionally, 34.7% (n = 53) of these isolates were associated with farmers, as documented in the pathogenic bacteria listed in NEMESISdb. Farmers PHPB isolates were dominated by *Escherichia coli* (27.8%), *Proteus mirabilis* (22.2%), and *Klebsiella pneumoniae* (16.7%) (Figure 3.2A). Moreover, 3 PHPB isolates of *Escherichia coli*, *Klebsiella pneumoniae*, and *Bacillus subtilis*, were found on all compartments, accounted for 15.3% (n = 19), 11.3% (n = 14) and 4% (n = 5) of the total identified isolates. All of the 124 identified isolates were further tested for their phenotypic antibiotic resistances. The overall mean MAR index of our isolates was 0.26 ± 0.20, with 54% of the total isolates exceeding a MAR value of 0.2, suggesting a high prevalence of multidrug resistance (Figure 3.2B). Bacterial isolates from human exhibited MAR indices on an average of 0.31 ± 0.22, while animals and water samples posed with lower values at 0.24 ± 0.17 and 0.20 ± 0.22, respectively. Seasonality significantly affected MAR indices, where rainy season of December 2023 isolates exhibited higher values than the other seasons (Kruskal-Wallis test, $p < 0.05$, Figure 3.2B).

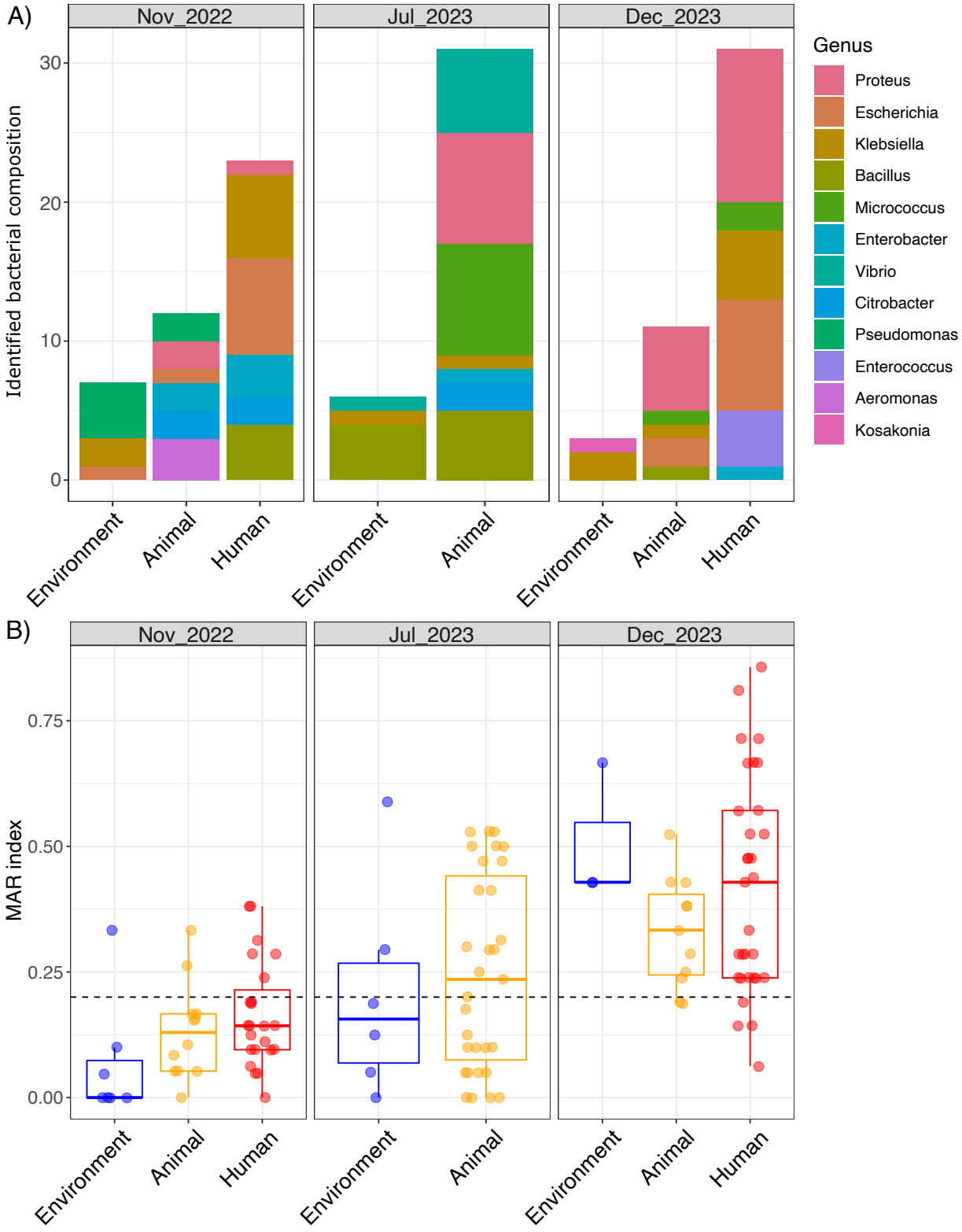


Figure 3.2: A: Number of identified bacterial isolates between compartments and season. B: MAR index of bacterial isolates between compartments and season. Dot-line represents the threshold for multiple antibiotics resistance (0.2).

Furthermore, phenotypic resistance indicated that multidrug-resistant (MDR) bac-

teria comprised 73.4% of the total antibiotic susceptibility tested isolates (Figure 3.3). Specifically, certain strains such as *Proteus mirabilis*, *Enterobacter hormaechei*, *Escherichia coli* and *Klebsiella variicola* among Gram-negative bacteria, along with *Enterococcus faecalis* and *Bacillus cerius* among Gram-positive bacteria, demonstrated resistance to over ten different antibiotics (Figure 3.3). Seasonal influences and differences in sample types had a significant impact on the MAR index and phenotypic resistance patterns of the Gram-negative isolates (Kruskal-Wallis test, $p < 0.05$, Permanova test, $p < 0.05$; Figure 3.2 & Figure 3.3) (Supplementary Material SM3-5 & SM3-6). Bacterial isolates obtained during the rainy season showed high levels of resistance to the penicillin group such as amoxicillin–clavulanic acid (49% of isolates, n = 37), ticarcillin (46.7% of isolates, n = 35) and, ticarcillin-clavulanic acid (26.7% of isolates, n = 20) (Figure 3.3A). Surprisingly, isolates from rainy season of December 2023 also exhibited high levels of resistance to cephalosporin (ceftazidime & cefotaxime), tigecycline, fosfomycin, aztreonam, and trimethoprim antibiotics, compared to lower resistance in rainy season of November 2022. Conversely, gram-negative bacteria identified in the dry season of July 2023 only exhibited resistance to a few antibiotics, including amoxicillin–clavulanic acid, ceftazidime, cefotaxime and aztreonam (Figure 3.3A). Between different compartments, farmer's isolates dominated the phenotypic resistance, with 72.3% (n = 32) of the isolates showed resistance to more than 3 antibiotic classes (Figure 3.3). Environmental isolates, compared to farmers and animal counterparts, displayed much lower resistant patterns. Interestingly, several isolates cultured from all compartments during the rainy season in December 2023, primarily *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Escherichia coli*, demonstrated similar phenotypic resistance patterns to a range of antibiotics. These included antibiotic drugs from the penicillin group, such as amoxicillin with clavulanic acid, ticarcillin, and ticarcillin with clavulanic acid, as well as cephalosporins like cefepime, ceftazidime, and cefotaxime. Additionally, their resistances were against other antibiotic classes, including tigecycline, fosfomycin, cotrimoxazole, and aztreonam (Figure 3.3A). On the other hand, no significant differences of season variability or sample types were observed in Gram-positive isolates phenotypic resistance (Permanova test, $p > 0.05$, respectively; Figure 3B) (Supplementary Material SM3-5). However, these bacteria particularly showed high resistance to commonly used antibiotics in aquacultural practices such as tetracycline (79.3%, n = 23)

and rifampicin (37.9% of isolates, n = 11), along with erythromycin (65.5% of isolates, n = 19), trimethoprim (55.2%, n = 16), linezolid (44.8%, n = 13), and fosfomycin (41.4% of isolates, n = 12) (Figure 3.3B).

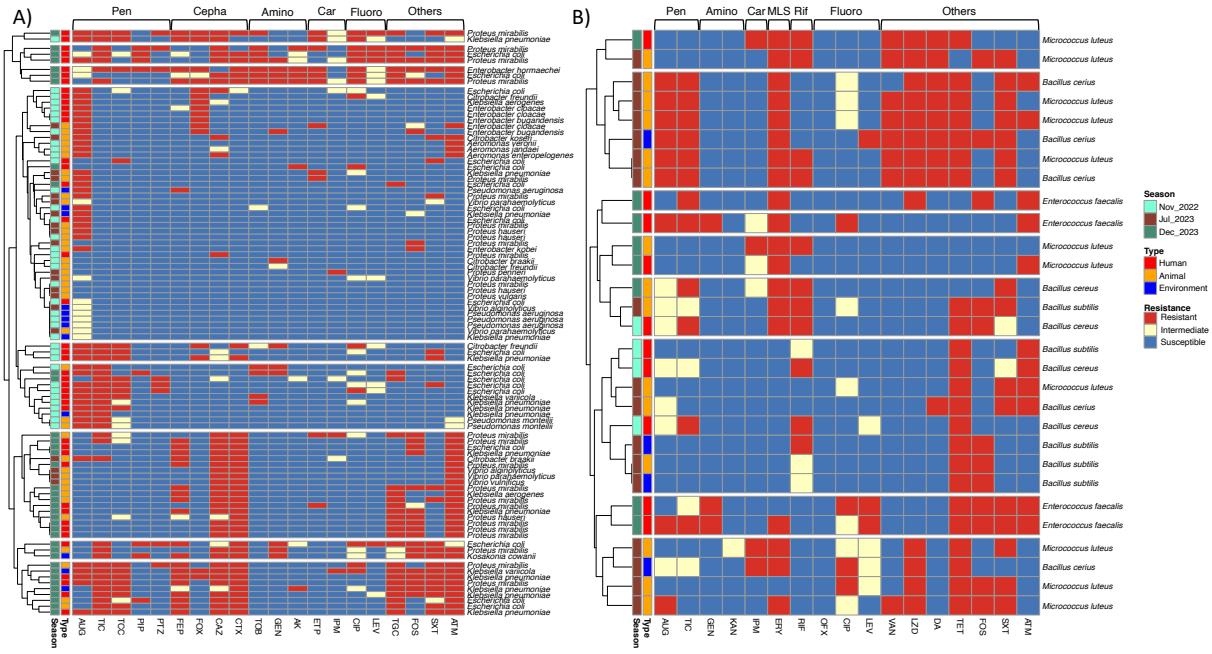


Figure 3.3: Antibiotic susceptibility test on A: gram-negative bacteria, B: gram-positive bacteria. The breakpoints of each antibiotic were determined according to EUCAST's guidance. Samples are clustered by resistant distance matrix. Antibiotic class abbreviations are: Pen: Penicillins, Cepha: Cephalosporins, Amino: Aminoglycosides, Car: Carbapenems, Fluoro: Fluoroquinolones, MLS: Macrolide–Lincosamide–Streptogramin B. Antibiotic abbreviations are: ATM - Aztreonam; AUG - Amoxicillin with Clavulanic acid; CAZ - Ceftazidime; CIP - Ciprofloxacin; CTX - Cefotaxime; DA – Clindamycin; ETP - Ertapenem; ERY - Erythromycin; FEP - Cefepime; FOX - Cefoxitin; FOS - Fosfomycin; GEN - Gentamicin; IPM - Imipenem; KAN - Kanamycin; LEV - Levofloxacin; LZD – Linezolid; OFX - Ofloxacin; PIP - Piperacillin; PTZ - Piperacillin with Tazobactam; RIF - Rifampicin; SXT - Cotrimoxazole; TCC - Ticarcillin with Clavulanic acid; TIC - Ticarcillin; TOB - Tobramycin; TET – Tetracycline; TGC – Tigecycline; VAN – Vancomycin

1.3.4 Resistome profiling in different compartments

In general, we obtained a total of 538 ARGs spanning across 26 antibiotic classes. The animal and environmental resistomes showed lower ARG richness and diversity compared to the gut resistomes of both human groups (Figure 3.4A). However, the animal and environmental resistomes exhibited a higher uniqueness of ARGs, as indi-

cated by the LCBD index (Figure 3.4A). Principal Coordinate Analysis further highlighted differences in resistome compositions, primarily driven by sample type (Permanova test, $R^2 = 0.08$, $p < 0.05$; Figure 3.4B & Supplementary Material SM3-5). In contrast to these compartmental differences, seasonal variations in the region did not affect the resistome composition, as shown by Permanova tests ($R^2 = 0.03$, $p > 0.05$) (Supplementary Material SM3-5). Additionally, we observed a higher relative abundance of ARGs (RPKM) in farmers (154.7 ± 233) and non-farmers (128 ± 226) compared to animal (13 ± 24) and environment samples (5 ± 6) (Figure 3.4C). Highest ARGs relative abundance belong to *tet*Q (21.5%), *Cfx*A6 (17.7%), *tet*W (5.0%), *tet*O (4.2%) and *Erm*F (4.1%). The most abundant ARG classes among all resistomes are tetracycline (47.7%), beta-lactam (21.8%), MLS (11.9%) and multidrug (7.4%). The shared ARGs were dominated by multidrug, tetracycline, beta-lactam, and MLS drug classes (Figure 3.4D) and the relative abundance of these shared resistome together accounted for 85.6% of the total ARGs abundance. Notably, most of the ARGs associated with aminocoumarin, tetracycline, fosfomycin, nucleoside, beta-lactam, and MLS antibiotic classes were enriched in the human resistome compared to animal resistome (Figure 3.4E). In contrast, we also found that over 20% of the total ARGs richness (108/538) belonging to 11 antibiotic classes was shared across compartments within the floating farms setting. Moreover, we found a total of 3481 unique pairs of highly correlated ARGs ($r > 0.7$, $p < 0.05$) in our data. Among these highly correlated ARGs, more than 60% were pairs of genes providing resistance to multidrug antibiotics with highest percentage of ARGs pairs are linked to resistance within multidrug ARGs (17.9%), multidrug and peptide ARGs (7.2%), multidrug and tetracycline ARGs (5.3%), multidrug and beta-lactam ARGs (5.1%), and multidrug and MLS ARGs (4.9%). Overall, the correlation of the top 10 most abundant ARG classes (resistance against multidrug, peptide, fluoroquinolone, aminoglycoside, sulfonamide, phenicol, MLS, diaminopyrimidine, tetracycline and beta-lactam antibiotics) is described in Figure 3.4F.

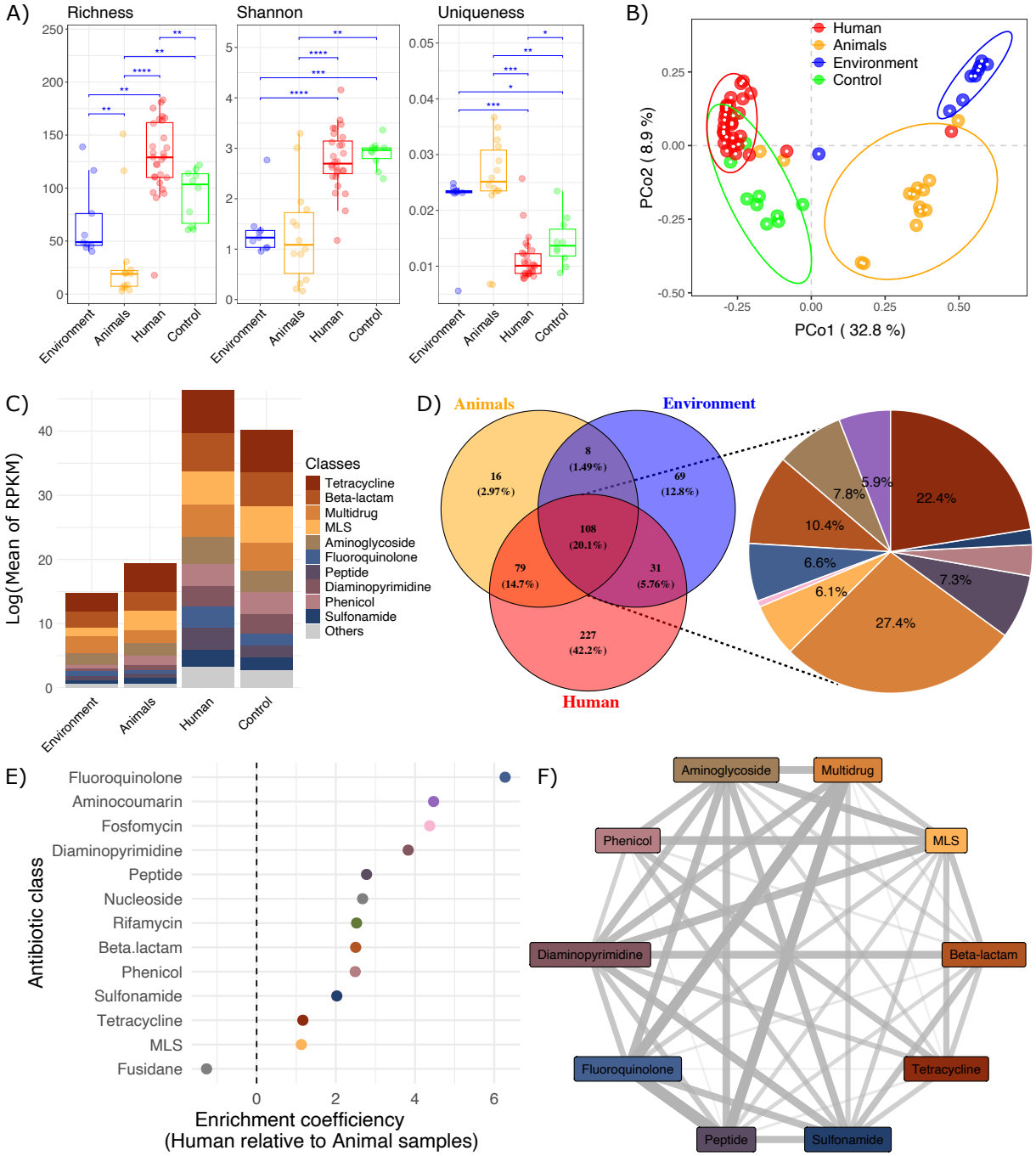


Figure 3.4: Functional metagenomic resistome profiling. A: Indices of resistome richness, diversity, and originality across compartments. B: Principal coordinate analysis of Bray–Curtis dissimilarities in the resistome compositions. C: Log of mean relative abundances (RPKM) and composition of resistome for farmers, water column (environment), animals, and non-farmers. The top 10 antibiotic classes, based on combined relative abundances (RPKMs) of corresponding ARGs, are highlighted with colors. D: Venn diagram showing ARGs richness and the shared ARGs composition between compartments in the floating farms setting. Pie colors of ARG classes are shown as in C. E: Antibiotic classes that are significantly represented (combined RPKMs of corresponding ARGs) in the human resistome compared to animal resistome. F: Network of correlating ARGs, where nodes represent antibiotic classes corresponding to ARGs and are colored as shown in C.

1.3.5 Resistome risk assessments and Metagenome-assembled genomes (MAGs) analysis of different compartments in floating cage farms

Results from MetaCompare2 contig-based analysis revealed the average risk score for resistomes in the farmers and non-farmers groups were 31.1 and 31.3, respectively, while the scores for animals and the environment were 11.6 and 2.9, respectively (Figure 3.5A). This trend was confirmed by the 3D hazard space, where the proportion of contigs carrying ARGs (x-axis), ARGs and MGEs (z-axis), ARGs and MGEs and pathogens (y-axis) for animals and environmental resistomes were in a lower position compared to farmers and non-farmer resistomes (Figure 3.5C).

Furthermore, the genetic context of AMR was examined by construction of metagenome assembled genomes (MAGs) in different compartments of the floating cage farms to evaluate potential resistomes risk. In brief, we constructed a total of 1,507 bacterial metagenome assembled genomes, with highest number of MAGs were found in farmers compared to water and animal samples, with averages of 37, 30, and 15 MAGs per sample for each compartment, respectively. The analysis of the constructed Metagenome-assembled Genomes (MAGs) revealed that the five most abundant bacterial families were *Lachnospiraceae* (16.5%), *Bacteroidaceae* (7.1%) and *Prevotellaceae* (4.4%), *Oscillospiraceae* (4.1%), and *Ruminococcaceae* (3.3%). Among these MAGs, the bacterial families *Bacteroidaceae*, *Prevotellaceae*, *Burkholderiaceae*, *Coriobacteriaceae* and *Peptostreptococcaceae* were identified as common across all three compartments in the floating cage farms. These findings align with data obtained from metabarcoding data. Importantly, out of all the MAGs analyzed, 9.2% (n = 138) were identified as potential human pathogenic bacteria according to the NEMESIS database. The most prevalent potential pathogens were *Escherichia coli* (n = 14) and *Parabacteroides distasonis* (n = 14). By combining the MAG taxonomy with the relative abundance of ARGs, we identified several bacteria from the families *Lachnospiraceae*, *Bacteroidaceae*, *Prevotellaceae*, *Tannerellaceae*, and *Enterobacteriaceae* that showed a high abundance of ARGs related to tetracycline, multidrug resistance, beta-lactam, rifamycin, and peptides drug classes (Figure 3.5D).

We also observed several ARGs and MGEs with high similarity between the contigs of different MAGs (Figure 3.5B & Figure 3.5E). Notably, among the beta-lactam drug group, ARG family *bla*OXA-347, known for its resistance to many antibiotics including aztreonam, and piperacillin (classified as the highest risk of AMR, Supplementary Material SM3-7), was showing 100% similarity in contigs from various human and animal samples. Notably, the majority of these contigs shared the same taxonomy (i.e.; *Chryseobacterium* sp. POL2) and multiple MGEs were found in close proximality of the ARGs (Figure 3.5B). In a similar finding, we were able to observe ARGs from the tetracycline drug class such as *tet*A, *tet*A(P), *tet*B(P), and *tet*R also displaying high similarity across samples from human, animals and environment compartments, especially within PHPB species of Escherichia coli and Peptacetobacter hiranonis (Figure 3.5E). Our results also highlighted these ARGs (*bla*OXA-347, *tet*A, *tet*A(P), *tet*B(P), and *tet*R) were associated with high antimicrobial resistance (AMR) risk, as outlined in the WHO List of Medically Important Antimicrobials (Supplementary Material SM3-7) (Organization et al., 2024). Based on the social data we collected, 75% of the farms in the village utilized antibiotics for treatment and prevention of diseases for both human but mostly the livestock. On an average, the farmers typically use at least 4 different drugs at the same time and the most widely used antibiotics are tetracycline, rifampicin and trimethoprim, as described in Supplementary Material SM3-8. These antibiotics were mainly bought at the local pharmacy and were intended for human use only (classified as highly and critically important). In general, the relative abundance of ARGs reflected the antibiotic usage on the farms. Tetracycline drug class dominated the resistome in all of our samples (Figure 3.4C) and also corresponded to dominant bacterial families as illustrated in Figure 3.5D.

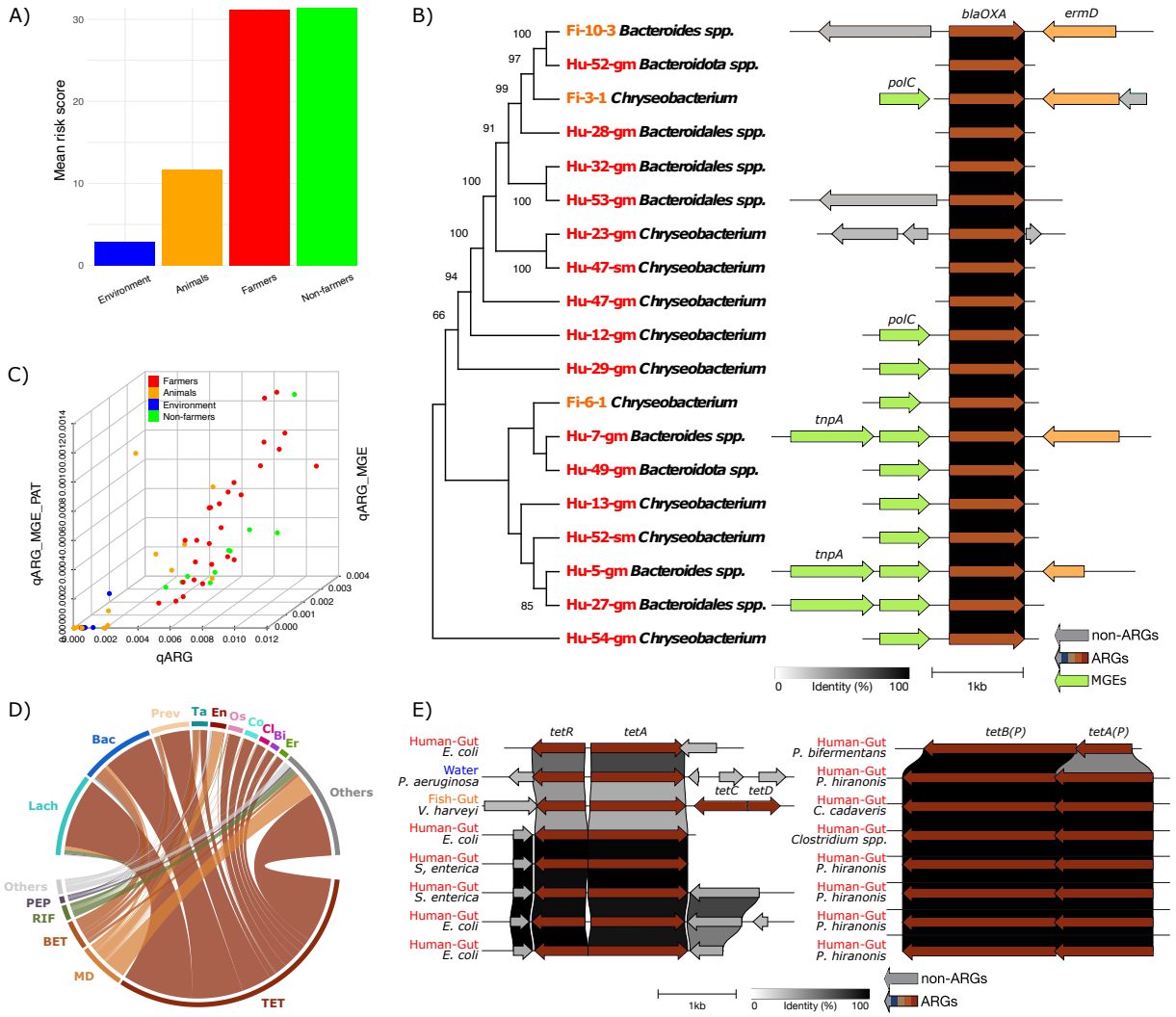


Figure 3.5: Risk of the resistomes. A: Average risk scores of the resistome between compartments. B: Phylogenetic tree of similar ARG carrying contigs and ARG synteny analysis with all variants of blaOXA-347 were aligned and centered. C: 3D hazard space diagram of the risk ratios calculated for all samples. Color codes are similar to A. D: Chord diagram showing the relationship between the ARG classes and MAGs bacterial families. Upper half indicates top 10 bacterial families and others (Lach = Lachnospiraceae, Bac = Bacteroidaceae, Prev = Prevotellaceae, Ta = Tannerellaceae, En = Enterobacteriaceae, Os = Oscillospiraceae, Co = Coriobacteriaceae, Cl = Clostridiaceae, Bi = Bifidobacteriaceae, Er = Erysipelotrichaceae), lower half indicates top 5 ARG classes and others (TET = Tetracycline, MD = Multidrug, BET = Beta-lactam, RIF = Rifamycin, PEP = Peptide). E: Tetracycline ARGs synteny analysis. All variants of the same genes were aligned and centered.

1.4 Discussion

1.4.1 Floating cages ecosystems promote circulation of pathogens and ARGs between farmers, aquatic animals and the surrounding water

Floating cages system in aquaculture has demonstrated its substantial advantages and benefits for the local and global aquaculture productions (Food & Organization, 2022; VASEP, 2023). However, such system with close interactions between people, livestock and waters, can promote incidences of bacteria and antibiotic resistance genes circulation between compartments. Cage structures are often concentrated with feces, wasted feed, and antibiotic residues. This combination accumulates ARGs, MGEs, and selects antibiotic resistant bacteria (ARB), indicating strong local selection pressure in the net cage ecosystem (M. Wang et al., 2024). Notably, global aquaculture antibiotic consumption is projected to increase by 30% in 2030. This trend will amplifies the selection pressure, largely in South East Asia where floating cages are widely utilized (Schar et al., 2020). Therefore, human, animals and environment can play critical role as reservoirs and conduits for pathogens and resistance gene dissemination, especially in regions influenced by anthropogenic activities such as effluent discharge and agricultural runoff (O. S. Ali et al., 2022; Y. Yang et al., 2024).

Horizontal gene transfer (HGT) are widely recognized as one of the main factors that spread antibiotic resistance genes (ARGs) between microbes (W. Li & Zhang, 2022). Specifically, mobile genetic elements (MGEs) play a crucial role in aiding bacteria adaptation to their surroundings and are key to transferring ARGs, especially under selective pressures such as aquaculture environments (H. Fang, Ye, Huang, Yu, & Zhang, 2021). Mobile genetic elements (MGEs) such as class-1 integrons (*intI*1) are central to ARG exchange and is widely used as a proxy of anthropogenic AMR pressure and correlates with human impacts in aquatic environments (Gillings et al., 2015). Antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria, including pathogens that can be transmitted from animals to humans (zoonose), have been found in water, animals,

and among farmers in aquaculture systems worldwide (Cabello et al., 2013; Miranda, Tello, & Keen, 2013; Grema, Geidam, Suleiman, Gulani, & Birma, 2015; Chuah, Effarizah, Goni, & Rusul, 2016; Watts et al., 2017; Santos & Ramos, 2018; Shen et al., 2018). PHPB such as *Klebsiella pneumoniae* can serves as a major carrier of antibiotic resistance genes from environment to human or animal pathogens (Wyres & Holt, 2018). The presence of ARGs, MGEs and pathogenic bacteria between human, animal, and environment further highlights the importance of the One Health approach in monitoring ecosystem health. Nevertheless, there is currently a limited understanding of the circulation and mechanism of pathogens and antibiotic resistance between compartments in aquaculture systems. Some reports suggest substantial active microbial and genetic exchange between animals, humans, and environmental reservoirs, facilitated by close physical contact and shared exposure to antibiotics (Mahmud et al., 2024). In contrast, another study by Herrero et al. (2022) found no evidence of significant cross-compartmental transfer, instead suggesting only limited circulation under specific environmental conditions (Herrero et al., 2022).

Within our floating cage ecosystems, we found that 45 PHPB species were shared among farmers, animals and environment water, accounting for 11.5% of the total PHPB species richness (Figure 1D). Most of the shared PHPB species belonged to the *Prevotella*, *Faecalibacterium*, and *Vibrio* genera, which also served as core ASVs (Figure 3.1B). Consistently, Bacteroidaceae and Prevotellaceae were also idenfied as shared bacterial families from MAGs analysis. The Prevotella lineage was detected to co-occur in both farmers and animals guts from the same study by Mahmud, suggesting a high likelihood of circulation of this genus between different human and animals (Mahmud et al., 2024). Other pathogens such as *Aeromonas* spp, although presented with lower abundance within our 16*S rRNA* dataset, still found presented within all three compartments. *Aeromonas* species are often presented in freshwater, estuarine, and saltwater habitats. However, these organisms have gained many research interest due to their ability to function as pathogens affecting a diverse spectrum of hosts, including fish, amphibians, reptiles, and even mammals, such as humans (Janda & Abbott, 2010; Plumb & Hanson, 2010). The consumption of contaminated water and aquatic products are considered the main sources of *Aeromonas* infection in human (Hoel, Vad-

stein, & Jakobsen, 2019; Nhinh et al., 2021). Interestingly, *Vibrio aestuarianus*, which is often claimed to be oyster-associated pathogen and non-colonizable in humans, was enriched in water and also detected on the aquaculture farmers (Coyle et al., 2023; C. Lupo et al., 2019). This finding suggests that, although there may not be an immediate risk, there is a possibility for environmental pathogenic bacteria to circulate and colonize the human microbiota and cause disease in the future.

A similar pattern was observed when 20% of the total ARG richness was shared among the three compartments of the floating cages farms setting (Figure 3.4D). Most of the shared ARGs belonged to the multidrug, tetracycline, MLS, and beta-lactam drug classes, showed strong correlations with each other, and were also more enriched in the human resistome. The highest numbers of shared pathogens and ARGs were observed between farmers and animals (Figure 3.1D & 3.2D), indicating a potential circulation of ARG-carrying pathogens between hosts. This result also suggests that, although the environment can act as a reservoirs for pathogens, its influence on ARGs is only temporary (Permanova test, $R^2 = 0.03$, $p > 0.05$, Supplementary Material SM3-6). Furthermore, a report by Wang and colleagues had also highlighted the connections between several bacterial phyla with ARGs and MGEs. In his study, *Bacillota* has been identified as key role in carrying and transferring ARGs. For instance, *Planctomycetes* are linked to aminoglycoside, while *Chloroflexi* and *Bacteroidetes* show strong associations with tetracycline and MLS. On the other hand, *Synergistetes* is closely related to *bla*CTX-M, *ere*A, *tet*G, and *sul*1 (S. Wang et al., 2021). However, unlike other phyla, it was suggested that *Pseudomonadota* was not strongly connected to ARGs and/or MGEs in previous study (J. Zhang et al., 2016). In our study, analyzing these highly correlated genes, we found that beta-lactam, multidrug, MLS, peptide, and tetracycline drug classes were predominant in many potential hosts, but mostly in *Pseudomonadota* and *Bacillota* phyla. Hence, our findings provided additional information about the relationship between *Pseudomonadota* species with ARGs, potentially support the reveal of more pathogens and antibiotic resistance genes circulation between compartments. Moreover, the phenotypic resistance of bacterial isolates also demonstrated similar resistance patterns, especially from animals and farmers isolates to amoxicillin–clavulanic acid, ceftazidime, cefotaxime, tetracycline and fosfomycin

(Figure 3.3). This finding is also consistent with a recent study of Reverter in 2020 that revealed the phenotypic resistance and multiple antibiotic resistance index (MAR) of aquaculture animal-derived bacteria correlates strongly with the same index of human isolates, especially in LMICs (Reverter et al., 2020). The potential circulation of pathogens and ARGs can also be observed from the genomic context of different compartments. The majority of farmer and animal contigs were 100% identical and annotated as the same species of *Chryseobacterium* sp. POL2, with multiple MGEs were found in close proximality of the *bla*OXA gene (Figure 3.5B). The bacteria species *Chryseobacterium* sp. POL2 has been indicated as a contributor for the dissemination of multiple ARGs through horizontal gene transfer conjugation mechanism (Fu et al., 2021). Other ARGs from tetracycline drug class (*tet*A, *tet*A(P), *tet*B(P), and *tet*R) also displayed high similarity across contigs from several pathogens such as *Escherichia coli*, *Salmonella enterica*, and *Peptacetobacter hiranonis* of samples from human, animals and environment compartments. These pathogenic bacteria were known to harbor multiple ARGs in both human and animals (Hammerum & Heuer, 2009; J. Fernández, Guerra, & Rodicio, 2018; X.-J. Chen et al., 2020). Taken altogether, floating cage ecosystems where exposure rates are intensified can increase selection pressures, and horizontal gene transfer across humans, aquatic livestock, and surrounding waters.

1.4.2 Potential risk of bacterial infection and antimicrobial resistance spread from farmers to animals and environment in the floating cages ecosystems

The burden of antibiotic resistance genes (ARGs) varied significantly across different compartments of the aquatic environment, reflecting the complex interactions among humans, animals, and their shared surrounding water. Our hypothesis is that aquaculture farmers potentially face a higher burden of antimicrobial resistance (AMR) and bacterial infections, likely because of the repeated, direct exposure to concentrated, pathogen-rich environment and antibiotic residues for a long period of time. However, there are currently lack of studies focus on the pathobiome and resistome in different compartments of aquaculture settings. Most of current studies primarily focus on specific, well-known pathogenic bacteria that affect humans but they of-

ten overlook anthropogenic factors, particularly those related to practices in aquaculture (Wachsmuth et al., 1994; Finkelstein et al., 2002; Arab et al., 2020; Håkonsholm et al., 2020; Tian et al., 2024). Noteworthy, a study on Korea aquaculture indicated that fecal *Escherichia coli* in fishery workers showed significantly higher resistance to tetracycline, cephalothin, and trimethoprim-sulfamethoxazole than non-aquaculture workers (H.-H. Shin & Cho, 2013). Other pathogens have been repeatedly reported in aquaculture and are potentially relevant to farmers exposure, including *Vibrio parahaemolyticus* (T. H. T. Tran et al., 2018; Hong To et al., 2020), *Aeromonas hydrophila* with multidrug-resistant profiles (Nhinh et al., 2021), ESBL *Escherichia coli* (Sivaraman et al., 2021; Uhland et al., 2023), *mcr*-1–positive *Escherichia coli* from farmed fish (Hassan et al., 2020), and MRSA detected in shrimp-farm settings (Rajan et al., 2022). This situation of antibiotic resistance bacteria is likely resulted from the extensive and sometimes inappropriate use of antibiotics in both daily routine and aquacultural practices, which was already reported in numerous studies (Carrique-Mas et al., 2020; Hedberg et al., 2018). Another study on antimicrobial usage in aquaculture exposed that antimicrobial agents used in humans were also commonly used in animals feed for purposes like growth promotion, disease prevention, and treatment (Angulo, Baker, Olsen, Anderson, & Barrett, 2004). The consequences of these misuse and overused of antibiotics were further supported by a study of Ravi and colleagues, who observed significant changes in the gut microbiota of severely ill patients, where, despite the use of broad-spectrum antimicrobial agents like meropenem, pathogenic strains continued to dominate over commensals, disrupting gut microbiota diversity (Ravi et al., 2019).

Overall, our study findings indicated that the relative abundance of both PHPB and ARGs were consistently higher in farmers compared to those from animals and water column (Figure 3.4B & Figure 3.5C). All of the highest abundant ARG classes, such as aminocoumarin, tetracycline, fosfomycin, nucleoside, beta-lactam, and MLS were found more enriched in the human resistome compared to animal resistome, confirming human hosts under great burden of antimicrobial resistance (Figure 3.4C&E). High correlation between different antibiotic classes (Figure 3.4F) also reflecting the uses of multiple antibiotics on the farms, which exerts selective pressure on the human microbiome, fostering the persistence and proliferation of potential multidrug-resistant

bacteria (R. Davies & Wales, 2019). Moreover, results from phenotypic resistances of isolates from farmer samples showed highest MAR index (0.31 ± 0.22) (Figure 3.2B), mostly from resistance to antibiotic class of penicillin, tetracycline and fosfomycin (Figure 3.3). In contrast, animals on the farms, while also exposed to antibiotics through mix-ed feed and prophylactic treatments, generally exhibit lower ARG abundance than human counterpart (Figure 3.4C). Their respective MAR index and phenotypic resistance were also lower compared to human counterparts. This observation has been reported, possibly due to differences in host microbial composition (Mahmud et al., 2024). On the other hand, environmental water samples typically harbor the lowest ARG burden compared to human and animals, as also illustrated in Figure 3.4C. These observations may be biased due to most existing ARG databases are heavily focused toward antibiotic resistance genes characterized from commensal or pathogenic bacteria relevant to human, with an underrepresentation of ARGs from other environments (C. Lee et al., 2023). However, novel antibiotic resistant bacteria and ARGs in water or animals can transfer toward humans, either through horizontal gene transfer or seafood consumption, especially in communities that depend on aquaculture for food and income like the floating cages setting, intensified the risk of contamination (Cabello, Godfrey, Buschmann, & Dölz, 2016; T. H. T. Tran et al., 2018). In deed, contaminated water and aquatic food products are regarded as the primary causes of human *Aeromonas* infection (Hoel et al., 2019). Therefore, the risk of antimicrobial resistance in aquaculture farmers is not to be underestimated.

In addition, higher PHPB species richness as well as diversity from ASVs in farmers compared to the animals and environment also suggests a risk of bacterial infection in human compartment (Figure 3.3 & Figure 3.4). The increasing number of bacteria especially PHPB in human gut microbiota along with the presence of antibiotic resistance genes from authorized for human used only antibiotics, severely pose a high risk of bacterial infections and also making antimicrobial resistance significantly more challenging. Overall, most of the core ASVs in our data were enriched in farmers, mainly from *Prevotella copri* and *Faecalibacterium prausnizii*. Both of these two species are known as human fecal markers, with *Prevotella copri* recognized as enteric opportunistic genera (C. Lee et al., 2012; Koskey et al., 2014), and *Faecalibacterium praus-*

nizii as the most common bacterial species found in human intestinal (Saarela, 2019). The proliferated presence of a diverse range of PHPB in the farmers' pathobiome, particularly *Prevotella copri*, *Faecalibacterium prausnitzii*, *Prevotella stercorea*, and *Bacteroides vulgatus*, indicates that these pathogens are not rare in the human gut microbiota as expected and observed in other studies (Han et al., 2024; Shimasaki et al., 2019; Magruder et al., 2019). In fact, they are abundant and significantly enriched in humans compared to animals or the water column (Figure 3.1E&G). This finding also suggests that our method for identifying potential pathogenic bacteria in the microbiome using 16*S rRNA* sequencing and NEMESISdb is effective and accurate. Details from bacterial culture also indicated high prevalence of pathogens isolated from farmers, with 34.7% of the identified isolates taxa classified as PHPB in farmers. The highest abundant of these human PHPB isolates were mainly from *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae*. These PHPB species are often associated with several risk factors and infections (MacKinnon et al., 2020; Fox-Moon & Shirtliff, 2024; D. Chang, Sharma, Dela Cruz, & Zhang, 2021), indicating potential infections in aquaculture farmers.

Furthermore, Metacompare2 results revealed that human's resistomes (farmers and non-farmers) accounted for the highest risk scores compared to the rick scores in animals and the environmental resistomes (Figure 3.5A). Although in the floating cage aquaculture settings, farmers generally don't consume antibiotics directly on daily basis as reported from the interview, the resistome risk score of these farmers were highly identical to the resistome risk score of non-farmers (31.1 vs 31.3, respectively), indicating the strong influence of human host as well as anthropogenic activities on ARGs profiles. Result from the study of Ali, even though only focused only on the resistome risk of wastewater from different sources, also pointed out that intensive anthropogenic activities was a strong driver for the risk score (O. S. Ali et al., 2022). These findings further indicate human are preferred reservoirs for pathogens and ARGs, and are not only facing a higher burden but also propagating risk of antimicrobial resistance and bacterial infections in floating cage farm ecosystems.

1.5 Conclusion

In conclusion, this study exposed floating-cage aquaculture in Nha Trang Bay as a concentrated hotspot of fecal contamination, antibiotic resistance genes (ARGs), and an opportunist-dominated pathobiome. Accumulated feces, wasted feed, and residual antibiotics within cages create strong, sustained selection that fosters potentially pathogenic bacteria (PPB) and amplifies ARG proliferation across the human–animal–environment interface. Microbial exchange among compartments was substantial, with human fecal markers such as *Prevotella copri* and *Faecalibacterium prausnizii* circulating between all aquaculture compartments. High *intI*1 abundance and co-occurrence with multiple mobile genetic elements suggest active horizontal gene transfer and elevated dissemination potential. The resistome composition also mirrored antibiotic usages in aquaculture practices, with prominent tetracycline, multidrug, and beta-lactam signatures. Ecologically, difference in sample type was the primary driver of both pathobiome and resistome patterns, with farmers consistently bearing higher pathogen and resistance burdens than animals or surrounding water, highlighting disproportionate occupational risk. Overall, floating-cage systems concentrate exposure, selection pressure, and gene exchange, thereby intensifying the enrichment and circulation of pathogens and ARGs across humans, livestock, and the environment.

General Discussion

Infectious diseases (IDs) affecting humans and wildlife, including domestic and livestock animals, as well as antimicrobial resistance (AMR), represent a significant and increasing threat to both global health and biodiversity (Read & Woods, 2014; Vayssier-Taussat et al., 2014; Aslam et al., 2024). Several studies had shown escalating frequencies of IDs and AMR, alongside with their socio-economic effects (Figure 3) (K. E. Jones et al., 2008; Keesing et al., 2010). However, our understanding of their emergence regarding environmental, ecological, and socio-economic factors recognized as possible contributors is still limited (Allen et al., 2010). Moreover, the majority of research has concentrated on inland ecosystems, while marine environments, which can serve as reservoirs for potentially pathogenic bacteria (PPB) and AMRs due to the influx of bacteria from various origins, have received less attention (Patz et al., 2004).

To address the research gaps related to the pathobiome and resistome dynamics in coastal and aquacultural ecosystems, this thesis offers insights to help us better understand the health risks from marine pathogens and antimicrobial resistance (AMR) in contrasted areas of the Vietnamese coast. The results from the studies conducted in this thesis not only underscore the need for a well-structured 16*S rRNA* pathogenic dataset for identifying potentially pathogenic bacteria, but also reveal the inventory and the effectiveness of the circulation of potentially pathogenic bacteria and antibiotic resistance genes between human, animal and environment compartments (Figure 14).

Development of a curated dataset of pathogenic bacterial 16*S rRNA* gene

Recent advances in high-throughput sequencing enable large-scale detection of potentially pathogenic bacteria (PPB) using 16*S rRNA* gene surveys. Yet, resources suitable for precise PPB identification across human, marine animal, and environmental compartments within a One Health framework remain limited. Existing datasets frequently suffer from inconsistent/unstable taxonomy, provide only partial 16*S rRNA* regions, or focus on a single host group, hindering cross-system comparisons. To address these gaps, we developed NEMESISdb with three curated full-length 16*S rRNA* reference datasets for humans, fishes, and crustaceans, designed to support both short- and long-read sequencing across variable regions and to improve PPB detection accuracy.

NEMESISdb was assembled fromcpublished pathogen lists and detection pipelines (16SPIP, FAPROTAX, MPD, MBPD) (Wardeh et al., 2015a; Miao et al., 2017; Louca et al., 2016; T. Zhang et al., 2018; X. Yang et al., 2023). The resource comprises 13 files: three FASTA files containing full-length 16*S rRNA* sequences for human, fish, and crustacean PPB; tab-separated genus–species lists for each host group; and Excel files documenting data sources, taxonomic synonyms, and the curated species inventories (Chapter 1-1). In total, the crustacean, fish, and human datasets contain 34481, 80761, and 196770 sequences, representing 65, 223, and 1757 PPB species, respectively. Among the enrire dataset, PPB sequences are dominated by *Bacillota* and *Pseudomonadota* (on average 50.83% and 42.66%, respectively). NEMESISdb, particularly the potentially pathogenic bacteria for human host, substantially expands PPB species coverage relative to existing resources, spanning 1757 species versus 878 (Wardeh), 295 (16SPIP), 212 (FAPROTAX), 143 (MPD), and 739 (MBPD).

Applications of the NEMESISdb on different sets of data in Mayotte (Chapter 1-2) and Nha Trang Bay (Chapter 2 & Chapter 3) reveal significant differences of PPB composition between transects, sample types, substrates and seasons. Notably, we found PHPB significantly enriched and more abundant in human and animal compared to

the environment, which consistent with other studies that demonstrating pathogens are not rare in human gut microbiota (Shimasaki et al., 2019; Magruder et al., 2019; Han et al., 2024). Overall, our studies indicate the accuracy and benefit of the NEMESISdb.

The other side of the coin where marine holobionts also act as a hidden reservoirs of potential pathogenic bacteria

The "sustaining the rare" framework uncovers a striking paradox with significant consequences for ecosystem services and public health. Often seen as a way to bolster microbial biodiversity by maintaining rare taxa and the vital yet largely unexamined functions they might perform, our research indicated that this ecological process can also act as a double-edged sword. Again, we showed that for the first time, this same mechanism can unintentionally aid in the survival and even the growth of potential human pathogenic bacteria (PHPB) in marine holobionts (Chapter 1-2).

PHPB found in the ocean can be autochthonous, such as some *Vibrio* and *Aeromonas* strains, or allochthonous, like *Mycobacterium marinum* and *Erysipelothrix rhusiopathiae*, which are emerging health threats (Doni et al., 2023; Landrigan et al., 2020). External sources typically include species such as *Helicobacter pylori* and *Escherichia coli*, introduced through runoff or human activities (Wanjugi et al., 2016). These pathogens can cause various diseases, including diarrhea, respiratory infections, and wound infections (Landrigan et al., 2020). Although they are typically part of the rare biosphere in marine environments, recent surveys indicate that many PHPB are more widespread than previously thought, prompting questions about their persistence (Marc Trousselier, 2017).

Our study showed that 22 PHPB genera were significantly enriched in marine holobionts compared to the surrounding environment with 7.5% and 28.3% of these enriched genera transitioned from being rare to dominant in surface and gut microbiotas, respectively. Moreover, the number of exclusive PHPB genera to animal surfaces or gut (i.e., absent in plankton or sediment) accounted for over 48% of the total

PHPB genera richness while environmental microbial communities did not harbor any unique PHPB genera. Many of the enriched PHPB were largely autochthonous opportunists such as *Shewanella* (Fernandes et al., 2023) and many are already established as members of the marine holobionts, notably *Vibrio parahaemolyticus* and *Vibrio vulnificus* (Gilliss et al., 2013). Allochthonous *Enterobacteriaceae* (e.g., *Escherichia*, *Klebsiella*, and *Enterobacter*) were mainly enriched in fish guts, suggesting host-associated microbiotas may be more sensitive than environmental samples for early contamination. Overall, PHPB relative abundance was 2 times higher in marine holobionts than in environmental samples (12% of total reads), with fishes showing the highest proportion (15.6 ± 12.3%), indicating that mobile marine animals can act as vectors that disseminate PHPB across habitats (Troussellier et al., 2017; Khurana et al., 2020; Mills et al., 2022). Although molecular detection does not confirm pathogenicity and absolute pathogen loads were not measured, the observed diversity, enrichment, and rare-to-dominant shifts within marine hosts point to an ecological transition that merits attention (Day et al., 2001). Collectively, these patterns suggest marine holobionts can not only harbor, concentrate, but also amplify, and potentially disseminate both indigenous and external pathogens.

The discovery that marine holobionts can act as hidden reservoirs and amplifiers of potential human pathogenic bacteria (PHPB) has profound implications when considered in the context of floating aquaculture systems in South Asia, which often operate in close proximity to coral reef ecosystems. Such spatial overlap creates ecological interfaces where wild marine holobionts (corals, fish, invertebrates) and farm-raised livestock are exposed to similar microbial pools, facilitating the exchange of pathogens and resistance genes. In these environments, aquaculture species—often kept at high densities and subjected to antibiotic use—may act as amplification hubs for PHPB and antibiotic resistance genes (ARGs). Coral reef holobionts, already shown in our study to harbor enriched communities of PHPB, could serve as natural reservoirs where these bacteria persist and evolve. This raises the risk of two-way circulation:

• From aquaculture livestock to wild hosts, through effluents, waste, or escaped animals;

• From wild holobionts to cultured fish, providing opportunities for cross-colonization

and microbial exchange.

Such interactions may accelerate the emergence of multi-host pathogens and promote the horizontal transfer of resistance genes across compartments (livestock, wildlife, environment). In turn, this may undermine both ecosystem health (via increased disease outbreaks in corals, sponges, or wild fish) and aquaculture sustainability (through higher pathogen loads and reduced treatment efficacy).

From a human health perspective, the risks are multifold. PHPB enriched in holobionts—such as *Vibrio parahaemolyticus*, *Vibrio vulnificus*, or *Enterobacteriaceae*—include well-documented agents of foodborne and waterborne infections. Their persistence in both wild and farmed animals could enhance the probability of direct transmission to humans, either through seafood consumption, occupational exposure (e.g., farmers, fishers), or recreational contact with contaminated waters. Moreover, if resistance genes circulate between wild and farm-associated microbiotas, this could foster the emergence of antibiotic-resistant pathogens with reduced treatment options, echoing global concerns under the One Health framework.

In summary, the close proximity of floating aquaculture to coral reef holobionts in South Asia represents a critical ecological and epidemiological nexus. Our findings suggest that these interactions may not only compromise reef biodiversity and aquaculture productivity but also pose significant public health risks through the amplification and dissemination of both pathogens and resistance genes. Addressing these challenges will require integrated monitoring programs, prudent antibiotic stewardship in aquaculture, and the incorporation of microbial risk assessments into marine spatial planning.

Exploring the hidden diversity of the pathobiome and resistome within Nha Trang Bay

Antimicrobial resistance (AMR) is a natural evolutionary process, but its pace and spread are profoundly amplified by antibiotic usage across clinical, veterinary, food-

animal, and aquaculture sectors (Köck et al., 2018; Prestinaci et al., 2015; Suyamud et al., 2024). Although potentially pathogenic bacteria (PPB) and ARGs dynamics are often documented in humans and inland livestock, aquatic environments, especially marine coastal systems, remain underexplored despite acting as integrative hubs where human, animal, and environmental microbiomes can co-exist (Köck et al., 2018; Reverter et al., 2020; Siri et al., 2023). Addressing this gap, our study provided comprehensive phenotypic and genotypic evidences that both urban human activities and aquaculture practices can shape the pathobiome and resistome of Nha Trang Bay, a tropical coastal system located in the South of Vietnam, subjected to strong seasonal hydrology and intense local anthropogenic pressures.

MALDI-ToF identified 67.6% of the total identified isolates from both dry and rainy seasons across transects as PHPB. Potentially pathogenic bacteria from *Enterobacteriaceae* family were also detected in 44.8% of cultured isolates in the rainy season across the three transects, falling into risk group 2, with *Escherichia coli*, *Klebsiella pneumoniae*, *Vibrio alginolyticus*, and *Pseudomonas aeruginosa* were presented in all transects (Chapter 2-1). These results align with Southeast Asian studies identifying *Escherichia coli* and *Vibrio* as AMR contamination indicators (Siri et al., 2023), while *Aeromonas* was not isolated here, likely due to methodological differences and freshwater bias in regional studies (Naudet et al., 2023; Siri et al., 2023). Using our NEMESISdb (Chapter 1-1), we were also able to find 485 PHPB ASVs across 49 genera and 98 species, comprising 0.28% of the microbial community. This PHPB richness of the Nha Trang Bay is similar to the PHPB richnes found in other studies in wastewater and coastal waters, which observed an average of 16-50 PHPB genera (Cui et al., 2019; Y. Guo et al., 2022). PHPB communities in Nha Trang Bay primarily consisted of native opportunistic species like *Vibrio*, *Staphylococcus*, *Bacillus*, *Acinetobacter*, *Pseudomonas*, and *Aeromonas* (Chapter 2-2). While *Vibrio* and *Bacillus* are common in marine settings (Y.-J. Wang et al., 2020), enteric opportunistic genera *Prevotella*, *Arcobacter*, and *Enterobacter* dominated the PHPB microbiome, with *Prevotella* prevalent during the rainy season. These genera indicate human fecal contamination (C. Lee et al., 2012; Koskey et al., 2014), suggesting human-related inputs into Nha Trang Bay. This diversity nearly doubles previous findings (F. Guo et al., 2016), despite their higher bacterial community contribution (0.13-

24.65%). Our stringent criteria may explain this abundance discrepancy, highlighting the need for standardized approaches. Our research confirms the effectiveness of 16*S rRNA* amplicon sequencing for genus-level PHPB diversity assessment (T. Fang et al., 2018).

Testing phenotypic resistance, the mean MAR index across seasons was 0.29 ± 0.31, with 51.7% of isolates exceeding 0.2 threshold (Chapter 2-2). This is comparable to values in Vietnam (0.39), Indonesia (0.35), and India/Sri Lanka (0.36), and higher than African coastal waters (0.19 South Africa; 0.15 Mauritius) (Suyamud et al., 2024; Naudet et al., 2023; Reverter et al., 2020). Lower MAR means likely reflect freshwater/pond overrepresentation in meta-analyses, showing stronger MDR than seawater systems (Siri et al., 2023; P. T. P. Hoa et al., 2011). In general, dry-season isolates demonstrated broader resistance profiles and higher MAR, corresponding with reduced water flow and elevated temperature. Gram-positive bacteria showed high resistance to rifampicin (60%), which also reflected clinical tuberculosis treatment and aquaculture use (2,114 g per ton of lobster) (Hedberg et al., 2018; Mason et al., 2020). On the other hand, quantitative PCR also confirmed phenotypic resistance patterns, with *sul*1, *sul*2, *bla*KPC abundance at higher levels than other study in Asia (Ng et al., 2018) (Chapter 2-1). Beta-lactam resistance dominated phenotypically and was reflected by *bla*KPC and *bla*VIM genes. The prevalence of beta-lactam and multidrug resistance genes aligns with other coastal environments (F. Guo et al., 2016; Zhu et al., 2017; L. Zhou et al., 2022; Peng et al., 2024; H. S. Tran et al., 2025), likely entering through river discharge and human activities (Su et al., 2023). *IntI*1, indicating anthropogenic pollution, occurred in 95.8% of samples and correlated with *bla*KPC, suggesting active gene transfer platforms (Chapter 2-1).

Comprehensively, the pathobiome and resistome of Nha Trang Bay represent similar trends and patterns recognized in other studies in the same systems, highlighted the proliferation of opportunistic taxa such as *Virbio*, *Bacillus*, *Aeromonas* and *Klebsiella*, along with ARGs to the widely used antibiotic in aquaculture practices (tetracycline, rifampicin, beta-lactam, and multidrug) (Hedberg et al., 2018; Cui et al., 2019; Y. Guo et al., 2022).

Seasonality and anthropogenic inputs as drivers for the proliferation of pathogens and antibiotic resistance genes in Nha Trang Bay

Our findings indicated coastal pathobiome and resistome were influenced by hydrological regimes, nutrients, and human activities. During the rainy season, increased rainfall and river discharge diluted coastal microbial assemblages, reducing the PHPB diversity (Chapter 2-1). The rainy season transported enteric markers (e.g., *Prevotella* spp.) into previously unaffected areas, indicating bay-wide human influences. In the dry season, warmer, saltier water favored diversification of autochthonous opportunists and increased multidrug resistance traits. Spatially, dry-season patterns revealed anthropogenic impacts. In fish-farm areas, PHPB were enriched with *Vibrionaceae*, with isolates showing high resistance to penicillin and beta-lactams, matching local aquaculture antibiotic use (Hedberg et al., 2018; Naudet et al., 2023). Rifampicin resistance was prominent in Gram-positive isolates and sediment resistomes, reflecting medical and aquaculture applications (Chapter 2-2). Hedberg et al. (2018) reported high rifampicin use in lobster cages, potentially driving pathogen and ARG selection. In urban areas, pathogens and ARG richness dominated, with elevated beta-lactamases and fecal-associated taxa. These patterns resulted from untreated sewage discharge via the Cai River, while tourism and aquaculture introduced antibiotics and nutrients, promoting microbial growth. Overall, in tropical coastal settings, dry seasons present the highest exposure to multidrug resistance bacteria and ARGs (Chapter 2-1 & 2-2).

Pronounced spatial heterogeneity characterized the resistome and pathobiome, with the strongest contrasts between fish farm/recovery and urban transects. Under limited rainfall and reduced river discharge (conditions that restrict hydrological mixing) inputs from aquaculture and urban sewage exert localized, persistent effects on microbial and ARG communities (Chapters 2-1 and 2-2). This segregation yields aquaculture-associated assemblages shaped by veterinary antibiotic pressures alongside urban assemblages influenced by human-derived sewage. In the fish-farm transect, opportunistic *Vibrionaceae* (*Vibrio alginolyticus* and *Vibrio parahaemolyticus*) were prevalent and enriched (17.8% of the PHPB community), consistent with dense marine livestock in floating cages (Chapter 2-2). *Vibrionaceae* are prominent in aquaculture and include

species pathogenic to humans and farmed animals (Naudet et al., 2023, 2025; J. Lu et al., 2019; X. Sun et al., 2020; Rivas et al., 2013; Mustapha et al., 2013; Schröttner et al., 2020; Letchumanan et al., 2019; Aus der Beek et al., 2016; Stentiford et al., 2017; Ina-Salwany et al., 2019). Elevated MAR indices in the fish farm and recovery transects likely reflect intensive antibiotic use in floating cage farms. In Vietnam, antibiotic inputs per ton of fish or lobster are 100 to 1000 time higher than other nations, spanning 13 drug classes, prominently sulfamethoxazole, tetracycline, trimethoprim, and rifampicin, with 11 classified by WHO as "highly important" to "critical," and applied therapeutically, prophylactically, and as growth promoters (Hedberg et al., 2018; P. T. P. Hoa et al., 2011; Pham et al., 2015; Rico et al., 2013). Residues of tetracyclines, sulfonamides, and beta-lactams are frequently detected in ponds and effluents across Vietnam and the region, imposing strong selective pressures on microbial communities (Cabello et al., 2013; Henriksson et al., 2018). Such pressures enrich multidrug-resistant strains and facilitate horizontal gene transfer, with aquaculture environments recognized as hotspots for plasmid-mediated ARG dissemination (Shah et al., 2014; Watts et al., 2017). Consistent with this, dry-season isolates from fish and recovery transects exhibited higher MAR and ARG signatures related to rifamycins and trimethoprim, indicating that aquaculture acts as a reservoir with potential spillover to human pathogens (Chapter 2-1 & 2-2). Notably, similarities between fish-farm and recovery resistomes/pathobiomes suggest impacts can persist well after farming ceases.

In contrast, the urban transect showed the highest occurrence and diversity of pathogenic taxa and the greatest ARG richness and diversity (mean 2,985 ± 108 ARG hits), indicating strong human influence on the local pathobiome and resistome (Chapter 2-2). The resistome was notably enriched in beta-lactamase genes and coincided with elevated *Arcobacteraceae* (*Arcobacter butzleri*, *Arcobacter cryaerophilus*) and *Moraxellaceae* (*Acinetobacter soli*), recognized fecal-associated pathogens (C. Lee et al., 2012). These patterns are consistent with discharge of untreated or insufficiently treated sewage to the Cai River and into Nha Trang Bay, supported by a distance-to-shore dilution of PHPB. Our results align with studies showing urban effluents increase pathogen and ARG loads (Cui et al., 2019; Powers et al., 2020; F. Guo et al., 2016; Jurelevicius et al., 2021; Peng et al., 2024). Given limited wastewater treatment in Nha Trang, urban dis-

charges likely constitute a major source of fecal bacteria, clinically relevant pathogens (*Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus*, and *Acinetobacter*), and residual antibiotics (Rizzo et al., 2013; Hendriksen et al., 2019). Nutrient inputs further stimulate growth and interactions, facilitating HGT and ARG mobilization (Jacobs et al., 2009; F. Guo et al., 2016).

Overall, our results show that Nha Trang Bay bears a clear seasonality and anthropogenic imprint on both the pathobiome and resistome. Several ARGs were more frequent in water samples, indicating exposure risk and pelagic-benthic exchange. Seasonal factors also significantly influenced ARG and PHPB patterns, showing hydrology-driven bacterial and resistance dissemination. Anthropogenic drivers are most decisive where urban and fish farm transects collectively structure the pathobiome and resistome of Nha Trang Bay, yielding high PHPB prevalence, elevated MAR indices, and abundant ARGs, especially beta-lactamases ARGs, underpinned by the widespread of *intI*1.

Aquaculture practices using floating cages facilitates the transfer of pathogens and antimicrobial resistance genes (ARGs) among farmers, aquatic species, and the surrounding water

The floating cage system in aquaculture offers significant advantages for local and global production (Food & Organization, 2022; VASEP, 2023). However, the close interactions between human, animals, and surrounding water in such systems can lead to increased circulation of pathogenic bacteria and antibiotic resistance genes (ARGs). Enrichment in feces, wasted feed, and antibiotic residues in floating cage structures create environments that promote antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) proliferation, indicating strong selection pressure (M. Wang et al., 2024). With global aquaculture antibiotic consumption projected to rise by 30% by 2030, particularly in South East Asia, the risk of pathogens and antimicrobial resistance dissemination in aquaculture increases (Schar et al., 2020).

Horizontal gene transfer (HGT) significantly contributes to the spread of ARGs,

with MGEs like class-1 integrons serving as key players in ARG exchange (W. Li & Zhang, 2022; Gillings et al., 2015; H. Fang et al., 2021). Pathogenic bacteria and ARGs have been identified across various compartments in aquaculture systems, linking environmental and human health (Cabello et al., 2013; Miranda et al., 2013). According to Wyres and Holt (2018), potential pathogens like *Klebsiella pneumoniae*, can be a significant vector for antibiotic resistance genes that are transferred from the environment to human or animal infections (Wyres & Holt, 2018). The necessity of the One Health method in monitoring ecosystem health is further highlighted by the prevalence of pathogenic bacteria, MGEs, and ARGs across humans, animals, and the environment. However, the dissemination and transmission of pathogens and antimicrobial resistance between aquaculture compartments are currently poorly understood and sometimes contradicting (Munita & Arias, 2016; Herrero et al., 2022).

Therefore, our study provides insights on the potential circulation of pathobiome and resistome between compartments over time, with a focus on aquaculture practices in Nha Trang, Vietnam (Chapter 3). Our work revealed 45 shared potential human pathogenic bacteria (PHPB) species among farmers, animals, and water, comprising 11.5% of total PHPB richness. Most belonged to the *Prevotella*, *Faecalibacterium*, and *Vibrio* genera, indicating substantial microbial exchange (Mahmud et al., 2024). *Aeromonas* spp., although less abundant, were present in all compartments and pose a risk of infection through contaminated water and seafood (Hoel et al., 2019; Nhinh et al., 2021). The detection of *Vibrio aestuarianus* on farmers also highlights the potential for environmental pathogens to enter the human microbiome and pose future health risks.

An identical pattern showed that 20% of total ARG richness was shared among floating cage farm compartments, with multidrug, tetracycline, aminoglycoside, and beta-lactam classes predominating, particularly in the human resistome. The most significant sharing of ARGs and pathogens was between farmers and animals, suggesting potential circulation of ARG-carrying pathogens. While the environment can act as a pathogen reservoir, its influence on ARGs seems to be temporary. A research by Wang and colleagues indicated that *Bacillota* and *Planctomycetes* are notably involved in carrying ARGs, while *Pseudomonadota* had weaker associations (J. Zhang et al., 2016;

Y.-J. Wang et al., 2020). However, beta-lactam, multidrug, MLS, peptide, and tetracycline drug classes were found to be prevalent in our study, primarily in the phyla *Bacillota* and *Pseudomonadota*. Therefore, our results provide more information on the connection between *Pseudomonadota* species and ARGs and may help identify other infections and antibiotic resistance genes that move between compartments. Our findings also revealed prevalent phenotypic resistance patterns to amoxicillin–clavulanic acid, ceftazidime, cefotaxime, tetracycline and fosfomycin among bacterial isolates from farmers and animals, consistent with earlier studies on aquaculture bacteria in LMICs (Reverter et al., 2020). Genomic analysis also showed 100% similarity in contigs from different MAGs of farmers, animals, and environment water to Beta-lactam (*bla*OXA) and Tetracycline (*tet*A, *tet*A(P), *tet*B(P), and *tet*R) ARGs. Overall, floating cage ecosystems promote exposure rates, increasing selection pressures and circulation of both pathogens and antibiotic resistance genes between humans, animals, and the surrounding environment.

Floating cage farmers as potential reservoirs for bacterial infections and the transmission of antimicrobial resistance to animals and the surrounding environment

The burden of antimicrobial resistance (AMR) varies significantly across aquatic environments, influenced by complex interactions among humans, animals, and environment. We hypothesize that aquaculture farmers face a higher burden of antimicrobial resistance (AMR) and bacterial infections due to prolonged exposure to pathogen-rich environments and antibiotic residues. However, there is a lack of studies on the pathobiome and resistome in aquaculture settings, with most research focusing on specific pathogenic bacteria affecting humans while overlooking relevant anthropogenic factors (Wachsmuth et al., 1994; Finkelstein et al., 2002; Arab et al., 2020; Håkonsholm et al., 2020; Tian et al., 2024). An interesting study in Korea found that fecal *Escherichia coli* in fishery workers had significantly higher resistance to tetracycline and other antibiotics compared to non-aquaculture workers (H.-H. Shin & Cho, 2013). This antibiotic resistance is likely linked to the extensive and inappropriate use of antibiotics in aquaculture practices, as reported in several studies (Carrique-Mas et

al., 2020; Hedberg et al., 2018). Moreover, antimicrobial agents used in humans are commonly applied in animal feed for growth promotion and disease prevention. The misuse of antibiotics has disrupted gut microbiota diversity in severely ill patients, allowing pathogenic strains to dominate despite broad-spectrum antibiotic treatments (Angulo et al., 2004; Ravi et al., 2019).

Our study revealed that the relative abundance of PHPB and ARGs was consistently higher in farmers than in animals and environmental water samples (Chapter 3). Notably, the most abundant ARG classes, including aminocoumarin, tetracycline, and beta-lactam, were more enriched in the human resistome, highlighting a significant burden of antimicrobial resistance in humans. The high correlation between various antibiotic classes also underscores the selective pressure on the human microbiome from multiple antibiotic uses on farms, potentially fostering multidrug-resistant bacteria (R. Davies & Wales, 2019). Phenotypic resistance analysis showed that farmers had a higher MAR index (0.31 ± 0.22) and resistance primarily to penicillin, tetracycline, and fosfomycin. Animals exhibited lower ARG abundance, possibly due to differences in microbial composition. These findings might be biased because the majority of ARG databases now in existence are mostly concentrated on antibiotic resistance genes identified from human-relevant commensal or pathogenic bacteria, with a lack of ARGs from diverse settings (C. Lee et al., 2023). Therefore, environmental water samples despite had the least ARG burden, or animals with lower ARG abundance can still pose a risk due to potential transfer of resistant bacteria to humans through gene transfer or seafood consumption (Cabello et al., 2016; T. H. T. Tran et al., 2018; Hoel et al., 2019).

Moreover, farmers exhibited higher species richness and diversity of PHPB, indicating a greater risk of bacterial infections. The presence of antibiotic resistance genes from approved for human use only and the growing number of bacteria, particularly PHPB, in the human gut microbiota greatly increase the risk of bacterial infections and complicate the treatment of antibiotic. More than half of the core ASVs were found enriched in farmers, with notable markers like *Prevotella copri* and *Faecalibacterium prausnizii*, both associated with human fecal matter (C. Lee et al., 2012; Koskey et al., 2014;

Saarela, 2019) (Chapter 3). These PHPB are abundant and significantly enriched in human gut microbiota compared to animals and water, indicating the identification of PHPB using 16*S rRNA* sequencing and our curated NEMESISdb is effective and accurate. Furthermore, bacterial isolation results showed a 34.7% prevalence of PHPB isolates in farmers, mainly from *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae*, posing potential potential infections risk to farmers. Metacompare2 analysis also indicated that human resistomes had the highest risk scores compared to animals and environmental samples. Despite not consuming antibiotics daily, farmers' resistome scores were closely aligned with non-farmers (31.1 vs 31.3, respectively), emphasizing the impact of human as host and anthropogenic activities on ARG profiles. These results demonstrate that humans are favored hosts for pathogens and ARGs, increasing the risk of bacterial infections and antibiotic resistance dissemination in floating cage farm environments while also bearing a greater burden.

General Conclusion

In general, this thesis advances in both methodology and knowledge of marine pathobiomes and resistomes across distinct socio-systemic contexts. We utilized NEMESISdb, a curated full-length 16*S rRNA* dataset tailored to identify human, fish and crustacean potential pathogenic bacteria (PPB), compatible with both long and short read amplicons. Applied to Mayotte coral reef holobionts, the database revealed the dual ecological role of marine hosts as potential reservoirs and also dispersal vectors for rare and/or potential pathogenic bacteria. Under accelerating biodiversity loss, global warming, and anthropogenic pressure, disturbances to host–microbiota relationships may erode this buffering capacity and favor pathogen emergence and transmission.

On the other hand, the same framework applied in Nha Trang Bay, Viet Nam, exposed a coastal hotspot characterized by fecal contamination, abundant ARGs, and a diverse pathobiome dominated by opportunistic taxa. Seasonal rainfall, riverine inputs, and aquaculture intensity structured spatial–temporal patterns, with different compartments (farmers, animals, and environment) and diferent anthropogenic inputs (urban, fish farm, and recovery transects) emerging as the primary drivers of both pathobiome and resistome abundance, diversity and composition. Specifically, aquaculture farmers exhibited consistently higher pathogenic bacteria and antibiotic resistance burdens than animals and surrounding environment samples. High surface-water *int*I abundance also suggests active horizontal gene transfer potential, while frequent detection of tetracycline, multidrug, beta-lactam and rifampicin resistance, alongside taxa from *Prevotellaceae*, *Vibrionaceae* and *Ruminococcaceae*, signals elevated risk of ARG circulation between the human–animals–environment. Notably, seasonality influences the pathobiome composition, whereas the resistome showed lower temporal variability. In contrary, different sample types affect both pathogenic bacteria communities and antibiotic resistance genes, underscoring different ecological controls

on pathogens versus resistance genes.

Collectively, this study demonstrates the advanced use of the full-length 16*S rRNA* pathogenic dataset (NEMESISdb) within One Health schematic to provide significant surveillance benefits on samples across different compartments. It helps identifying potential reservoirs, clarifying environmental drivers (e.g., water nutrients, pH, temperature) and also providing strong platform for future studies on 16S screening, shotgun metagenomics for strain-level resolution to detect potential human disease outbreaks and risks. Furthermore, results of antibiotic resistance genes profiles in our dataset also reflect aquacultural practices and suggest antimicrobial resistance risk to human communities. Evidently, our findings also furnish concrete evidences to support the promulgate of stricter antibiotic stewardship in aquaculture (including the removal/replacement of ineffective antibiotics such as rifampicin, tetracycline). By conducting these studies in an under-developed South East Asia country like Viet Nam and especially to coastal regions, we further highlight the potential circulation of pathogens and antimicrobial burden from farmers to animals and surrounding environment in the aquaculture ecosystems.

Résumé de la Thèse

INTRODUCTION

La résistance aux antimicrobiens (RAM) survient lorsque des bactéries, des virus, des champignons ou des parasites acquièrent des mécanismes rendant les thérapies existantes inefficaces. En 2019, on estime que la RAM a causé directement 1,27 million de décès et a contribué à près de 5 millions de décès supplémentaires, dépassant la mortalité liée au VIH/sida et au paludisme. Parallèlement, les zoonoses émergentes et ré-émergentes (p. ex., COVID-19, Ebola, Nipah, Monkeypox) mettent en évidence des lacunes persistantes en matière de surveillance, de diagnostic rapide et de réponse coordonnée. Dans un monde interconnecté, la propagation transfrontalière rapide de nouveaux agents pathogènesset de la RAM souligne la nécessité d'une action internationale rapide, du partage des données et du développement de contre-mesures efficaces.

Malgré l'essor rapide de la recherche sur les maladies infectieuses (MI) et la RAM, les efforts se sont surtout concentrés sur les systèmes continentaux, alors que les milieux marins ont reçu relativement peu d'attention. Les environnements côtiers fonctionnent comme des zones de convergence pour les microbes d'origine terrestre et marine, où les apports anthropiques, tels que les polluants et les résidus pharmaceutiques, peuvent remodeler les interactions, sélectionner la résistance et favoriser l'émergence de maladies. La transmission d'infections et de la RAM via les eaux marines et les produits de la mer constitue donc un enjeu majeur pour la santé publique et la sécurité alimentaire. D'importantes lacunes persistent : profils incomplets des bactéries potentiellement pathogènes, connaissances limitées sur l'usage des antibiotiques et la perception du risque en aquaculture, impacts encore mal élucidés de ces pratiques sur les microbiomes environnants, et insuffisance de preuves concernant la circulation

des bactéries potentiellement pathogènes (PPB) et des gènes de résistance aux antibiotiques (ARG) entre milieux marins, animaux et agriculteurs.

Pour combler ces lacunes, cette thèse applique un cadre interdisciplinaire intégrant des perspectives écologiques, microbiologiques et sociales afin d'évaluer les risques sanitaires posés par les pathogènes marins et la RAM le long des côtes vietnamiennes. Le premier objectif est de développer un jeu de données 16*S rRNA* pleine longueur (NEMESISdb) permettant la détection et le suivi des bactéries potentiellement pathogènes chez l'humain, les poissons et les crustacés. Le deuxième objectif caractérise le pathobiome et le résistome dans la baie de Nha Trang, influencée par les activités humaines, en tenant compte de la saisonnalité et des apports anthropiques. Le dernier objectif s'intéressse à la circulation des PPB et des ARG entre l'humain, les animaux et les environnements adjacents.

RÉSULTATS ET DISCUSSION

Création d'une base de donnée 16S de bactéries potentiellement pathogènes

La précision et l'étendue de la détection des pathogènes par le séquençage 16S dépendent largement des bases de données de référence utilisée. Cependant, les jeux de données nécessaires pour identifier précisément les PPB circulant entre les compartiments humains, environnement marin et animaux marins, dans une approche one health, restent encore largement sous-développés.

Pour répondre à ce manque, nous avons développé NEMESISdb qui est un jeu de données de séquences 16*S rRNA* pleine longueur, soigneusement curé, conçu pour permettre l'identification et le suivi des bactéries potentiellement pathogènes (PPB) pour l'humain, les poissons et les crustacés. Il répond au manque d'attention porté aux environnements marins et côtiers en tant que réservoirs clés de PPB, où coexistent des bactéries d'origines variées : terrestre, marine et animale. En s'appuyant sur les récents progrès du séquençage haut débit, NEMESISdb constitue une ressource fiable

pour la détection des PPB dans les données de métabarcoding ou de métagénomique ciblant le gène 16*S rRNA*. La base regroupe trois ensembles de données correspondant aux hôtes humains, poissons et crustacés, contenant respectivement 1 703, 222 et 64 espèces de PPB, soit plus de 150 000 séquences 16*S rRNA* pleine longueur, rigoureusement vérifiées pour garantir leur exactitude. Cette ressource a été construite à partir de l'extraction de séquences de la base SILVA 138.2 SSU Ref NR99, puis affinée grâce à un pipeline de curation strict assurant la cohérence taxonomique et éliminant les erreurs de classification. Les jeux de données obtenus sont optimisés pour une utilisation avec des outils populaires tels que BLAST ou divers logiciels de classification, permettant une détection rapide et précise des PPB dans les données de métabarcoding et de métagénomique.

NEMESISdb soutient un large éventail d'applications, notamment la surveillance des pathogènes dans les écosystèmes aquatiques, l'étude des facteurs environnementaux influençant la dynamique des PPB et le développement de stratégies ciblées pour limiter l'impact des pathogènes en aquaculture. De plus, il facilite les recherches dans le cadre du concept « One Health » en reliant la circulation des PPB entre les compartiments environnementaux, animaux et humains.

Les Holobiontes marins: Un reservoir et vecteur meconnu d'especes rares et de pathogenes

Afin de tester le cadre conceptuel du «maintien du rare» («sustaining the rare»), nous avons examiné si les microbiotes de surface et intestinaux des holobiontes marins représentaient des points chauds pour les taxons microbiens rares présents dans les communautés planctoniques et benthiques environnantes. Nous en avons profité pour tester la base de données de PPB développée précédemment et explorer si ce cadre pouvait également s'appliquer aux bactéries potentiellement pathogènes pour l'homme (PHPB).

Dans cette étude, nous avons échantillonné les microbiomes de surface et intestinaux de 265 animaux récifaux appartenant à 74 taxons différents, comprenant respec-

tivement 32 genres de poissons téléostéens et 18 genres d'anthozoaires (coraux durs, coraux mous et anémones), ainsi que 12 taxons de crustacés, échinodermes, mollusques et éponges, provenant d'un seul écosystème récifal: le lagon de mayotte (océan indien). Nous montrons que ces holobiontes constituent des réservoirs majeurs de richesse microbienne, représentant jusqu'à 98.4% de la richesse totale en genres dans les écosystèmes récifaux, les microbiotes de surface contribuant davantage que les communautés intestinales. De manière importante, les holobiontes soutiennent de façon disproportionnée les microbes rares de l'environnement, validant ainsi l'hypothèse du « maintien du rare ». Nous montrons que 74.5% des genres rares de l'environnement sont hébergés par les holobiontes, dont 22.2% deviennent enrichis, soulignant leur rôle dans la promotion des taxons conditionnellement rares (CRT), incluant des symbiotes et des copiotrophes opportunistes. Cependant, ce mécanisme d'enrichissement favorise également les bactéries pathogènes humaines potentielles (PHPB), dont certaines passent de la rareté à la dominance au sein des microbiotes des hôtes (*Hermaniimonas* en surface et *Delftia* dans l'intestin). Plus de 48% des genres totaux de PHPB ont été trouvés exclusivement dans les microbiotes associés aux hôtes, et leurs abondances relatives étaient près de deux fois plus élevées chez les holobiontes que dans l'environnement.

Ces résultats révèlent un double rôle écologique des holobiontes marins : à la fois refuges pour des microbes rares bénéfiques et pour des pathogènes potentiellement nuisibles. Cette dualité souligne la nécessité de prendre en compte la dynamique holobionte–microbiote dans les cadres de conservation comme de santé publique, en particulier dans le contexte du changement environnemental global.

L'aquaculture et les activites humaines alimentent la dissemination des pathogenes et des genes de resistance dans une baie tropicale

Les écosystèmes côtiers sont de plus en plus menacés par des polluants microbiens, notamment des bactéries potentiellement pathogènes pour l'homme (PHPB) et des bactéries résistantes aux antibiotiques (ARB), qui représentent un risque majeur pour la santé à l'interface entre l'homme et l'océan.

Nous avons combiné des approches par métabarcoding du gène 16*S rRNA*, de qPCR, de métagénomique avec des approches de culture pour caractériser la diversité, la composition et les profils de résistance du pathobiome et du résistome dans la baie de Nha Trang (Vietnam). La diversité du pathobiome s'est révélée élevée, avec 485 variants de séquences d'amplicons (ASV) de PHPB appartenant à 49 genres et 98 espèces. Les communautés de PHPB étaient dominées par des pathogènes opportunistes autochtones (*Staphylococcus*, *Bacillus*, *Acinetobacter*, *Pseudomonas* et *Aeromonas*), mais incluaient également des pathogènes entériques (par exemple *Prevotella* et *Arcobacter*) révélateurs d'une contamination fécale.

Les données issues à la fois des tests phénotypiques de résistance et de l'analyse du résistome ont montré des niveaux élevés de multirésistance, la résistance à la rifampicine étant particulièrement répandue, ce qui est cohérent avec son usage massif en aquaculture et dans le traitement de la tuberculose. En outre, huit gènes de résistance aux antibiotiques (ARG), *sul*1, *sul*2, *sul*3, *tet*Q, *tet*B, *mec*A, *bla*VIM et *bla*KPC, ont été détectés en forte abondance dans la majorité des échantillons d'eau et de sédiments. Fait notable, le gène de l'intégrase de l'intégron de classe 1 (*intI*1) était largement répandu (95.8%) dans tous les échantillons, avec une abondance absolue totale de 2.9×10^5 copies/ml d'eau et 9.7×10^7 copies/g de sédiment.

Les variations saisonnières et spatiales ont mis en évidence la forte influence des paramètres hydrologiques (précipitations et apports fluviaux) et des pressions anthropiques (pratiques aquacoles et tourisme) sur la structure du pathobiome et du résistome. Dans l'ensemble, ces résultats montrent que la baie de Nha Trang constitue un véritable foyer de dissémination de pathogènes et de gènes de résistance, soulevant des préoccupations pour les populations locales et pour la santé mondiale. Les stratégies d'atténuation devraient donner la priorité à la réduction de la pollution fécale, à une réglementation plus stricte de l'usage des antibiotiques en aquaculture, et à la mise en place de cadres intégrés de surveillance « One Health » pour gérer les risques microbiens dans les écosystèmes côtiers.

Dynamiques longitudinales du resistome et du pathobiome chez les eleveurs, le betail et l'environnement dans les fermes flottantes d'aquaculture a Nha Trang, Vietnam

L'aquaculture est l'un des secteurs de l'élevage animal qui connaît la plus forte croissance et est le principal moyen de subsistance de 22 millions de personnes dans le monde, principalement (95%) en Asie. De plus en plus de données montrent que les systèmes aquacoles constituent des réservoirs de bactéries pathogènes et de gènes de résistance aux antibiotiques (ARG). Ainsi, à l'instar de l'élevage terrestre, l'aquaculture peut exposer les travailleurs à des risques professionnels ayant un impact direct sur leur santé et jouer un rôle clé dans la dissémination de la résistance aux antimicrobiens. Malgré l'importance de cette problématique pour la santé publique mondiale, l'impact des activités aquacoles sur la circulation des bactéries potentiellement pathogènes (PPB) et des gènes de résistance (ARG) entre les microbiotes des pisciculteurs, des animaux d'élevage et de l'environnement reste encore mal compris.

Pour explorer cette problématique, nous avons mené un suivi longitudinal de 18 mois (couvrant la saison sèche et la saison des pluies) des microbiotes fécaux et cutanés de pisciculteurs de la baie de Nha Trang, au Vietnam. Afin d'examiner la connectivité entre compartiments, nous avons comparé le microbiote des pisciculteurs à celui des poissons d'élevage et de l'eau à l'aide d'approches phénotypiques et génomiques.

L'analyse des ASV (amplicon sequence variants) a révélé que les lignées de bactéries potentiellement pathogènes (PPB) les plus dominantes appartenaient aux familles *Bacillaceae* et *Lactobacillaceae*, et ce dans tous les échantillons. Les résultats issus du métabarcoding et des isolats bactériens ont montré une possible circulation des PPB : 16.5% du total des ASV de PPB et 7% des isolats de PPB étaient partagés entre les trois compartiments. Ces PPB communs appartenaient principalement aux genres *Bacteroides*, *Prevotella*, *Klebsiella* et *Vibrio*. Par ailleurs, l'analyse métagénomique a détecté un total de 1216 gènes de résistance aux antibiotiques (ARG) appartenant à 26 classes, dont 315 ARG étaient partagés entre les compartiments, indiquant également

une circulation active de ces gènes. L'abondance relative du résistome partagé reflétait les principales classes d'antibiotiques utilisées dans les fermes, avec une prédominance des ARG liés aux tétracyclines, bêta-lactamines, macrolides-lincosamides-streptogramines et à la multirésistance, représentant respectivement 86.5% des ARG partagés et 16.9% du total des ARG. En cohérence avec ces résultats, une forte résistance phénotypique aux bêta-lactamines (pénicillines, céphalosporines, monobactames) a également été observée.

Un résultat particulièrement original réside dans le contexte génomique des MAG reconstittués. Plusieurs contigs issus des échantillons de pisciculteurs et d'animaux étaient identiques à 100%, annotés à la même souche *Chryseobacterium* sp. POL2, et portaient des éléments génétiques mobiles à proximité immédiate du gène *bla*OXA. Étant donné que *Chryseobacterium* sp. POL2 a été impliqué dans la dissémination de multiples gènes de résistance aux antibiotiques (ARG) par conjugaison, cette découverte met en évidence un mécanisme concret de transfert inter-compartiments, rarement documenté dans les systèmes aquacoles. De même, les gènes de résistance aux tétracyclines (*tet*A, *tet*A(P), *tet*B(P) et *tet*R) ont montré une conservation remarquable de séquence sur des contigs provenant d'espèces pathogènes telles que *Escherichia coli*, *Salmonella enterica* et *Peptacetobacter hiranonis*, couvrant les échantillons humains, animaux et environnementaux. La détection de ces signatures ARG partagées entre compartiments fournit une solide preuve d'un résistome interconnecté, où les pathogènes circulent au-delà des frontières écologiques traditionnelles.

Dans l'ensemble, nos résultats démontrent de manière unique que les écosystèmes aquacoles en cages flottantes non seulement intensifient les pressions de sélection, mais créent également un foyer de transfert horizontal de gènes, reliant les humains, le bétail aquatique et l'environnement environnant. Cela met en évidence le rôle de l'aquaculture comme nœud clé dans la dissémination mondiale de la résistance aux antibiotiques.

CONCLUSION

Dans leur ensemble, ces travaux dressent un tableau cohérent et inédit de la manière dont les écosystèmes marins et côtiers participent à la circulation des bactéries potentiellement pathogènes (PPB) et des gènes de résistance aux antibiotiques (ARG), en intégrant une perspective « One Health ».

Premièrement, la création de NEMESISdb comble un manque méthodologique majeur : cette base de données 16*S rRNA* pleine longueur, spécifiquement dédiée aux environnements côtiers et marins, offre un outil robuste et standardisé pour identifier et suivre les PPB dans les analyses de métabarcoding et de métagénomique. Elle ouvre la voie à une surveillance plus fine et mieux intégrée des pathogènes dans les socio-écosystèmes marins.

Deuxièmement, l'étude des holobiontes récifaux révèle leur double rôle écologique. Ces hôtes marins constituent à la fois des réservoirs clés de la richesse microbienne rare et des vecteurs de pathogènes potentiels. Ils démontrent ainsi que le « maintien du rare » favorise non seulement des symbiotes bénéfiques mais aussi des bactéries opportunistes et potentiellement pathogènes susceptibles de devenir dominantes. Ce constat souligne que les efforts de conservation et de gestion de la biodiversité récifale doivent intégrer les dimensions sanitaires du microbiote.

Troisièmement, l'exploration des baies tropicales soumises à de fortes pressions anthropiques, en particulier la baie de Nha Trang, met en lumière un véritable foyer de dissémination de pathogènes et d'ARG. La co-occurrence d'apports fécaux, de pratiques aquacoles intensives et d'une circulation saisonnière des nutriments favorise une multirésistance préoccupante, illustrée par la diffusion massive du gène intI1 et d'ARG cliniquement critiques. Ces résultats renforcent la nécessité d'une régulation stricte de l'usage des antibiotiques et de dispositifs de surveillance microbiologique intégrés.

Enfin, le suivi longitudinal dans les fermes aquacoles flottantes révèle une intercon-

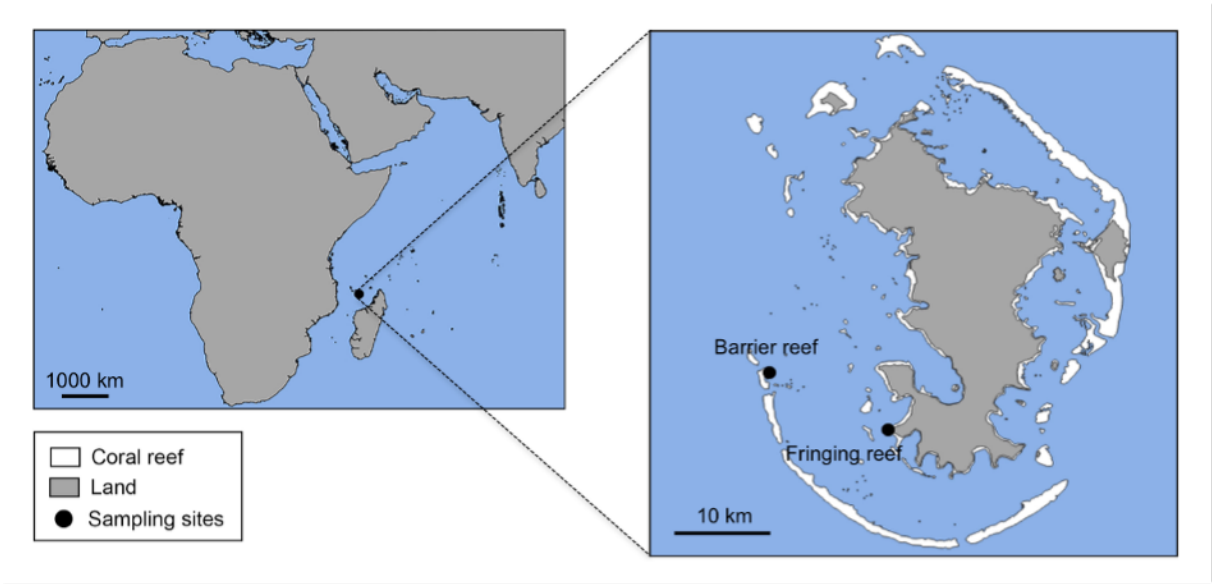
nexion directe entre microbiotes humains, animaux d'élevage et environnementaux. La mise en évidence de contigs identiques, porteurs de gènes de résistance, entre pisciculteurs et poissons démontre un mécanisme tangible de transfert horizontal rarement documenté. Ces écosystèmes en cages flottantes apparaissent comme de véritables « hotspots » de sélection et de propagation des ARG, positionnant l'aquaculture comme un nœud central de la dissémination mondiale de la résistance aux antimicrobiens.

Pris ensemble, ces résultats appellent à une approche intégrée et transdisciplinaire, combinant écologie microbienne, santé publique et politiques de gestion durable des écosystèmes côtiers. Ils soulignent l'urgence d'actions coordonnées, allant de la limitation des rejets de pathogènes et de l'usage des antibiotiques jusqu'à la mise en place de programmes de surveillance « One Health » à l'échelle locale et internationale, afin de réduire les risques sanitaires pour les populations humaines et les écosystèmes marins.

Appendix

Supplementary Materials

Supplementary Materials for Chapter 1



Supplementary Material SM1-1: Sampling sites of the study

Supplementary Material SM1-2: Overview of samples included in this study

Coral reef compartment	Animal group	Number of taxa	Number of samples	Details
Animals (surface & gut)	Teleost fish	32 genera	265	7 orders and 18 families
	Anthozoa	18 genera	84	14 hard coral genera, 2 soft coral genera, 1 gorgoniidae and 1 anemone
	Other invertebrates	12 genera	43	2 crustaceans, 7 echinoderms, 1 mollusk, and 2 sponges
Planktons	—	—	35	17 surface samples and 18 samples from the bottom of the water column
Benthos	—	—	14	14 sediment samples
Unidentified taxa	—	—	19	—
		Total	**460**	

Supplementary Material SM1-3: Host identification of animal surface microbiomes sampled, number of samples and average number of prokaryotic sequences per sample for each host species and sample type.

Taxonomic	Identification	Nb of samples	Nb of sequence	Sample
Teleostei	*Acanthurus leucosternon*	5	29,932±7,256	Swab
Teleostei	*Acanthurus lineatus*	3	30,341±8,084	Swab
Teleostei	*Ctenochaetus striatus*	7	26,233±8,997	Swab
Teleostei	*Naso unicornis*	1	33,537±0	Swab
Teleostei	*Balistapus undulatus*	3	32,256±4,517	Swab
Teleostei	*Sufflamen chrysopterum*	6	35,720±10,541	Swab
Teleostei	*Pterocaesio tile*	1	24,143±0	Swab
Teleostei	*Pterocaesio trilienata*	2	21,478±1,976	Swab
Teleostei	*Caranx melampygus*	3	31,387±3,962	Swab
Teleostei	*Chaetodon auriga*	2	38,509±3,270	Swab
Teleostei	*Chaetodon falcula*	6	38,307±4,457	Swab
Teleostei	*Chaetodon lunula*	4	41,056±6,002	Swab
Teleostei	*Chaetodon meyeri*	3	32,528±10,371	Swab
Teleostei	*Chaetodon trifascialis*	3	28,593±1,1001	Swab
Teleostei	*Forcipiger flavissimus*	3	17,279±8,906	Swab
Teleostei	*Arothron nigropunctatus*	1	27,160±0	Swab
Teleostei	*Platax orbicularis*	5	36,061±4,462	Swab
Teleostei	*Platax teira*	1	30,904±0	Swab
Teleostei	*Myripristis murdjan*	2	11,040±547	Swab
Teleostei	*Myripristis violacea*	1	13,339±0	Swab
Teleostei	*Kyphosus vaigiensis*	3	23,444±7,943	Swab
Teleostei	*Cheilinus fasciatus*	3	28,826±2,059	Swab
Teleostei	*Hemigymnus fasciatus*	3	32,492±7,692	Swab
Teleostei	*Thalassoma hebraicum*	3	25,292±4,830	Swab
Teleostei	*Monotaxis grandoculis*	5	27,976±6,574	Swab
Teleostei	*Cantherhines pardalis*	1	26,705±0	Swab
Teleostei	*Parupeneus cyclostomus*	2	28,694±330	Swab

Supplementary Material SM1-3 (continued)

Taxonomic	Identification	Nb of samples	Nb of sequence	Sample
Teleostei	*Parupeneus trifasciatus*	3	47,428±8,361	Swab
Teleostei	*Parapercis hexophtalma*	3	30,863±17,123	Swab
Teleostei	*Pomacanthus imperator*	3	27,737±1,367	Swab
Teleostei	*Pygoplites diacanthus*	6	33,359±8,607	Swab
Teleostei	*Abudefduf sexfasciatus*	3	30,278±4,838	Swab
Teleostei	*Abudefduf sparoides*	4	20,760±5,460	Swab
Teleostei	*Amphiprion akallopisos*	3	17,918±3,065	Swab
Teleostei	*Chlorurus sordidus*	6	30,907±8,560	Swab
Teleostei	*Scarus caudofasciatus*	4	28,528±5,374	Swab
Teleostei	*Scarus russelii*	1	25,953±0	Swab
Teleostei	*Pterois miles*	2	33,932±7,141	Swab
Teleostei	*Pterois radiata*	1	36,857±0	Swab
Teleostei	*Cephalopholis argus*	6	28,950±5,919	Swab
Teleostei	*Cephalopholis boenak*	1	25,014±0	Swab
Teleostei	*Sphyraena barracuda*	1	31,282±0	Swab
Teleostei	*Corythoichthys flavofasciatus*	3	33,704±6,798	Swab
Teleostei	*Zanclus cornutus*	6	34,144±6,764	Swab
	Total Teleostei	**138**		
Anthozoa	*Acropora cf. clathrata*	2	34,522±10,470	Mucus
Anthozoa	*Acropora cf. cytherea*	3	37,382±696	Mucus
Anthozoa	*Acropora cf. elseyi*	3	41,414±6,475	Swab
Anthozoa	*Acropora cf. intermedia*	3	37,819±4,619	Mucus
Anthozoa	*Acropora cf. latistella*	3	48,336±4,605	Mucus
Anthozoa	*Acropora cf. muricata*	3	47,805±7,040	Mucus
Anthozoa	*Acropora cf. nasuta*	2	14,122±3,166	Mucus
Anthozoa	*Echinopora sp*	2	25,023±15,517	Mucus
Anthozoa	*Favia sp*	3	32,391±6,855	Mucus
Anthozoa	*Favites sp*	2	34,703±5,087	Mucus
Anthozoa	*Fungia sp*	6	37,228±6,121	Mucus

Supplementary Material SM1-3 (continued)

Taxonomic	Identification	Nb of samples	Nb of sequence	Sample
Anthozoa	*Goniopora sp*	3	20,844±11,947	1 mucus 2 swabs
Anthozoa	*Herpolitha sp*	3	41,341±3,965	Mucus
Anthozoa	*Isopora sp*	2	34,294±4,865	2 mucus 1 swab
Anthozoa	*Lobophyllia sp*	2	32,916±1,200	Swab
Anthozoa	*Montastrea sp*	3	19,874±4,191	Mucus
Anthozoa	*Montipora sp*	3	44,104±804	2 mucus 1 swab
Anthozoa	*Platygyra sp*	6	31,001±5,973	Mucus
Anthozoa	*Porites sp(branching)*	3	26,557±2,720	Mucus
Anthozoa	*Porites sp(massive)*	5	22,255±8,224	3 mucus 2 swabs
Anthozoa	*Gorgonidae*	5	29,813±4,911	Swab
Anthozoa	*Sarcophyton*	9	19,736±9,111	4 mucus 5 swabs
Anthozoa	*Sinularia*	4	32,282±8,382	Swab
Anthozoa	*Heteractis magnifica*	2	12,722±3,738	Swab
	Total Anthozoa	**82**		
Malacostraca	*Paguroidea sp*	5	32,158±1,820	Swab
Malacostraca	*Panulirus versicolor*	3	32,210±6,702	Swab
Crinoidea	*Comatulida sp*	3	28,669±6,726	Swab
Holothuroidea	*Bohadschia sp*	3	21,861±10,657	Swab
Echinoidea	*Echinothrix diadema*	2	31,305±41	Swab
Holothuroidea	*Holothuria atra*	3	28,942±7,579	Swab
Asteroidea	*Linckia laevigata*	6	28,537±7,833	Swab
Asteroidea	*Linckia multifora*	2	30,908±10,423	Swab
Ophiuroidea	*Ophiuroidea sp*	6	37,436±5,155	Swab
Bivalvia	*Tridacna sp*	3	41,089±3,996	Swab
Demospongiae	*Spheciospongia cf. inconstans*	3	48,821±5,152	Swab
Demospongiae	*Cliona cf. vastifica*	4	41,612±3,243	Swab
	Total Other Invertebrates	**43**		
	TOTAL Animal Surfaces	**263**		
	Plankton	35	39,433±3,526	Filter

Supplementary Material SM1-3 (continued)

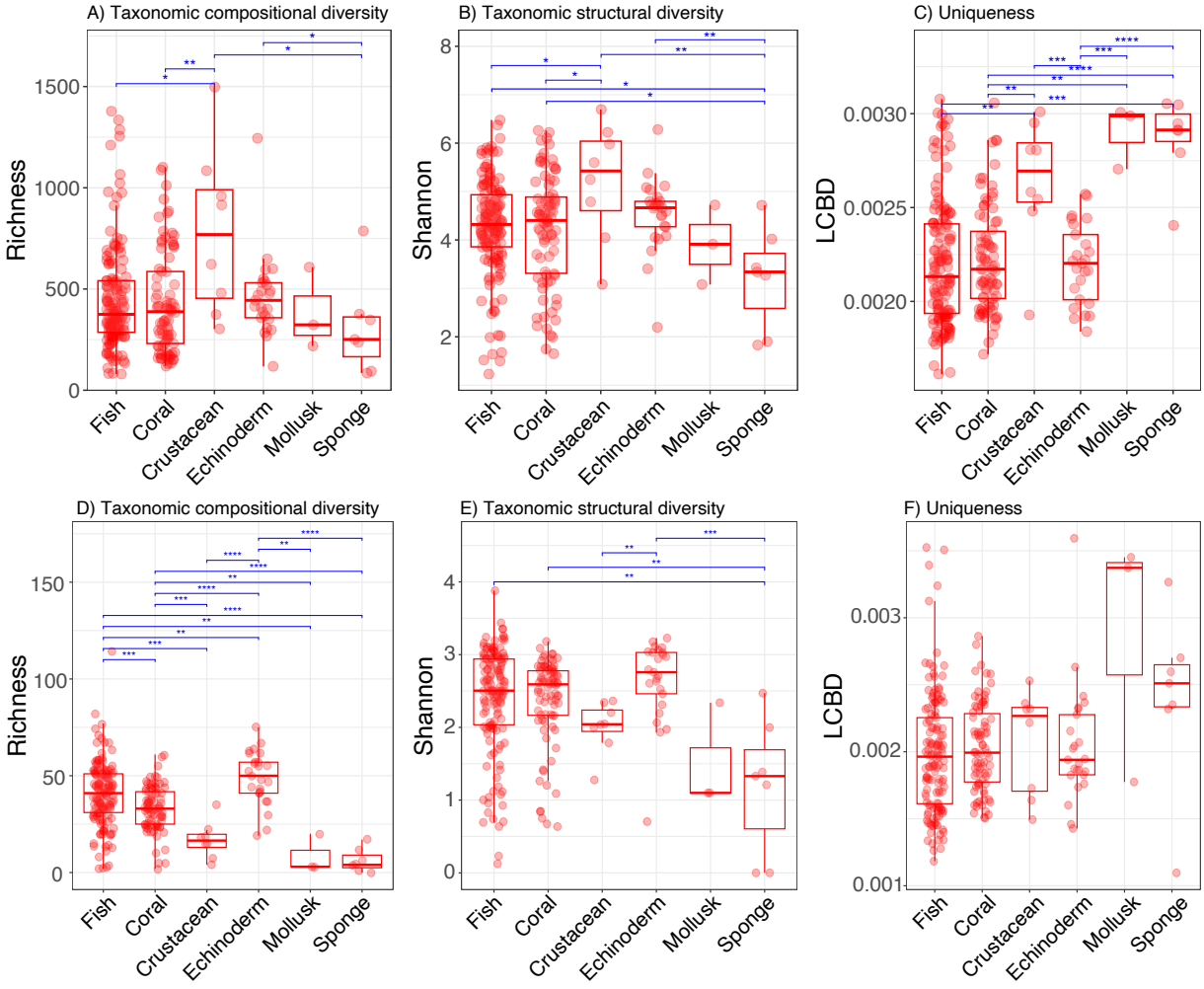
Taxonomic	Identification	Nb of samples	Nb of sequence	Sample
	TOTAL SAMPLES	**298**		

Supplementary Material SM1-4: DADA2 script

(Due to the large size, the full script of DADA2 will be available online upon completion of the submission process.)

Supplementary Material SM1-5: Post-hoc Dunn test p-value of alpha diversity indices between MH and environment for whole bacteria community

Comparison	Shannon			Observed			LCBD		
	Z	P_{unadj}	P_{adj}	Z	P_{unadj}	P_{adj}	Z	P_{unadj}	P_{adj}
Gut – Sediment	-79.489	0.0000	0.0000	-84.327	0.0000	0.0000	0.8094	0.4182	0.4182
Gut – Surface	-64.639	0.0000	0.0000	-86.389	0.0000	0.0000	10.9060	0.0000	0.0000
Sediment – Surface	54.538	0.0000	0.0000	49.934	0.0000	0.0000	40.0220	0.0000	0.0000
Gut – Water	-39.263	0.0000	0.0001	-65.273	0.0000	0.0000	12.3490	0.0000	0.0000
Sediment – Water	47.484	0.0000	0.0000	35.493	0.0003	0.0004	70.4380	0.0000	0.0000
Surface – Water	0.0339	0.9728	0.9728	-13.726	0.1698	0.1698	62.8470	0.0000	0.0000



Supplementary Material SM1-6: Alpha diversity of the whole bacterial community (A,B,C) and PHPB community (D,E,F) between marine holobionts. A,D: Species richness; B,E: Shannon diversity; C,F: LCBD index. Dunn post-hoc test between holobionts (****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$).

Supplementary Material SM1-7: Post-hoc Dunn test p-value of alpha diversity indices between MH and environment for PHPB communities

Comparison	Shannon			Observed			LCBD		
	Z	P_{unadj}	P_{adj}	Z	P_{unadj}	P_{adj}	Z	P_{unadj}	P_{adj}
Gut – Sediment	-2.5220	1.16e-02	1.74e-02	-5.0423	4.59e-07	1.37e-06	3.8647	1.11e-04	2.22e-04
Gut – Surface	-12.426	1.88e-35	1.12e-34	-11.981	4.46e-33	2.68e-32	14.561	4.95e-48	2.97e-47
Sediment – Surface	-2.8417	4.48e-03	8.97e-03	0.0000	1.00e+00	1.00e+00	2.3755	1.75e-02	2.10e-02
Gut – Water	-1.7917	7.31e-02	8.78e-02	-4.3847	1.16e-05	2.32e-05	6.3334	2.39e-10	7.19e-10
Sediment – Water	1.1626	2.44e-01	2.44e-01	1.8189	6.89e-02	8.27e-02	0.4785	6.32e-01	6.32e-01
Surface – Water	6.3958	1.59e-10	4.78e-10	3.2114	1.32e-03	1.98e-03	-2.7859	5.33e-03	8.00e-03

Supplementary Material SM1-8: Pairwise PERMANOVA test detail between groups

Pairs	SumsOfSqs	F.Model	R^2	p.value	p.adjusted
Sediment vs Water	1.483463	4.944664	0.09519098	0.000999001	0.001198801
Sediment vs Surface	1.049749	3.257891	0.01074297	0.001998002	0.001998002
Sediment vs Gut	4.473285	13.911291	0.09599867	0.000999001	0.001198801
Water vs Surface	4.793576	15.145367	0.04505600	0.000999001	0.001198801
Water vs Gut	11.159827	36.047820	0.19169497	0.000999001	0.001198801
Surface vs Gut	30.719321	95.982032	0.19158777	0.000999001	0.001198801
Coral vs Echinoderm	1.0353445	3.4806786	0.03208570	0.000999001	0.001498501
Coral vs Sponge	1.6194970	5.3378788	0.05780812	0.000999001	0.001498501
Coral vs Mollusk	0.8647454	2.8869024	0.03361284	0.002997003	0.003746254
Coral vs Crustacean	1.0453560	3.4915487	0.03816253	0.000999001	0.001498501
Coral vs Fish	1.9004720	6.4202819	0.02786335	0.000999001	0.001498501
Echinoderm vs Sponge	1.6770335	5.3691645	0.15180354	0.000999001	0.001498501
Echinoderm vs Mollusk	0.9420918	3.1255968	0.10731443	0.002997003	0.003746254
Echinoderm vs Crustacean	1.3368059	4.4456628	0.12542191	0.000999001	0.001498501
Echinoderm vs Fish	1.3718647	4.6488598	0.02708355	0.000999001	0.001498501
Sponge vs Mollusk	0.2750015	0.7334586	0.08398261	0.820179820	0.820179820
Sponge vs Crustacean	0.5584046	1.6187578	0.11073156	0.076923077	0.088757396
Sponge vs Fish	1.8162655	6.0890908	0.03926189	0.000999001	0.001498501
Mollusk vs Crustacean	0.3605426	1.0996587	0.10888078	0.388611389	0.416369345
Mollusk vs Fish	0.8512837	2.8766055	0.01945274	0.000999001	0.001498501
Crustacean vs Fish	1.0683089	3.6095331	0.02349811	0.000999001	0.001498501
Barrier vs Fringing	1.591929	4.040517	0.00882131	0.001998002	0.001998002

Supplementary Material SM1-9: Detail classification of enriched bacterial genera in MH microbiotas. Opportunistic classification was performed by searching bacterial genera against multiple literatures. Symbiotic classification was based on Symbiotic Genomes Database (SymGenDB). Potential human pathogens were based on NEMESISdb. Only classified bacteria with at least 1 category are provided.

Genera	**Opportunists**	**Symbiotics[1]**	**Potential pathogens[2]**
Achromobacter	x	x	x
Actinomyces	x	x	
Akkermansia	x	x	
Aliivibrio	x	x	
Alishewanella	x		
Alistipes	x	x	
Alteromonas	x		
Anaerococcus	x	x	
Anaerofilum	x		
Aquabacterium	x		
Aquimarina	x		
Arcobacter	x	x	
Bacteroides	x	x	x
Blautia		x	
Bradyrhizobium	x		
Brevibacterium	x		
Brevundimonas	x	x	x
Burkholderia-Caballeronia-Paraburkholderia	x		
Caulobacter	x		
Cellulomonas	x	x	
Chryseobacterium	x	x	
Clostridium	x	x	
Comamonas	x	x	x
Corynebacterium	x	x	x

Supplementary Material SM1-9 (continued)

Genera	Opportunists	Symbiotics[1]	Potential pathogens[2]
Cutibacterium	x	x	x
Cyclobacterium		x	
Delftia	x		x
Desulfovibrio	x	x	
Devosia	x	x	
Dietzia	x		
Dolosigranulum	x		
Endozoicomonas	x		
Exiguobacterium	x		
Flavobacterium	x	x	
Flavonifractor		x	
Fuerstia		x	
Fusobacterium	x	x	
Gardnerella	x	x	
Gemella	x	x	
Grimontia	x	x	
Haemophilus	x	x	x
Halomonas	x		
Herbaspirillum	x		
Herminiimonas			x
Intestinimonas		x	
Klebsiella	x	x	x
Kocuria	x	x	
Lachnoclostridium		x	
Lactobacillus	x	x	
Lawsonella		x	x
Legionella	x	x	
Leuconostoc	x	x	

Supplementary Material SM1-9 (continued)

Genera	Opportunists	Symbiotics[1]	Potential pathogens[2]
Litoricola	x		
Litorimonas	x		
Lyngbya	x		
Marinicella	x		
Marinobacter	x		
Massilia	x		x
Mesorhizobium			x
Methylobacterium	x		
Micrococcus	x		x
Moraxella	x	x	
Myroides	x	x	
Neisseria	x	x	
Odoribacter	x	x	
Ornithobacterium	x	x	
Paenibacillus	x	x	
Parabacteroides		x	
Paracoccus	x	x	x
Pelagibius	x		
Peptococcus	x		
Phenylobacterium		x	
Photobacterium	x	x	
Polaribacter		x	
Prevotella	x	x	x
Pseudoalteromonas	x	x	
Pseudomonas	x	x	
Pseudovibrio	x		
Psychrobacter	x	x	
Ralstonia	x		x

Supplementary Material SM1-9 (continued)

Genera	**Opportunists**	**Symbiotics[1]**	**Potential pathogens[2]**
Rhodococcus	x	x	
Roseomonas	x	x	
Ruminiclostridium		x	
Ruminococcus		x	
Salinicola	x		
Shewanella	x	x	x
Sphaerochaeta	x	x	
Sphingobacterium	x	x	
Sphingomonas	x		x
Spongiibacter		x	
Staphylococcus	x	x	
Stenotrophomonas	x	x	x
Synechococcus	x		
Synergistes	x		
Tenacibaculum	x	x	
Terrisporobacter	x		
Treponema		x	
Truepera	x		
Turicella	x		
Turicibacter		x	
Veillonella	x	x	x
Vibrio	x	x	

[1] Symbiotics source: `http://symbiogenomesdb.uv.es/database.html`

[2] Pathogens source: NEMESISdb — Tran et al., in revision

Supplementary Material SM1-10: Taxonomic detail of enriched PHPB biomarkers in surface and gut pathobiome

Phylum	Class	Order	Family	PHPB genera	Enriched group
Pseudomona-dota	*Gammaproteo-bacteria*	*Burkholder-iales*	*Alcaligena-ceae*	*Achromo-bacter*	Gut
Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Bacteroida-ceae*	*Bacteroides*	Gut
Pseudomona-dota	*Alphaproteo-bacteria*	*Caulobacter-ales*	*Caulobactera-ceae*	*Brevundi-monas*	Surface, Gut
Pseudomona-dota	*Gammaproteo-bacteria*	*Burkholder-iales*	*Comamona-daceae*	*Comamonas*	Gut
Actinomycet-ota	*Actinobacteria*	*Mycobacter-iales*	*Corynebacter-iaceae*	*Corynebac-terium*	Surface
Actinomycet-ota	*Actinobacteria*	*Propionibac-teriales*	*Propionibac-teriaceae*	*Cutibacterium*	Gut
Pseudomona-dota	*Gammaproteo-bacteria*	*Burkholder-iales*	*Comamona-daceae*	*Delftia*	Gut
Pseudomona-dota	*Gammaproteo-bacteria*	*Enterobacter-ales*	*Pasteurellaceae*	*Haemophilus*	Gut
Pseudomona-dota	*Gammaproteo-bacteria*	*Burkholder-iales*	*Oxalobacter-aceae*	*Herminii-monas*	Surface
Pseudomona-dota	*Gammaproteo-bacteria*	*Enterobacter-ales*	*Enterobacter-iaceae*	*Klebsiella*	Surface, Gut
Actinomycet-ota	*Actinobacteria*	*Mycobacter-iales*	*Corynebacter-iaceae*	*Lawsonella*	Surface
Pseudomona-dota	*Gammaproteo-bacteria*	*Burkholder-iales*	*Oxalobacter-aceae*	*Massilia*	Surface
Pseudomona-dota	*Alphaproteo-bacteria*	*Hyphomicro-biales*	*Rhizobiaceae*	*Mesorhizobium*	Surface
Actinomyce-tota	*Actinobacteria*	*Micrococcales*	*Micrococcaceae*	*Micrococcus*	Surface, Gut
Pseudomona-dota	*Gammaproteo-bacteria*	*Burkholder-iales*	*Burkholder-iaceae*	*Paraburkhol-deria*	Surface
Pseudomona-dota	*Alphaproteo-bacteria*	*Rhodobacter-ales*	*Paracoccaceae*	*Paracoccus*	Surface
Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	Surface
Pseudomona-dota	*Gammaproteo-bacteria*	*Burkholder-iales*	*Burkholder-iaceae*	*Ralstonia*	Surface, Gut
Pseudomona-dota	*Gammaproteo-bacteria*	*Enterobacter-ales*	*Shewanell-aceae*	*Shewanella*	Gut
Pseudomona-dota	*Alphaproteo-bacteria*	*Sphingomona-dales*	*Sphingomona-daceae*	*Sphingomonas*	Surface, Gut
Pseudomona-dota	*Gammaproteo-bacteria*	*Lysobacter-ales*	*Lysobacter-aceae*	*Stenotro-phomonas*	Gut
Bacillota	*Negativicutes*	*Veillonellales-Selenomona-dales*	*Veillonell-aceae*	*Veillonella*	Surface

Supplementary Materials for Chapter 2

Supplementary Material SM2-1: Metadata of the sampling sites

Parameter	**Urban**			
Station	1	2	3	4
Location_ID	U1	U2	U3	U4
Sampling_date	31/10/2022	31/10/2022	31/10/2022	31/10/2022
Weather	sunny	sunny	sunny	sunny
Wind_speed (m/s)	5	5	5	10
Wave_height (cm)	200	200	200	150
Depth (m)	5.6	12.7	15.8	15.5
Sample_types	water, sediment	water, sediment	water, sediment	water, sediment
Type_of_storage	cooler with ice	cooler with ice	cooler with ice	cooler with ice
Latitude	12.26242	12.26251	12.262272	12.26277
Longitude	109.20664	109.21583	109.22510	109.23428
pH	8.28	8.27	8.28	8.31
Temperature (°C)	27.6	27.9	27.6	27.4
Salinity (‰)	30.8	30.0	30.1	30.7
DO (mg/L)	6.58	6.56	6.57	6.47
NH_4 (µg/L)	15.7	18.245	13.155	11.245
NO_3 (µg/L)	31.515	75.42	61.295	46.79
PO_4 (µg/L)	9.81	16.375	14.185	15.64
Chl-a (µg/L)	0.46	0.695	0.545	0.485
Parameter	**Fish Farm**			
Station	1	2	3	4
Location_ID	F1	F2	F3	F4
Sampling_date	11/1/22	11/1/22	11/1/22	11/1/22
Weather	sunny	sunny	sunny	sunny
Wind_speed (m/s)	3	3	3	3
Wave_height (cm)	50	50	50	50
Depth (m)	9.1	13.4	16.1	17.2
Sample_types	water, sediment	water, sediment	water, sediment	water, sediment

Supplementary Material SM2-1 (continued)

Parameter	**Fish Farm**			
Type_of_storage	cooler with ice	cooler with ice	cooler with ice	cooler with ice
Latitude	12.18342	12.16751	12.16264	12.15778
Longitude	109.22221	109.23179	109.23951	109.24724
pH	8.26	8.29	8.29	8.32
Temperature (°C)	27.2	27.3	27.4	27.0
Salinity (‰)	29.3	30.1	30.3	29.6
DO (mg/L)	6.48	6.84	6.74	6.76
NH_4 (µg/L)	70.085	14.43	24.29	24.29
NO_3 (µg/L)	83.435	48.315	42.585	62.44
PO_4 (µg/L)	17.83	14.91	15.64	17.10
Chl-a (µg/L)	0.76	0.70	0.555	0.545
Parameter	**Recovery**			
Station	1	2	3	4
Location_ID	R1	R2	R3	R4
Sampling_date	11/2/22	11/2/22	11/2/22	11/2/22
Weather	sunny	sunny	cloudy	cloudy
Wind_speed (m/s)	15	15	15	15
Wave_height (cm)	200	200	200	200
Depth (m)	21	22.5	25.9	27.7
Sample_types	water, sediment	water, sediment	water, sediment	water, sediment
Type_of_storage	cooler with ice	cooler with ice	cooler with ice	cooler with ice
Latitude	12.34267	12.33462	12.32607	12.31768
Longitude	109.29292	109.29635	109.29979	109.30339
pH	8.28	8.28	8.30	8.32
Temperature (°C)	27.4	27.3	27.2	27.1
Salinity (‰)	31.4	31.4	31.2	31.1
DO (mg/L)	6.58	6.59	6.63	6.68
NH_4 (µg/L)	9.34	23.015	21.425	11.25

Supplementary Material SM2-1 (continued)

Parameter	**Fish Farm**			
NO_3 (µg/L)	23.225	22.54	38.685	42.125
PO_4 (µg/L)	61.65	54.35	10.54	12.725
Chl-a (µg/L)	0.27	0.105	0.105	0.25

Supplementary Material SM2-2: Details of bacterial isolates

Family	No	Species	Risk	# isolates	Sampling site
***Enterobacteriaceae* family (Water)**					
Ent	1	*Citrobacter freundii*	2	1	Recovery 2 (Water)
	2	*Enterobacter asburiae*	2	1	Urban 2
	3	*Enterobacter bugandensis*	NA	1	Urban 1
	4	*Escherichia coli*	2	3	Urban 1,2 & Farm 3
	5	*Klebsiella pneumoniae*	2	5	Farm 1,2; Recovery 3; Urban 3,4
	6	*Klebsiella variicola*	NA	2	Recovery 2 & Urban 1
	7	*Kluyvera ascorbata*	2	1	Recovery 2
			Total (Water)	14	
Non-*Enterobacteriaceae* family (Water)					
Non-Ent.	1	*Acinetobacter baumannii*	2	1	Recovery 3
	2	*Acinetobacter nosocomialis*	NA	1	Recovery 1
	3	*Aeromonas caviae*	NA	1	Urban 2
	4	*Aeromonas hydrophila*	NA	2	Urban 3,4
	5	*Bacillus halosaccharovorans*	NA	1	Urban 2
	6	*Bacillus horikoshii*	NA	1	Farm 3
	7	*Clostridium baratii*	2	1	Recovery 3
	8	*Exiguobacterium* sp. [3]	NA	1	Recovery 2
	9	*Microbacterium oleivorans*	NA	1	Recovery 1
	10	*Microbacterium paraoxydans*	NA	1	Urban 4
	11	*Micrococcus flavus*	NA	1	Recovery 1
	12	*Ochrobactrum intermedium*	NA	1	Recovery 4
	13	*Paenibacillus urinalis*	NA	1	Farm 4
	14	*Pantoea stewartii*	1	1	Urban 3
	15	*Pseudomonas aeruginosa*	2	7	Farm 1,3,4; Recovery 1–4
	16	*Pseudomonas alcaligenes*	2	1	Urban 3
	17	*Pseudomonas citronellolis*	NA	1	Urban 1
	18	*Pseudomonas nitroreducens*	NA	1	Recovery 2
	19	*Serratia marcescens*	2	1	Urban 3
	20	*Shewanella putrefaciens*	NA	1	Farm 2
	21	*Vibrio alginolyticus*	2	4	Farm 1; Recovery 1,2; Urban 1
	22	*Vibrio fluvialis*	2	1	Urban 2
	23	*Vibrio harveyi*	NA	1	Farm 2
	24	*Vibrio natriegens*	NA	1	Urban 4
			Total (Water)	34	
***Enterobacteriaceae* family (Sediment)**					
Ent.	1	*Citrobacter freundii*	2	3	Urban 1–3
	2	*Enterobacter bugandensis*	NA	1	Urban 2
	3	*Enterobacter cloacae*	2	1	Farm 3
	4	*Escherichia coli*	2	4	Farm 1; Urban 1,3,4
	5	*Klebsiella pneumoniae*	2	3	Farm 3; Urban 2,3

Supplementary Material SM2-2 (continued)

Family	No	Species	Risk	# isolates	Sampling site
			Total (Sediment)	12	
Non-Enterobacteriaceae family (Sediment)					
Non-Ent.	1	*Acinetobacter baylyi*	NA	1	Recovery 1
	2	*Acinetobacter haemolyticus*	2	1	Farm 3
	3	*Acinetobacter pittii*	NA	3	Recovery 1–3
	4	*Aeromonas caviae*	NA	2	Urban 2,3
	5	*Aeromonas hydrophila*	NA	2	Urban 1,4
	6	*Bacillus alcalophilus*	NA	1	Recovery 1
	7	*Bacillus altitudinis*	NA	1	Recovery 2
	8	*Bacillus amyloliquefaciens* ssp. plantarum	NA	1	Urban 1
	9	*Bacillus cereus*	2	1	Urban 3
	10	*Bacillus halosaccharovorans*	NA	1	Urban 2
	11	*Bacillus horikoshii*	NA	3	Urban 1,3
	12	*Bacillus licheniformis*	NA	1	Urban 3
	13	*Bacillus marisflavi*	NA	1	Recovery 3
	14	*Bacillus megaterium*	NA	2	Recovery 1; Urban 2
	15	*Bacillus vietnamensis*	NA	1	Farm 4
	16	*Brevundimonas diminuta*	1	1	Urban 4
	17	*Corynebacterium durum*	NA	1	Recovery 3
	18	*Kocuria marina*	NA	1	Recovery 3
	19	*Microbacterium paraoxydans*	NA	1	Urban 4
	20	*Micrococcus luteus*	NA	3	Recovery 1; Urban 3,4
	21	*Ochrobactrum tritici*	NA	1	Urban 4
	22	*Pantoea stewartii*	1	1	Urban 3
	23	*Pseudomonas aeruginosa*	2	2	Recovery 1; Urban 4
	24	*Pseudomonas citronellolis*	NA	2	Urban 1,4
	25	*Pseudomonas mendocina*	2	1	Farm 4
	26	*Pseudomonas putida*	NA	1	Urban 2
	27	*Pseudomonas stutzeri*	NA	1	Recovery 2
	28	*Shewanella algae*	2	1	Recovery 4
	29	*Shewanella putrefaciens*	NA	2	Urban 3,4
	30	*Vibrio alginolyticus*	2	3	Urban 1–3
	31	*Vibrio harveyi*	NA	1	Recovery 4
	32	*Vibrio natriegens*	NA	1	Urban 4
	33	*Vibrio parahaemolyticus*	2	1	—
			Total (Sediment)	47	

Supplementary Material SM2-3: Details of the statistical tests

Sources	Object	Compare	P-value
Relative abundance			
	*Sul*1	F – R	0,7525016
		F – U	0,4738879
		R – U	0,9151681
		Water – Sediment	3,80E-05
	*Sul*2	F – R	0,91007009
		F – U	0,0249001
		R – U	0,03678029
		Water – Sediment	0,024
	*tet*Q	F – R	0,9107094
		F – U	0,2278268
		R – U	0,1434536
		Water – Sediment	0,55
	*tet*B	F – R	0,1970504
		F – U	0,4924031
		R – U	0,4233864
		Water – Sediment	0,96
	*mec*A	F – R	0,9028158
		F – U	1
		R – U	1
		Water – Sediment	0,11
	*bla*KPC	F – R	0,3357511
		F – U	0,955374
		R – U	0,6548453
		Water – Sediment	0,032
	*bla*VIM	F – R	1
		F – U	0,08114348
		R – U	0,16228697

Supplementary Material SM2-3 (continued)

Sources	Object	Compare	P-value
		Water – Sediment	0,0016
	*IntI*1	F – R	0,65825341
		F – U	0,02270147
		R – U	0,0372999
		Water – Sediment	0,013
	*Sul*3	F – R	0,7745624
		F – U	0,9766037
		R – U	0,8928323
		Water – Sediment	0,0003267
Absolute abundance			
	*Sul*1	F – R	0,03312495
		F – U	0,76312506
		R – U	0,04688088
		Water – Sediment	0,064
	*Sul*2	F – R	0,96996671
		F – U	0,02035478
		R – U	0,01134903
		Water – Sediment	0,38
	*Sul*3	F – R	0,5553463
		F – U	0,6635776
		R – U	0,5427463
		Water – Sediment	1,50E-06
	*tet*Q	F – R	0,5010348
		F – U	0,2409064
		R – U	0,4077249
		Water – Sediment	0,14
	*tet*B	F – R	0,1527691
		F – U	0,3402607

Supplementary Material SM2-3 (continued)

Sources	Object	Compare	P-value
		R – U	0,4981541
		Water – Sediment	0,82
	*mec*A	F – R	0,9675337
		F – U	0,9980266
		R – U	1
		Water – Sediment	0,11
	*bla*KPC	F – R	0,8552709
		F – U	0,9918821
		R – U	0,4455042
		Water – Sediment	1,50E-06
	*bla*VIM	F – R	0,93092478
		F – U	0,08930715
		R – U	0,14722451
		Water – Sediment	0,0016
	*IntI*1	F – R	0,4609948
		F – U	0,3809032
		R – U	0,6235044
		Water – Sediment	4,00E-04
Log_{10}(Absolute abundance)			
	*Sul*1	F – R	0,03312495
		F – U	0,76312506
		R – U	0,04688088
		Water – Sediment	0,064
	*Sul*2	F – R	0,96996671
		F – U	0,02035478
		R – U	0,01134903
		Water – Sediment	0,38
	*Sul*3	F – R	0,5553463

Supplementary Material SM2-3 (continued)

Sources	Object	Compare	P-value
		F – U	0,6635776
		R – U	0,5427463
		Water – Sediment	1,50E-06
	*tet*Q	F – R	0,5010348
		F – U	0,2409064
		R – U	0,4077249
		Water – Sediment	0,14
	*tet*B	F – R	0,1527691
		F – U	0,3402607
		R – U	0,4981541
		Water – Sediment	0,82
	*mec*A	F – R	0,9675337
		F – U	0,9980266
		R – U	1
		Water – Sediment	0,11
	*bla*KPC	F – R	0,8552709
		F – U	0,9918821
		R – U	0,4455042
		Water – Sediment	1,50E-06
	*bla*VIM	F – R	0,93092478
		F – U	0,08930715
		R – U	0,14722451
		Water – Sediment	0,0016
	*IntI*1	F – R	0,4609948
		F – U	0,3809032
		R – U	0,6235044
		Water – Sediment	4,00E-04
	Water - Sediment	Permanova	9,99E-04
	Transect (Water + Sediment)	Permanova	3,23E-01

Supplementary Material SM2-3 (continued)

Sources	Object	Compare	P-value
	Transect (Water)	Permanova	6,19E-02
	Transect (Sediment)	Permanova	4,00E-03
Isolates composition			
	Total Strains	F – R	0,00509
		F – U	0,00056
		R – U	0,21109
		Water – Sediment	0,077
	Pathogenic Strains	F – R	0,58
		F – U	0,23
		R – U	0,56
		Water – Sediment	0,8
	Enterobacteriaceae	F – R	1
	Strains	F – U	0,137
		R – U	0,086
		Water – Sediment	0,66

Supplementary Material SM2-4: MAR indexes for the isolated bacteria between sample types and spieces

Transect	Average	Min	Max
U1 Water	0.19	0.05	0.33
U2 Water	0.05	0	0.10
U3 Water	0.24	0.24	0.24
U4 Water	0.24	0.24	0.24
U1 Sediment	0.07	0.05	0.10
U2 Sediment	0.13	0.05	0.14
U3 Sediment	0.19	0.14	0.24
U4 Sediment	0.05	0.05	0.05
F1 Water	0.00	0	0.00
F2 Water	0.10	0.10	0.10
F3 Water	0.05	0.05	0.05
F1 Sediment	0.24	0.24	0.24
F3 Sediment	0.12	0.05	0.19
R2 Water	0.22	0.14	0.33
R3 Water	0.14	0.14	0.14

Species	Average	Min	Max
Citrobacter freundii	0.14	0.05	0.33
Enterobacter asburiae	0.10	0.10	0.10
Enterobacter bugandensis	0.19	0.19	0.19
Enterobacter cloacae	0.05	0.05	0.05
Escherichia coli	0.09	0	0.24
Klebsiella pneumoniae	0.16	0	0.24
Klebsiella variicola	0.24	0.14	0.33
Kluyvera ascorbata	0.19	0.19	0.19

Supplementary Material SM2-5: PCR screening conditions and results

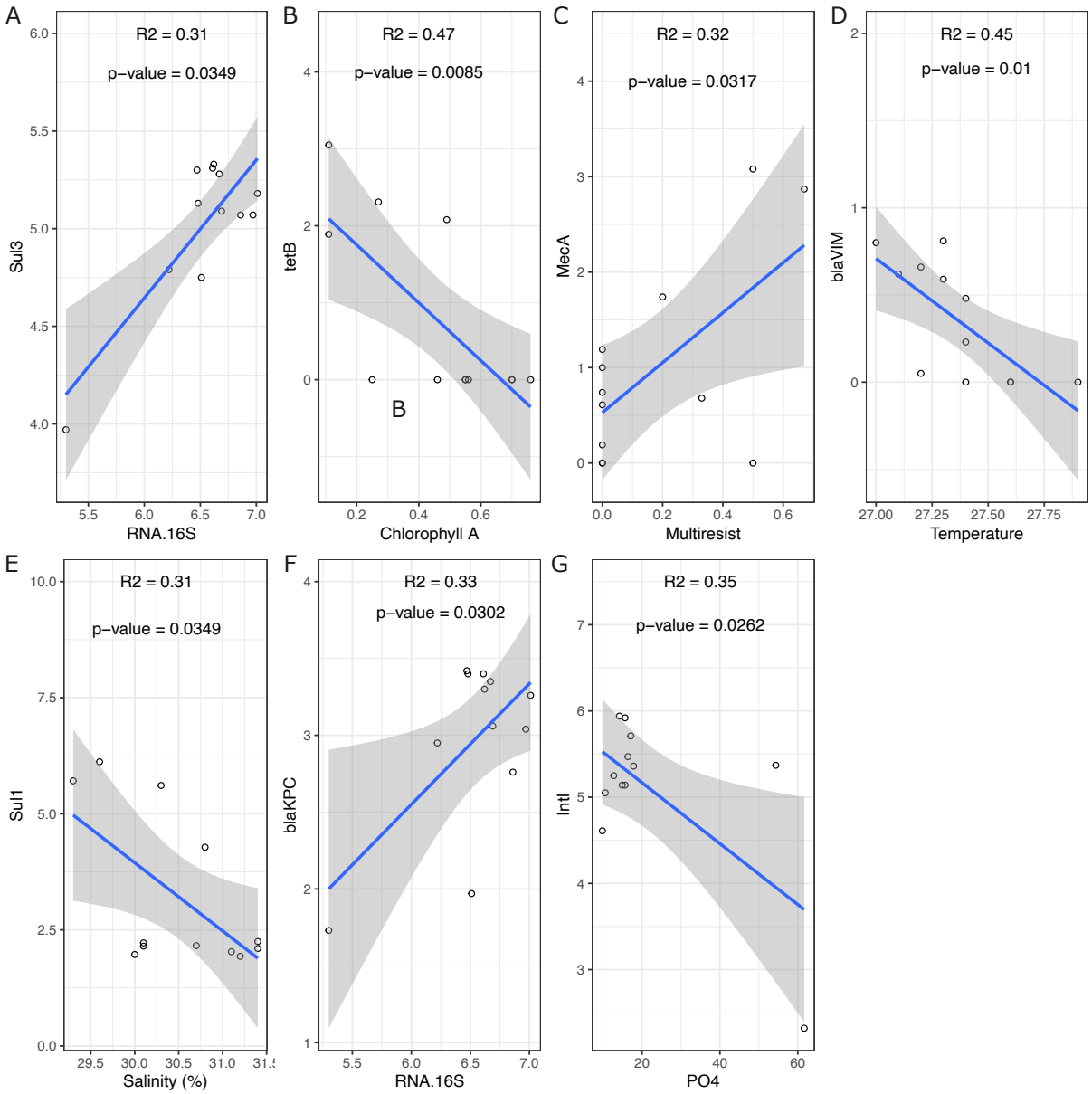
Drug classes	ARGs	Primer	Sequences	Amp	Ref.	Init. Den.	Den.	Ann.	Ext.	Final Ext.	PCR (+) results	PCR (–) results
Tetracycline resistance	*tet*M	*tet*M_FW	ACACGCCAGGACATATGGAT	126	(Aminov et al., 2004)	95°C, 15 min	95°C, 30s	57°C, 30s	72°C, 25s	79°C, 5 min	0	24
		*tet*M_RV	GGGAATCCCCATTTTCCTAA									
Tetracycline resistance	*tet*Q	*tet*Q_FW	CAAGGTGATATCCGCTCTGA	128	(Aminov et al., 2004)	95°C, 15 min	95°C, 30s	57°C, 30s	72°C, 25s	79°C, 5 min	16	8
		*tet*Q_RV	GGAAAATCGTTCTTCCAGCA									
Tetracycline resistance	*tet*X	*tet*X-FW	GGAAACCGGCTAATGGCAT	230	(Xexaki et al., 2023)	95°C, 3 min	95°C, 30s	55°C, 40s	72°C, 45s	72°C, 5 min	0	24
		*tet*X-R	AATCCTACAAATGACAACGTCG									
Sulfonamides resistance	*Sul*1	*Sul*1-F	CGCACCGGAAACATCGCTGCAC	163	(Pei et al., 2006)	95°C, 15 min	95°C, 15s	56°C, 30s	72°C, 30s	72°C, 7 min	13	11
		*Sul*1-R	TGAAGTTCCGCCGCAAGGCTCG									
Sulfonamides resistance	*Sul*2	*Sul*2-F	TCCGGTGGAGGCCGGTATCTGG	191	(Pei et al., 2006)	95°C, 15 min	95°C, 15s	61°C, 30s	72°C, 30s	72°C, 7 min	17	7
		*Sul*2-R	CGGGAATGCCATCTGCCTTGAG									
Sulfonamides resistance	*Sul*3	*Sul*3-F	GAGCAAGATTTTTGGAATCG	880	(Boerlin et al., 2005)	95°C, 4 min	95°C, 60s	51°C, 60s	72°C, 60s	72°C, 7 min	24	0
		*Sul*3-R	CATCTGCAGCTAACCTAGGGC									
TTTGGA												
Colistin resistance	*mcr*1	*mcr*-1F	TCCAAAATGCCCTACAGACC	320	(J. Liu et al., 2020)	94°C, 4 min	94°C, 5s	59°C, 15s	NA	72°C, 5 min	0	24
		mcr-1R	GCCACCACAGGCAGTAAAAT									
Carbapenem resistance	*bla*KPC	*bla*KPC-F	GATACCACGTTCCGTCTGG	796	(Hindiyeh et al., 2008)	95°C, 10 min	95°C, 15s	60°C, 60s	NA	NA	2	22
		*bla*KPC-R	GCAGGTTCCGGTTTTGCTC									
Carbapenem resistance	*bla*OXA	*bla*OXA-48F	AGGCACGTATGAGCAAGATG	743	(Teban-Man et al., 2022)	98°C, 2 min	95°C, 10s	60°C, 45s	NA	NA	0	24
		*bla*OXA-48R	TGGCTTGTTTGACAATACGC									
Carbapenem resistance	*bla*VIM	*bla*VIM-F	AGTGGTGAGTATCCGACAG	212	(Kaboré et al., 2023)	95°C, 5 min	95°C, 45s	52°C, 30s	72°C, 60s	72°C, 10 min	8	16
		*bla*VIM-R	TCAATCTCCGCGAGAAG									
Beta-lactam resistance	*bla*NDM	*bla*NDM_F	-TGGCAGCACACTTCCTATC	488	(Drk et al., 2023)	94°C, 2.5 min	94°C, 20s	58°C, 20s	72°C, 45s	72°C, 2 min	0	24
		*bla*NDM_R	AGATTGCCGAGCGACTTG									
Beta-lactam resistance	*bla*IMP	*bla*IMP-F	GGAATAGAGTGGCTTAAYTCTC	232	(Ellington et al., 2007)	94°C, 5 min	94°C, 30s	52°C, 40s	72°C, 50s	72°C, 5 min	0	24
		*bla*IMP-R	GGTTTAAYAAAACAACCACC									

Supplementary Material SM2-5 (continued).

Drug classes	ARGs.	Primer	Sequence	Amp	Ref.	Init. Den.	Den.	Ann.	Ext.	Final Ext.	PCR (+) results	PCR (–) results
Quinolone resistance	*Qnr*A	*Qnr*A-F-multi	AGAGGATTTCTCACGCCAGG	580	(Cattoir et al., 2007)	95°C, 10 min	95°C, 60s	54°C, 60s	72°C, 60s	72°C, 10 min	0	24
		*Qnr*A-R-multi	TGCCAGGCACAGATCTTGAC									
Methicillin resistance	*mec*A	*mec*A-F	TCCAGATTACAACTTCACCAGG	162	(Stegger et al., 2012)	94°C, 15 min	94°C, 30s	59°C, 60s	72°C, 60s	72°C, 10 min	10	14
		*mec*A-R	CCACTTCATATCTTGTAACG									
Macrolide resistance	*erm*B	*erm*B_F	AAGGGCATTTAACGACGAAACTG	438	(von Winters-dorff et al., 2014)	95°C, 3 min	95°C, 20s	60°C, 30s	72°C, 40s	72°C, 7 min	0	24
		*erm*B_R	ATTTATCTGGAACATCTGTGGTATG									
Class 1 Integrons	*IntI*1	*IntI*-B	CTCTATGGGCACTGTCCACATTG	592	(Rao et al., 2008)	94°C, 3 min	94°C, 30s	64°C, 30s	72°C, 60s	72°C, 7 min	3	21
		IntI-F	GCTGGATAGGTTAAGGGCGG									
Fluoro Quinolone resistance	*Qep*A	*Qep*A-F	CTGCAGGTACTGCGTCATG	403	(Heidary et al., 2017)	94°C, 5 min	94°C, 45s	51°C, 45s	72°C, 45s	72°C, 5 min	0	24
		*Qep*A-R	CGTGTTGCTGGAGTTCTTC									
Aminoglycoside resistance	*Rmt*B	*Rmt*B-multiF	ACTTTTACAATCCCTCAATAC	171	(Ramos et al., 2014)	94°C, 10 min	94°C, 45s	57°C, 30s	72°C, 25s	72°C, 3 min	0	24
		*Rmt*B-multiR	AAGTATATAAGTTCTGTTCCG									

Supplementary Material SM2-6: Detail of the abundances of ARGs

Gene / Copy type	U1W	U2W	U3W	U4W	R1W	R2W	R3W	R4W	F1W	F2W	F3W	F4W
16*S rRNA* (copies/1 µl reaction)	7.91E+06	3.97E+06	7.44E+06	6.50E+06	1.08E+07	7.96E+06	8.86E+06	1.50E+07	3.61E+07	7.84E+06	5.36E+06	4.99E+06
16*S rRNA* (copies/1 ml sample)	1.98E+05	9.92E+04	1.86E+05	1.63E+05	2.69E+05	1.99E+05	2.21E+05	3.75E+05	9.03E+05	1.96E+05	1.34E+05	1.25E+05
*Sul*1 (copies/1 µl reaction)	7.58E+05	3.42E+05	8.09E+05	8.36E+05	8.59E+05	1.12E+06	6.28E+05	1.83E+06	1.24E+06	6.31E+05	2.89E+05	4.59E+05
*Sul*1 (copies/1 ml sample)	1.90E+04	8.56E+03	2.02E+04	2.09E+04	2.15E+04	2.80E+04	1.57E+04	4.58E+04	3.10E+04	1.58E+04	7.22E+03	1.15E+04
*Sul*1 (Rel. Abun)	9.58E-02	8.63E-02	1.09E-01	1.29E-01	7.99E-02	1.41E-01	7.09E-02	1.22E-01	3.44E-02	8.04E-02	5.39E-02	9.18E-02
*Sul*2 (copies/1 µl reaction)	8.58E+04	2.37E+03	2.32E+05	1.50E+03	8.28E+02	4.68E+02	0.00E+00	1.60E+03	2.03E+03	5.16E+02	6.48E+02	2.36E+05
*Sul*2 (copies/1 ml sample)	2.14E+03	5.92E+01	5.81E+03	3.76E+01	2.07E+01	1.17E+01	0.00E+00	3.99E+01	5.08E+01	1.29E+01	1.62E+01	5.91E+03
*Sul*2 (Rel. Abun)	1.08E-02	5.96E-04	3.12E-02	2.31E-04	7.71E-05	5.87E-05	0.00E+00	1.06E-04	5.63E-05	6.58E-05	1.21E-04	4.73E-02
*Sul*3 (copies/1 µl reaction)	3.74E+05	1.41E+05	4.32E+05	5.12E+05	6.26E+05	5.27E+05	4.05E+05	7.74E+05	6.12E+05	5.51E+05	1.69E+05	3.29E+05
*Sul*3 (copies/1 ml sample)	9.35E+03	3.54E+03	1.08E+04	1.28E+04	1.56E+04	1.32E+04	1.01E+04	1.94E+04	1.53E+04	1.38E+04	4.21E+03	8.23E+03
*Sul*3 (Rel. Abun)	4.73E-02	3.57E-02	5.81E-02	7.87E-02	5.82E-02	6.62E-02	4.57E-02	5.16E-02	1.69E-02	7.02E-02	3.15E-02	6.59E-02
*tet*Q (copies/1 µl reaction)	3.61E+04	1.44E+04	0.00E+00	3.12E+04	0.00E+00	4.66E+04	8.09E+03	0.00E+00	3.73E+04	3.03E+04	2.69E+03	1.19E+04
*tet*Q (copies/1 ml sample)	9.01E+02	3.59E+02	0.00E+00	7.79E+02	0.00E+00	1.16E+03	2.02E+02	0.00E+00	9.34E+02	7.56E+02	6.73E+01	2.98E+02
*tet*Q (Rel. Abun)	4.56E-03	3.62E-03	0.00E+00	4.79E-03	0.00E+00	5.85E-03	9.14E-04	0.00E+00	1.03E-03	3.86E-03	5.03E-04	2.39E-03
*tet*B (copies/1 µl reaction)	0.00E+00	0.00E+00	0.00E+00	5.75E+05	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.39E+05	0.00E+00	0.00E+00
*tet*B (copies/1 ml sample)	0.00E+00	0.00E+00	0.00E+00	1.44E+04	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	5.98E+03	0.00E+00	0.00E+00
*tet*B (Rel. Abun)	0.00E+00	0.00E+00	0.00E+00	8.85E-02	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.05E-02	0.00E+00	0.00E+00
*mec*A (copies/1 µl reaction)	2.18E+03	0.00E+00	0.00E+00	0.00E+00	7.94E+02	0.00E+00	4.00E+03	9.50E+01	9.61E+03	9.31E+02	6.66E+02	1.19E+03
*mec*A (copies/1 ml sample)	5.46E+01	0.00E+00	0.00E+00	0.00E+00	1.99E+01	0.00E+00	1.00E+02	2.38E+00	2.40E+02	2.33E+01	1.67E+01	2.98E+01
*mec*A (Rel. Abun)	2.76E-04	0.00E+00	0.00E+00	0.00E+00	7.38E-05	0.00E+00	4.52E-04	6.33E-06	2.66E-04	1.19E-04	1.24E-04	2.39E-04
*bla*KPC (copies/1 µl reaction)	2.14E+03	7.48E+02	2.30E+03	3.46E+03	5.67E+03	2.66E+03	1.55E+03	3.90E+03	1.25E+02	2.24E+03	4.90E+02	3.57E+02
*bla*KPC (copies/1 ml sample)	5.34E+01	1.87E+01	5.75E+01	8.64E+01	1.42E+02	6.64E+01	3.87E+01	9.76E+01	3.12E+00	5.59E+01	1.23E+01	8.92E+00
*bla*KPC (Rel. Abun)	2.70E-04	1.89E-04	3.09E-04	5.32E-04	5.27E-04	3.34E-04	1.75E-04	2.60E-04	3.46E-06	2.85E-04	9.15E-05	7.15E-05
*bla*VIM (copies/1 µl reaction)	0.00E+00	0.00E+00	0.00E+00	0.00E+00	8.54E+02	6.11E+02	1.15E+02	1.61E+03	3.66E+02	1.63E+03	5.02E+01	7.10E+02
*bla*VIM (copies/1 ml sample)	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.14E+01	1.53E+01	2.86E+00	4.03E+01	9.15E+00	4.08E+01	1.26E+00	1.78E+01
*bla*VIM (Rel. Abun)	0.00E+00	0.00E+00	0.00E+00	0.00E+00	7.94E-05	7.68E-05	1.29E-05	1.07E-04	1.01E-05	2.08E-04	9.37E-06	1.42E-04
*IntI*1 (copies/1 µl reaction)	1.63E+06	8.52E+05	7.57E+05	8.22E+05	5.23E+05	3.94E+05	3.27E+05	2.81E+06	1.78E+06	6.08E+05	2.40E+05	7.54E+05
*IntI*1 (copies/1 ml sample)	4.08E+04	2.13E+04	1.89E+04	2.05E+04	1.31E+04	9.85E+03	8.18E+03	7.03E+04	4.45E+04	1.52E+04	6.01E+03	1.88E+04
*IntI*1 (Rel. Abun)	2.06E-01	2.15E-01	1.02E-01	1.26E-01	4.86E-02	4.95E-02	3.69E-02	1.88E-01	4.93E-02	7.75E-02	4.49E-02	1.51E-01
Gene / Copy type	**U1S**	**U2S**	**U3S**	**U4S**	**R1S**	**R2S**	**R3S**	**R4S**	**F1S**	**F2S**	**F3S**	**F4S**
16*S rRNA* (copies/1 µl reaction)	NA	5.58E+05	9.42E+06	1.29E+07	7.75E+05	1.65E+06	2.12E+06	2.77E+06	1.99E+05	2.25E+06	2.63E+06	1.40E+06
16*S rRNA* (copies/1 ml sample)	NA	2.79E+07	4.71E+08	6.45E+08	3.88E+07	8.27E+07	1.06E+08	1.38E+08	9.93E+06	1.13E+08	1.31E+08	7.02E+07
*Sul*1 (copies/1 µl reaction)	NA	0.00E+00	0.00E+00	0.00E+00	2.49E+05	0.00E+00	2.13E+05	7.47E+05	0.00E+00	0.00E+00	0.00E+00	0.00E+00
*Sul*1 (copies/1 ml sample)	NA	0.00E+00	0.00E+00	0.00E+00	1.24E+07	0.00E+00	1.06E+07	3.74E+07	0.00E+00	0.00E+00	0.00E+00	0.00E+00
*Sul*1 (Rel. Abun)	NA	0.00E+00	0.00E+00	0.00E+00	3.21E-02	0.00E+00	1.00E-02	2.70E-02	0.00E+00	0.00E+00	0.00E+00	0.00E+00
*Sul*2 (copies/1 µl reaction)	NA	2.66E+03	6.47E+02	1.05E+03	0.00E+00	0.00E+00	0.00E+00	9.26E+02	0.00E+00	0.00E+00	0.00E+00	0.00E+00
*Sul*2 (copies/1 ml sample)	NA	1.33E+05	3.23E+04	5.27E+04	0.00E+00	0.00E+00	0.00E+00	4.63E+04	0.00E+00	0.00E+00	0.00E+00	0.00E+00
*Sul*2 (Rel. Abun)	NA	4.77E-03	6.86E-05	8.17E-05	0.00E+00	0.00E+00	0.00E+00	3.34E-04	0.00E+00	0.00E+00	0.00E+00	0.00E+00
*Sul*3 (copies/1 µl reaction)	NA	2.17E+04	2.56E+04	3.62E+04	2.34E+04	5.40E+04	9.15E+04	4.19E+04	4.13E+03	5.95E+04	7.20E+04	3.35E+04
*Sul*3 (copies/1 ml sample)	NA	1.08E+06	1.28E+06	1.81E+06	1.17E+06	2.70E+06	4.57E+06	2.10E+06	2.07E+05	2.98E+06	3.60E+06	1.68E+06
*Sul*3 (Rel. Abun)	NA	3.88E-02	2.72E-03	2.81E-03	3.02E-02	3.27E-02	4.31E-02	1.51E-02	2.08E-02	2.65E-02	2.74E-02	2.39E-02
*tet*Q (copies/1 µl reaction)	NA	7.65E+03	2.85E+04	1.30E+04	1.86E+03	3.85E+03	4.02E+03	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.57E+03
*tet*Q (copies/1 ml sample)	NA	3.83E+05	1.43E+06	6.49E+05	9.31E+04	1.93E+05	2.01E+05	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.79E+05
*tet*Q (Rel. Abun)	NA	1.37E-02	3.02E-03	1.01E-03	2.40E-03	2.33E-03	1.90E-03	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.54E-03
*tet*B (copies/1 µl reaction)	NA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	8.34E+02	0.00E+00	2.54E+04	0.00E+00
*tet*B (copies/1 ml sample)	NA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	4.17E+04	0.00E+00	1.27E+06	0.00E+00
*tet*B (Rel. Abun)	NA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	4.20E-03	0.00E+00	9.68E-03	0.00E+00
*mec*A (copies/1 µl reaction)	NA	0.00E+00	1.08E+04	0.00E+00	1.44E+03	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
*mec*A (copies/1 ml sample)	NA	0.00E+00	5.40E+05	0.00E+00	7.21E+04	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
*mec*A (Rel. Abun)	NA	0.00E+00	1.15E-03	0.00E+00	1.86E-03	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
*bla*KPC (copies/1 µl reaction)	NA	8.34E+02	4.19E+02	7.57E+02	8.88E+02	1.51E+03	2.05E+03	1.39E+03	5.63E+01	2.23E+03	2.12E+03	7.34E+02
*bla*KPC (copies/1 ml sample)	NA	4.17E+04	2.09E+04	3.78E+04	4.44E+04	7.57E+04	1.03E+05	6.94E+04	2.82E+03	1.11E+05	1.06E+05	3.67E+04
*bla*KPC (Rel. Abun)	NA	1.49E-03	4.44E-05	5.87E-05	1.15E-03	9.16E-04	9.68E-04	5.01E-04	2.83E-04	9.89E-04	8.06E-04	5.23E-04
*bla*VIM (copies/1 µl reaction)	NA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
*bla*VIM (copies/1 ml sample)	NA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
*bla*VIM (Rel. Abun)	NA	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
*IntI*1 (copies/1 µl reaction)	NA	8.19E+04	7.91E+05	6.74E+05	8.10E+04	3.84E+04	4.65E+04	7.61E+04	0.00E+00	7.36E+04	4.20E+04	3.32E+04
*IntI*1 (copies/1 ml sample)	NA	4.09E+06	3.96E+07	3.37E+07	4.05E+06	1.92E+06	2.32E+06	3.80E+06	0.00E+00	3.68E+06	2.10E+06	1.66E+06
*IntI*1 (Rel. Abun)	NA	1.47E-01	8.39E-02	5.23E-02	1.04E-01	2.32E-02	2.19E-02	2.75E-02	0.00E+00	3.27E-02	1.60E-02	2.36E-02



Supplementary Material SM2-7: Multiple linear regression models describe the correlation between each ARG and the best-explained environmental variable. (A) *Sul*3 gene with 16S RNA gene abundance; (B) *tet*B gene with Chlorophyll A (µg/L); (C) *mec*A gene with multi-resist bacteria (ratio); (D) *bla*VIM gene with temperature (°C); (E) *Sul*1 gene with salinity (‰); (F) *bla*KPC gene with 16S RNA gene abundance; (G) *IntI*1 gene with PO_4 (µg/L).

Supplementary Material SM2-8: Correlations between ARGs and environmental variables

	16*S rRNA*	*Sul*1	*Sul*2	*Sul*3	*tet*B	*tet*Q	*bla*KPC	*bla*VIM	*IntI*1	*mec*A	pH	Temp	DO	NH_4	NO_3	PO_4	Chl.a	Salinity	Patho	Multi-resist
16*S rRNA*	NA	0.2885	0.573	0.0008	0.4010	0.754	0.0302	0.6108	0.3735	0.7088	0.2433	0.258	0.851	0.7926	0.77	0.824	0.68	0.922	0.8908	0.4780
*Sul*1	0.2885	NA	0.768	0.894	0.1194	0.395	0.608	0.3704	0.7274	0.3450	0.9204	0.313	0.595	0.0373	0.2721	0.48	0.15	0.0349	0.0441	0.9268
*Sul*2	0.573	0.7681	NA	0.199	0.3290	0.6448	0.485	0.1269	0.2412	0.8604	0.6752	0.148	0.33	0.3001	0.2961	0.25	0.413	0.453	0.5901	0.2610
*Sul*3	0.0008	0.8944	0.199	NA	0.7799	0.817	0.0000	0.1330	0.1813	0.4580	0.3250	0.0868	0.302	0.5482	0.533	0.849	0.947	0.499	0.7407	0.8866
*tet*B	0.4010	0.1194	0.329	0.7799	NA	0.208	0.794	0.7211	0.2544	0.4231	0.7062	0.635	0.314	0.4991	0.0620	0.114	0.0084	0.0125	0.5156	0.9646
*tet*Q	0.754	0.3946	0.6448	0.817	0.2079	NA	0.987	0.1816	0.4415	0.8115	0.7333	0.105	0.848	0.5434	0.5699	0.212	0.140	0.714	0.4391	0.6620
*bla*KPC	0.0302	0.6083	0.485	0.0000	0.7936	0.987	NA	0.2983	0.0106	0.5661	0.6220	0.254	0.515	0.2136	0.134	0.544	0.424	0.125	0.4911	0.5482
*bla*VIM	0.6108	0.3704	0.127	0.1330	0.7211	0.182	0.2983	NA	0.7526	0.8450	0.6485	0.01	0.226	0.2833	0.8770	0.158	0.755	0.789	0.7321	0.5476
*IntI*1	0.3735	0.7274	0.2412	0.1813	0.2544	0.442	0.0106	0.7526	NA	0.8658	0.4815	0.901	0.987	0.5456	0.0743	0.0262	0.311	0.109	0.5474	0.1867
*mec*A	0.7088	0.3450	0.8604	0.4580	0.4231	0.812	0.5661	0.8450	0.8658	NA	0.0618	0.812	0.169	0.0800	0.4285	0.937	0.573	0.414	0.0393	0.0316
pH	0.2433	0.9204	0.6752	0.3250	0.7062	0.733	0.622	0.6485	0.4815	0.0618	NA	0.0476	0.164	0.1302	0.4677	0.468	0.288	0.513	0.3947	0.3065
Temp	0.258	0.3126	0.148	0.0868	0.6352	0.105	0.254	0.0100	0.9008	0.8119	0.0476	NA	0.231	0.4035	0.7027	0.917	0.336	0.948	0.5213	0.6016
DO	0.851	0.5945	0.33	0.302	0.3144	0.848	0.515	0.2263	0.9867	0.1689	0.1635	0.231	NA	0.3947	0.630	0.611	0.876	0.770	0.3459	0.0201
NH_4	0.7926	0.0373	0.3	0.5482	0.4991	0.543	0.214	0.2833	0.5456	0.0800	0.1302	0.404	0.395	NA	0.0506	0.761	0.215	0.0474	0.0019	0.3534
NO_3	0.77	0.2721	0.2961	0.533	0.0620	0.57	0.134	0.8770	0.0743	0.4285	0.4677	0.703	0.631	0.0506	NA	0.0856	0.0026	0.0000	0.1246	0.4126
PO_4	0.824	0.4796	0.2504	0.849	0.1138	0.212	0.544	0.1576	0.0261	0.9366	0.4081	0.917	0.611	0.7608	0.0856	NA	0.142	0.103	0.5525	0.9137
Chl.a	0.68	0.1495	0.4131	0.947	0.0084	0.14	0.424	0.7551	0.3113	0.5727	0.2877	0.336	0.876	0.2150	0.0026	0.142	NA	0.0000	0.6999	0.5768
Salinity	0.9221	0.0349	0.4532	0.499	0.0124	0.714	0.125	0.7893	0.1094	0.4142	0.5128	0.948	0.770	0.0474	0.0000	0.103	0.0000	NA	0.2162	0.5102
Patho	0.8908	0.0441	0.5901	0.741	0.5156	0.439	0.491	0.7321	0.5474	0.0393	0.3947	0.521	0.346	0.0019	0.1246	0.553	0.700	0.216	NA	0.5226
Multiresist	0.4780	0.9268	0.2610	0.887	0.9646	0.662	0.548	0.5476	0.1867	0.0316	0.3065	0.602	0.0202	0.3534	0.4126	0.914	0.577	0.510	0.5226	NA

Supplementary Material SM2-9: Detail of the qPCR primers and procedure

Group	Gene	Primer	Primer sequence	Ta condition	Tm product	Product Size (bp)	Citation
House keeping gene (bacterial density)	16*S rRNA*	16S-F 16S-R	5′-TGTGTAGCGGTGAAATGCG-3′ 5′-CATCGTTTACGGCGTGGAC-3′	62°C	85°C	140	(S. Song et al., 2019)
Beta-lactams resistance	*bla*KPC	*bla*KPC-F *bla*KPC-R	5′-GATACCACGTTCCGTCTGG-3′ 5′-GCAGGTTCCGGTTTTGCTC-3′	58°C, 30s	87°C	246	(Tahmasebi et al., 2020)
	*bla*VIM	*bla*VIM-F *bla*VIM-R	5′-GAGTTGCTTTTGATTGATACAG-3′ 5′-TCGATGAGAGTCCTTCTAGA-3′	55°C, 37s	90°C	247	(Goudarzi et al., 2019)
Class 1 Integrons	*IntI*1	*IntI*1-F *IntI*1-R	5′-GCCTTGATGTTACCCGAGAG-3′ 5′-GATCGGTCGAATGCGTGT-3′	60°C, 60s	92°C	196	(Quintela-Baluja et al., 2021)
Methicillin resistance	*mec*A	*mec*A-F *mec*A-R	5′-TCCAGATTACAACTTCACCAGG-3′ 5′-CCACTTCATATCTTGTAACG-3′	58°C, 50s	74°C	162	(Koupahi et al., 2016)
Sulfonamides resistance	*Sul*1	*Sul*1-F *Sul*1-R	5′-CGCACCGGAAACATCGCTGCAC-3′ 5′-TGAAGTTCCGCCGCAAGGCTCG-3′	65°C, 30s	88°C	163	(Pei et al., 2006)
	*Sul*2	*Sul*2-F *Sul*2-R	5′-TCCGGTGGAGGCCGGTATCTGG-3′ 5′-CGGGAATGCCATCTGCCTTGAG-3′	57.5°C, 30s	90°C	191	(Pei et al., 2006)
	*Sul*3	*Sul*3-F *Sul*3-R	5′-TCCGTTCAGCGAATTGGTGCAG-3′ 5′-TTCGTTCACGCCTTACACCAGC-3′	61°C, 30s	90°C	128	(Pei et al., 2006)
Tetracycline resistance	*tet*Q	*tet*Q-F *tet*Q-R	5′-AGAATCTGCTGTTTGCCAGTG-3′ 5′-CGGAGTGTCAATGATATTGCA-3′	63°C, 35s	88°C	167	(Auerbach et al., 2007)
	*tet*B	*tet*B-F *tet*B-R	5′-TACGTGAATTTATTGCTTCGG-3′ 5′-ATACAGCATCCAAAGCGCAC-3′	61°C, 45s	83°C	206	(Y. Kang et al., 2018)

Supplementary Material SM2-10: Detail of the qPCR Limit of Detection (LOD)

Target gene	**Efficiency (%)**	**R^2**	**LOD (copies/µl)**	**LOQ (copies/µL)**
*Sul*1	98.8	0.996	0.66	2.21
*Sul*2	95.5	0.990	0.58	1.98
*Sul*3	100	0.971	2.91	9.70
*tet*Q	97.7	0.997	0.18	0.60
*tet*B	98.7	0.949	1.01	3.37
*bla*VIM	94.0	0.996	0.57	1.91
*bla*KPC	90.4	0.994	0.29	0.97
*mec*A	94.2	0.990	0.99	3.31
*IntI*1	90.6	0.993	0.76	2.55

Supplementary Material SM2-11: Environmental parameters (temperature, salinity, Chla and nutrient concentrations) documented between sampling stations

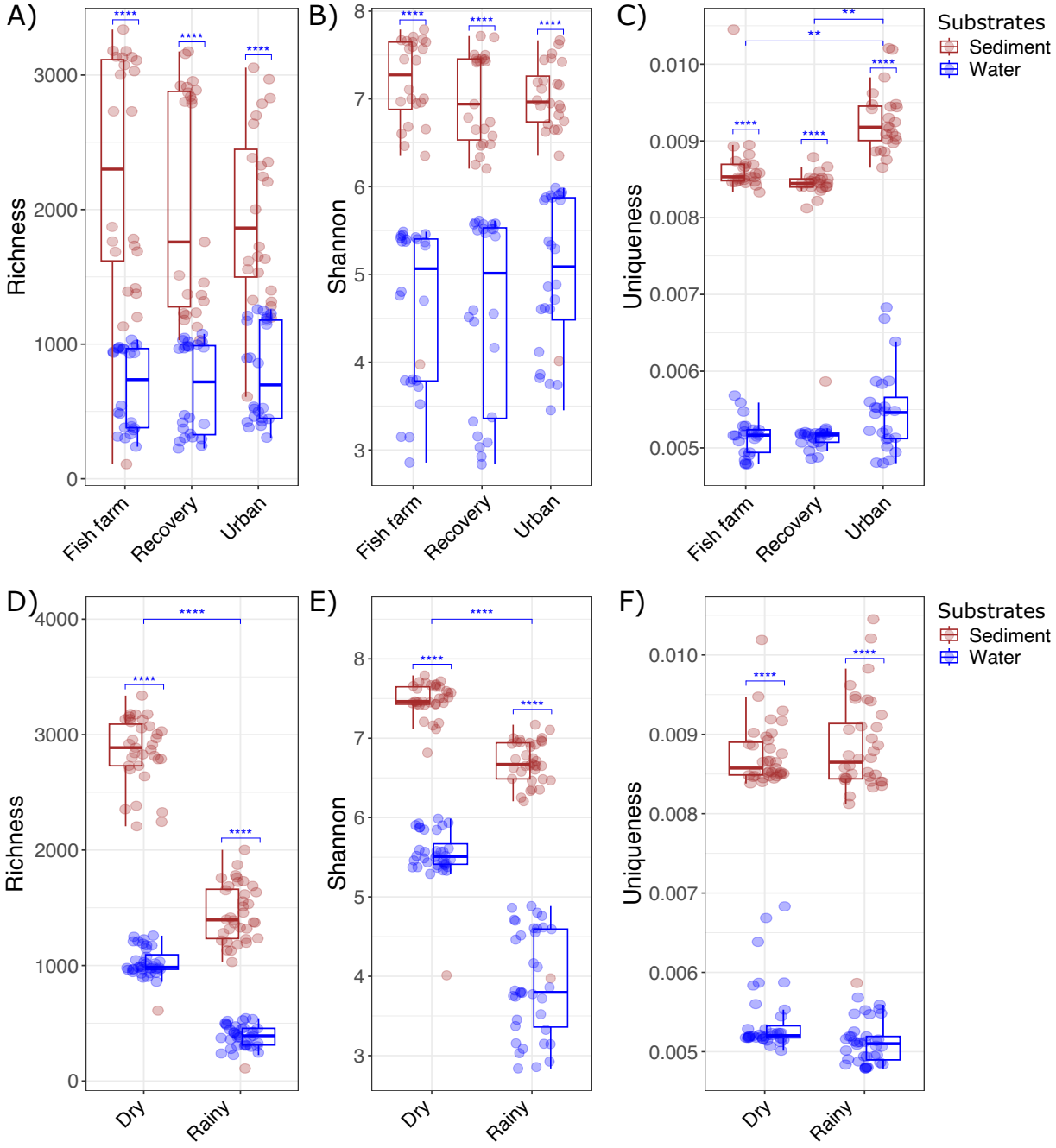
Station	Latitude	Longitude	Season	Temp (°C)	Salinity (‰)	NH_4 (µg/L)	NO_3 (µg/L)	PO_4 (µg/L)	Chl-a (µg/L)
U1	12,26242	109,20664	Dry	30,10	30,90	8,83	39,42	45,57	0,64
U2	12,26251	109,21583	Dry	29,70	32,10	7,99	46,27	42,76	0,77
U3	12,26227	109,22510	Dry	29,00	31,30	16,12	41,62	51,63	0,67
U4	12,26277	109,23428	Dry	29,00	32,90	6,75	32,56	44,95	0,32
F1	12,18342	109,22221	Dry	29,50	33,00	13,86	40,48	47,38	0,27
F2	12,16751	109,23179	Dry	29,50	33,00	11,42	39,39	18,11	0,37
F3	12,16264	109,23951	Dry	29,40	32,90	8,84	32,78	12,49	0,33
F4	12,15778	109,24724	Dry	29,00	32,50	7,39	13,71	9,04	0,34
R1	12,34267	109,29292	Dry	30,50	33,00	7,52	31,77	7,92	0,40
R2	12,33462	109,29635	Dry	30,00	32,90	6,43	11,67	9,79	0,39
R3	12,32607	109,29979	Dry	29,50	33,00	7,98	25,14	5,86	0,46
R4	12,31768	109,30339	Dry	29,70	33,00	8,82	46,08	8,01	0,43
U1	12,26242	109,20664	Rainy	27,60	30,80	15,70	31,52	9,81	0,46
U2	12,26251	109,21583	Rainy	27,90	30,00	18,25	75,42	16,38	0,70
U3	12,26227	109,22510	Rainy	27,60	30,10	13,16	61,30	14,19	0,55
U4	12,26277	109,23428	Rainy	27,40	30,70	11,25	46,79	15,64	0,49
F1	12,18342	109,22221	Rainy	27,20	29,30	70,09	83,44	17,83	0,76
F2	12,16751	109,23179	Rainy	27,30	30,10	14,43	48,32	14,91	0,70
F3	12,16264	109,23951	Rainy	27,40	30,30	24,29	42,59	15,64	0,56
F4	12,15778	109,24724	Rainy	27,00	29,60	24,29	62,44	17,10	0,55
R1	12,34267	109,29292	Rainy	27,40	31,40	9,34	23,23	61,65	0,27
R2	12,33462	109,29635	Rainy	27,30	31,40	23,02	22,54	54,35	0,11
R3	12,32607	109,29979	Rainy	27,20	31,20	21,43	38,69	10,54	0,11
R4	12,31768	109,30339	Rainy	27,10	31,10	11,25	42,13	12,73	0,25

Supplementary Material SM2-12: DADA2 script

(Due to the large size, the full script of DADA2 will be available online upon completion of the submission process.)

Supplementary Material SM2-13: Significance tests of whole bacteria and PHPB alpha diversity indices between season, substrates and MAR index of gram negative and gram positive isolates between seasons (Wilcoxon test)

Metric	group1	group2	p	p.adj	p.format	p.signif
Whole bacteria						
Observed index	Rainy	Dry	0.1124791	1.1e-07	1.1e-07	****
Observed index	Sediment	Water	4.2e-10	4.2e-22	<2e-16	****
Shannon index	Rainy	Dry	0.6759119	6.8e-07	6.8e-07	****
Shannon index	Sediment	Water	3.9e-11	3.9e-23	<2e-16	****
LCBD index	Rainy	Dry	0.1768402	0.18	0.18	ns
LCBD index	Sediment	Water	7.2e-13	7.2e-25	<2e-16	****
PHPB						
Observed index	Dry	Rainy	4.9e-06	4.9e-06	4.9e-06	****
Observed index	Sediment	Water	0.06427823	0.064	0.064	ns
Shannon index	Dry	Rainy	0.08258426	8.3e-08	8.3e-08	****
Shannon index	Sediment	Water	0.3282758	0.33	0.33	ns
LCBD index	Dry	Rainy	0.00000107	1.1e-12	1.1e-12	****
LCBD index	Sediment	Water	0.9387327	0.94	0.94	ns
Relative abundance	Dry	Rainy	0.2247338	0.22	0.22	ns
Relative abundance	Sediment	Water	0.05549429	0.055	0.055	ns
MAR index						
Gram (-) Fishfarm	Dry	Rainy	0.00088935	0.00089	0.00089	***
Gram (-) Recovery	Dry	Rainy	0.0024967	0.0025	0.0025	**
Gram (-) Urban	Dry	Rainy	0.4544935	0.45	0.45	ns
Gram (+) Fishfarm	Dry	Rainy	0.1461675	0.15	0.15	ns
Gram (+) Recovery	Dry	Rainy	1	1	1	ns
Gram (+) Urban	Dry	Rainy	0.2709632	0.27	0.27	ns



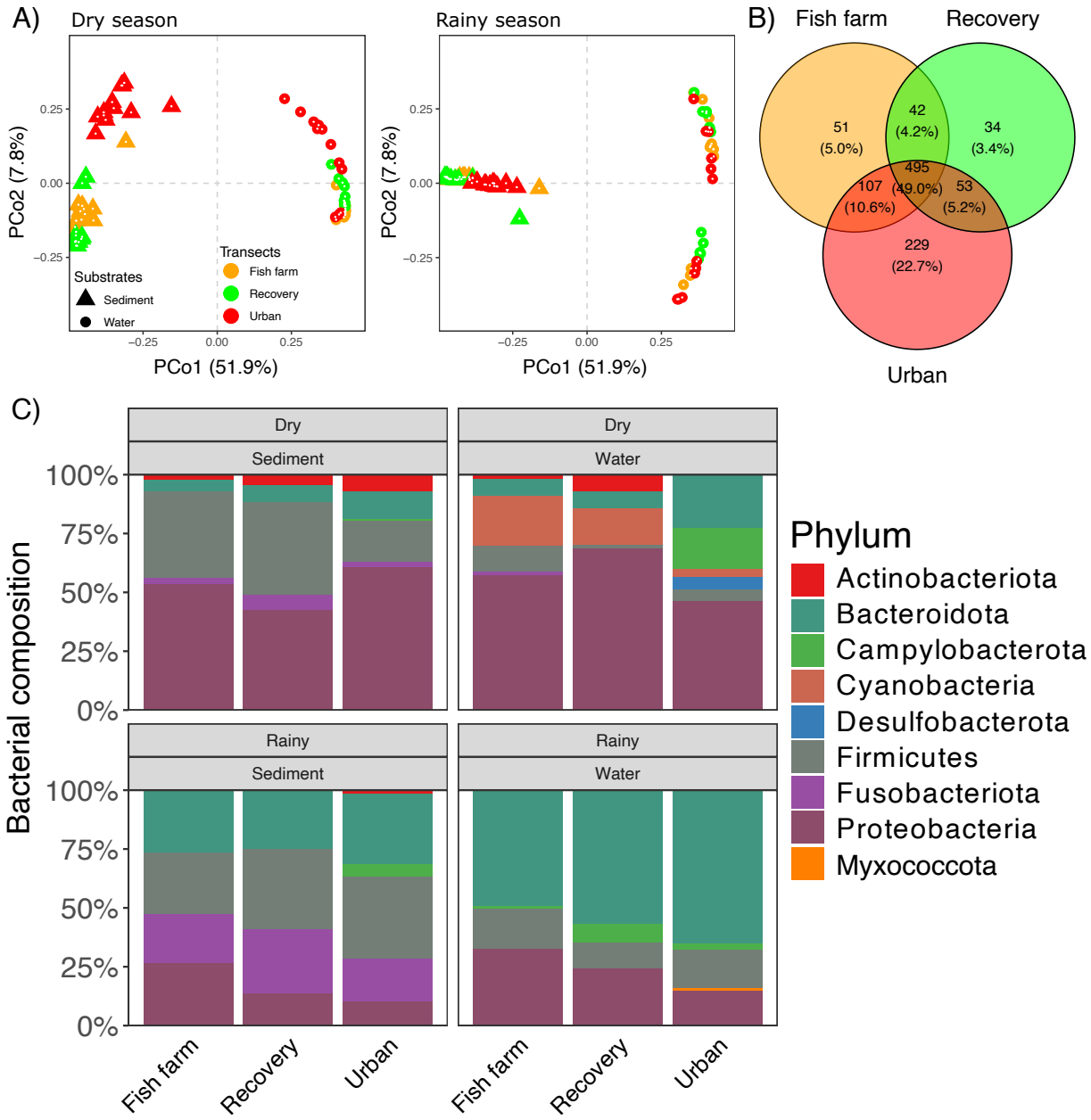
Supplementary Material SM2-14: Planktonic and benthic Alpha diversity indices of the whole bacteria communities across transects and seasons. Uniqueness of species is measured a the local contributor of beta-diversity (LCBD) index. Significant differences were observed with Dunn post-hoc test between groups (****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$).

Supplementary Material SM2-15: Significance tests of whole bacteria and PHPB for alpha diversity indices, relative abundance and total number of bacterial isolates between transects (Post hoc Dunn test)

Comparison	Z	P.unadj	P.adj
Observed index — Whole bacteria			
Fish farm – Recovery	0.3597476	0.7190359	1.0000000
Fish farm – Urban	-0.2587002	0.7958665	0.7958665
Recovery – Urban	-0.6170827	0.5371802	1.0000000
Observed index — PHPB			
Fish farm – Recovery	-0.4963361	0.6196572901	0.6196572901
Fish farm – Urban	-3.8764338	0.0001059986	0.0003179959
Recovery – Urban	-3.3596414	0.0007804369	0.0011706554
Shannon index — Whole bacteria			
Fish farm – Recovery	0.2646864	0.7912511	1.0000000
Fish farm – Urban	-0.1724661	0.8630711	0.8630711
Recovery – Urban	-0.4362424	0.6626609	1.0000000
Shannon index — PHPB			
Fish farm – Recovery	-0.1460553	0.8838777219	0.883877722
Fish farm – Urban	-3.4405034	0.0005806331	0.001741899
Recovery – Urban	-3.2762923	0.0010517965	0.001577695
LCBD index — Whole bacteria			
Fish farm – Recovery	1.055252	0.2913101368	0.291310137
Fish farm – Urban	-2.308582	0.0209667796	0.031450169
Recovery – Urban	-3.351651	0.0008033108	0.002409932
LCBD index — PHPB			
Fish farm – Recovery	0.8043368	0.4212025	0.6318037
Fish farm – Urban	1.0689118	0.2851094	0.8553282
Recovery – Urban	0.2589343	0.7956859	0.7956859
Total number of isolates — Transect			
Fish farm – Recovery	0.4738407	0.635613530	0.635613530
Fish farm – Urban	-3.2303247	0.001236497	0.001854745
Recovery – Urban	-3.2903051	0.001000788	0.003002364
Post hoc Dunn test — PHPB relative abundance (Transect)			
Fish farm – Recovery	-1.109659	2.671460e-01	2.671460e-01
Fish farm – Urban	-4.702660	2.567936e-06	7.703809e-06
Recovery – Urban	-3.568185	3.594629e-04	5.391944e-04

Supplementary Material SM2-16: Significance tests of whole bacteria and PHPB composition between transects, season, substrates and environmental parameters, and phenotypic resistance of bacterial isolates between transects (Permanova test)

Factor	R^2	F	$Pr(>F)$	Significance
Whole bacteria				
Transect	0.03378	23.602	0.000999	***
Substrate	0.05372	77.207	0.000999	***
Season	0.15185	24.349	0.000999	***
PHPB				
Transect	0.03378	23.602	0.000999	***
Substrate	0.05372	77.207	0.000999	***
Season	0.15185	24.349	0.000999	***
PHPB in dry season				
Transect	0.10114	38.255	0.000999	***
Substrate	0.13450	10.723	0.000999	***
PHPB in dry rainy				
Transect	0.05851	19.886	0.02897	*
Substrate	0.14297	10.843	0.000999	***
Gram(-)resistance				
Transect	0.09174	2121	0.06793	ns
Season	0.41321	30.28	0.000999	***
Gram(+)resistance				
Transect	0.11593	29.505	0.02897	*
Season	0.13124	69.489	0.001998	**
Environment variables				
Temperature	0.10581	15.619	0.000999	***
Salinity	0.06833	96.815	0.000999	***
NH_4	0.02708	36.735	0.000999	***
NO_3	0.06326	89.149	0.000999	***
PO_4	0.05051	70.216	0.000999	***
Chl-a	0.02071	2.791	0.000999	***



Supplementary Material SM2-17: A: PCoA plot with Bray-Curtis dissimilarities calculated for the whole bacteria communities. Transect are represented by colors and substrate type with different shapes. between transects and substrates in dry and rainy season. B: Venn diagram of unique and shared bacterial genera between each group. Percentage indicates the ratio of each group genera to the whole dataset genera. C: Bar plots representing the composition of the phylum in each seasons and substrates in separation by transects.

Supplementary Material SM2-18: Taxonomic detail of PHPB genera and species

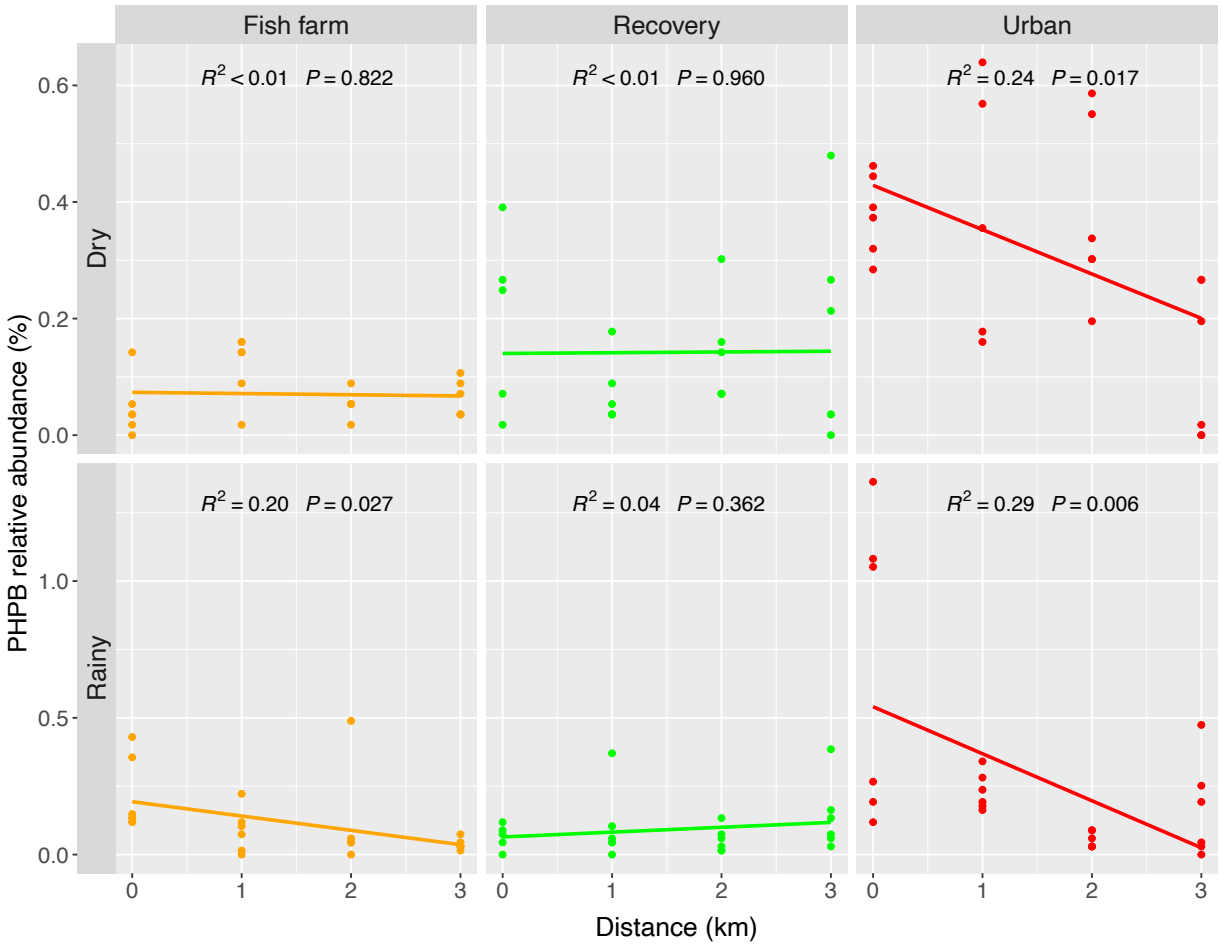
Phylum	Class	Order	Family	Genus	Species
Bacillota	*Clostridia*	*Peptostreptococcales-Tissierellales*	*Anaerovoracaceae*	*[Eubacterium]*	*sulci*
Bacillota	*Clostridia*	*Peptostreptococcales-Tissierellales*	*Peptostreptococcaceae*	*Acetoanaerobium*	*sticklandii*
Pseudomonadota	*Gammaproteobacteria*	*Burkholderiales*	*Alcaligenaceae*	*Achromobacter*	*xylosoxidans*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*baumannii*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*bouvetii*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*haemolyticus*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*johnsonii*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*junii*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*lwoffii*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*parvus*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*soli*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*towneri*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*venetianus*
Bacillota	*Bacilli*	*Lactobacillales*	*Aerococcaceae*	*Aerococcus*	*urinaeequi*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Aeromonadaceae*	*Aeromonas*	*caviae*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Aeromonadaceae*	*Aeromonas*	*salmonicida*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Aeromonadaceae*	*Aeromonas*	*veronii*
Pseudomonadota	*Alphaproteobacteria*	*Hyphomicrobiales*	*Xanthobacteraceae*	*Afipia*	*broomeae*
Pseudomonadota	*Alphaproteobacteria*	*Hyphomicrobiales*	*Rhizobiaceae*	*Agrobacterium*	*pusense*
Campylobacterota	*Campylobacteria*	*Campylobacterales*	*Arcobacteraceae*	*Arcobacter*	*butzleri*
Campylobacterota	*Campylobacteria*	*Campylobacterales*	*Arcobacteraceae*	*Arcobacter*	*cryaerophilus*
Bacillota	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	*anthracis*
Bacillota	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	*aquimaris*
Bacillota	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	*cereus*
Bacillota	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	*megaterium*
Bacillota	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	*mycoides*
Bacillota	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	*pumilus*
Bacillota	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	*subtilis*
Bacillota	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Blautia*	*wexlerae*
Pseudomonadota	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	*Brevundimonas*	*diminuta*
Bacillota	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	*acetobutylicum*
Bacillota	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	*beijerinckii*
Bacillota	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	*scatologenes*
Pseudomonadota	*Gammaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*	*testosteroni*
Actinomycetota	*Actinobacteria*	*Mycobacteriales*	*Corynebacteriaceae*	*Corynebacterium*	*accolens*
Actinomycetota	*Actinobacteria*	*Mycobacteriales*	*Corynebacteriaceae*	*Corynebacterium*	*aurimucosum*
Actinomycetota	*Actinobacteria*	*Mycobacteriales*	*Corynebacteriaceae*	*Corynebacterium*	*lipophiloflavum*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	*Cronobacter*	*dublinensis*
Actinomycetota	*Actinobacteria*	*Propionibacteriales*	*Propionibacteriaceae*	*Cutibacterium*	*avidum*
Pseudomonadota	*Gammaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Delftia*	*acidovorans*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	*Enterobacter*	*cloacae*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	*Escherichia*	*coli*
Bacillota	*Clostridia*	*Oscillospirales*	*Ruminococcaceae*	*Faecalibacterium*	*prausnitzii*
Actinomycetota	*Actinobacteria*	*Mycobacteriales*	*Nocardiaceae*	*Gordonia*	*terrae*

Supplementary Material SM2-18 (continued).

Phylum	Class	Order	Family	Genus	Species
Actinomycetota	*Actinobacteria*	*Kitasatosporales*	*Streptomycetaceae*	*Kitasatospora*	*psammotica*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	*Klebsiella*	*quasipneumoniae*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	*Klebsiella*	*variicola*
Bacillota	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	*johnsonii*
Bacillota	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	*reuteri*
Bacillota	*Bacilli*	*Lactobacillales*	*Listeriaceae*	*Listeria*	*monocytogenes*
Bacillota	*Bacilli*	*Bacillales*	*Planococcaceae*	*Lysinibacillus*	*sphaericus*
Bacillota	*Bacilli*	*Staphylococcales*	*Staphylococcaceae*	*Macrococcus*	*caseolyticus*
Pseudomonadota	*Alphaproteobacteria*	*Hyphomicrobiales*	*Beijerinckiaceae*	*Methylobacterium*	*radiotolerans*
Actinomycetota	*Actinobacteria*	*Micrococcales*	*Micrococcaceae*	*Micrococcus*	*luteus*
Actinomycetota	*Actinobacteria*	*Mycobacteriales*	*Mycobacteriaceae*	*Mycobacterium*	*bacteremicum*
Actinomycetota	*Actinobacteria*	*Mycobacteriales*	*Mycobacteriaceae*	*Mycobacterium*	*branderi*
Actinomycetota	*Actinobacteria*	*Mycobacteriales*	*Mycobacteriaceae*	*Mycobacterium*	*chubuense*
Actinomycetota	*Actinobacteria*	*Mycobacteriales*	*Mycobacteriaceae*	*Mycobacterium*	*malmoense*
Actinomycetota	*Actinobacteria*	*Mycobacteriales*	*Mycobacteriaceae*	*Mycobacterium*	*rhodesiae*
Pseudomonadota	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Novosphingobium*	*aromaticivorans*
Pseudomonadota	*Alphaproteobacteria*	*Hyphomicrobiales*	*Rhizobiaceae*	*Ochrobactrum*	*pituitosum*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Erwiniaceae*	*Pantoea*	*agglomerans*
Bacillota	*Clostridia*	*Peptostreptococcales-Tissierellales*	*Peptostreptococcaceae*	*Paraclostridium*	*bifermentans*
Pseudomonadota	*Alphaproteobacteria*	*Rhodobacterales*	*Paracoccaceae*	*Paracoccus*	*aminovorans*
Pseudomonadota	*Alphaproteobacteria*	*Rhodobacterales*	*Paracoccaceae*	*Paracoccus*	*marinus*
Pseudomonadota	*Alphaproteobacteria*	*Rhodobacterales*	*Paracoccaceae*	*Paracoccus*	*yeei*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Vibrionaceae*	*Photobacterium*	*damselae*
Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	*copri*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Morganellaceae*	*Providencia*	*alcalifaciens*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Morganellaceae*	*Providencia*	*rettgeri*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*anguilliseptica*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*brenneri*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*fragi*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*lundensis*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*mandelii*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*putida*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*stutzeri*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*toyotomiensis*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Psychrobacter*	*cibarius*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Psychrobacter*	*cryohalolentis*
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Psychrobacter*	*nivimaris*
Actinomycetota	*Actinobacteria*	*Micrococcales*	*Micrococcaceae*	*Rothia*	*dentocariosa*
Actinomycetota	*Actinobacteria*	*Micrococcales*	*Sanguibacteraceae*	*Sanguibacter*	*keddieii*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Shewanellaceae*	*Shewanella*	*baltica*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Shewanellaceae*	*Shewanella*	*bicestrii*
Bacillota	*Bacilli*	*Staphylococcales*	*Staphylococcaceae*	*Staphylococcus*	*epidermidis*
Bacillota	*Bacilli*	*Staphylococcales*	*Staphylococcaceae*	*Staphylococcus*	*succinus*
Bacillota	*Bacilli*	*Staphylococcales*	*Staphylococcaceae*	*Staphylococcus*	*warneri*

Supplementary Material SM2-18 (continued).

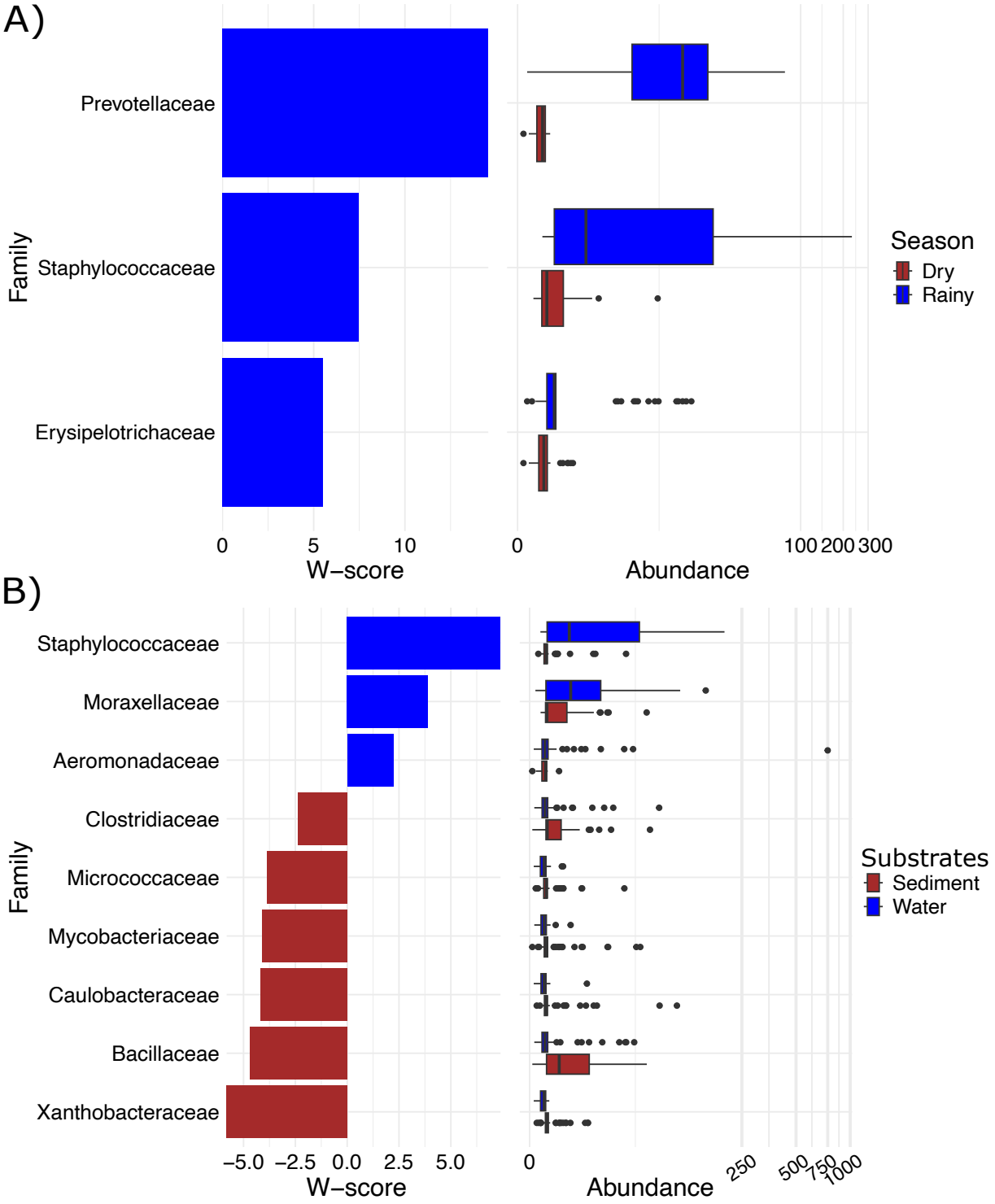
Phylum	Class	Order	Family	Genus	Species
Pseudomonadota	*Gammaproteobacteria*	*Lysobacterales*	*Lysobacteraceae*	*Stenotrophomonas*	*maltophilia*
Bacillota	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Streptococcus*	*equinus*
Bacillota	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Streptococcus*	*pneumoniae*
Bacillota	*Bacilli*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Turicibacter*	*sanguinis*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Vibrionaceae*	*Vibrio*	*alginolyticus*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Vibrionaceae*	*Vibrio*	*campbellii*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Vibrionaceae*	*Vibrio*	*furnissii*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Vibrionaceae*	*Vibrio*	*harveyi*
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Vibrionaceae*	*Vibrio*	*parahaemolyticus*



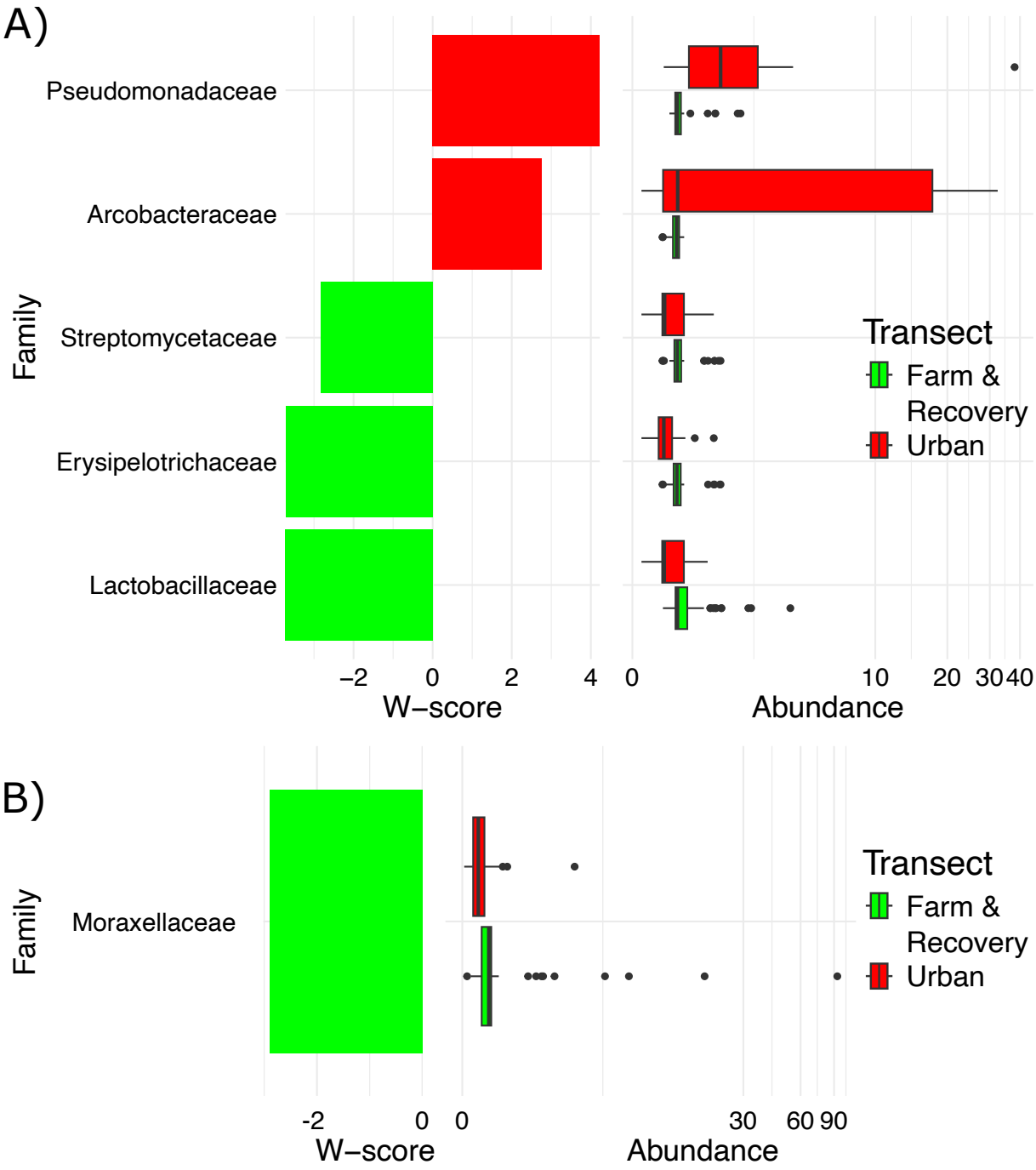
Supplementary Material SM2-19: Relative abundance of PHPB to whole bacterial community between transects and season according to distance to shore (km).

Supplementary Material SM2-20: Detail of core potential human pathogenic bacterial ASVs

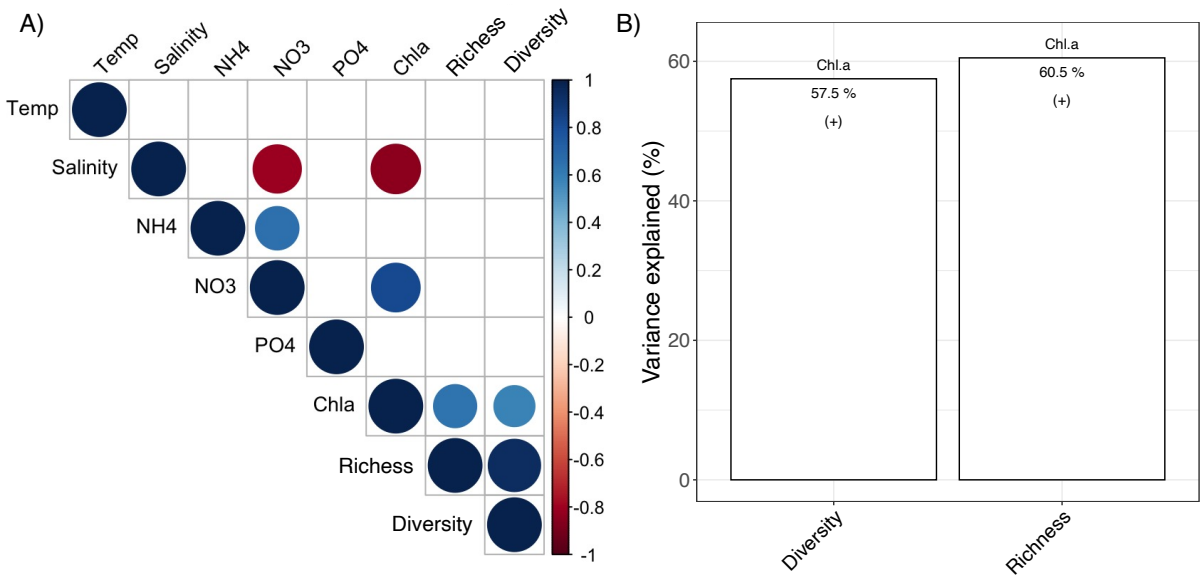
Phylum	Class	Order	Family	Genus	Prevalence (%)	Relative abundance (%)	Potential diseases
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Vibrionaceae*	*Vibrio*	47.4	8.2	Cholera, necrotizing infections, gastroenteritis, septicemia
Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	37.8	8.6	Periodontitis, bacterial vaginosis, rheumatoid arthritis
Bacillota	*Bacilli*	*Staphylococcales*	*Staphylococcaceae*	*Staphylococcus*	29.6	11.9	Skin infections, bacteremia, endocarditis, pneumonia, osteomyelitis
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	26.7	8.2	Bloodstream, urinary tract, and wound infections
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	17.0	5.2	Otitis externa and keratitis, infections
Campylobacterota	*Campylobacteria*	*Campylobacterales*	*Arcobacteraceae*	*Arcobacter*	8.9	6.1	Acute gastroenteritis, bacteremia
Pseudomonadota	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Psychrobacter*	8.1	2.7	Rare infections
Pseudomonadota	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	*Brevundimonas*	8.1	3.4	Bacteremia, endocarditis, septic arthritis, abscesses
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Aeromonadaceae*	*Aeromonas*	7.4	21.6	Gastroenteritis, infections, septicemia
Actinomycetota	*Actinobacteria*	*Mycobacteriales*	*Corynebacteriaceae*	*Corynebacterium*	6.7	1.1	Diphtheria, endocarditis
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	*Enterobacter*	6.7	1.1	Nosocomial, respiratory infections
Actinomycetota	*Actinobacteria*	*Micrococcales*	*Micrococcaceae*	*Rothia*	5.2	1.3	Endocarditis, bacteremia
Pseudomonadota	*Gammaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*	5.2	0.6	Opportunistic infections
Actinomycetota	*Actinobacteria*	*Mycobacteriales*	*Nocardiaceae*	*Gordonia*	4.4	1.0	Opportunistic infections
Actinomycetota	*Actinobacteria*	*Propionibacteriales*	*Propionibacteriaceae*	*Cutibacterium*	3.7	0.7	Acne vulgaris, infections
Pseudomonadota	*Alphaproteobacteria*	*Hyphomicrobiales*	*Rhizobiaceae*	*Ochrobactrum*	2.9	0.5	Bacteremia; peritonitis, endophthalmitis
Pseudomonadota	*Gammaproteobacteria*	*Enterobacterales*	*Shewanellaceae*	*Shewanella*	2.9	1.1	Bacteremia, infections
Pseudomonadota	*Gammaproteobacteria*	*Lysobacterales*	*Lysobacteraceae*	*Stenotrophomonas*	2.9	0.6	Bacteremia, respiratory infections



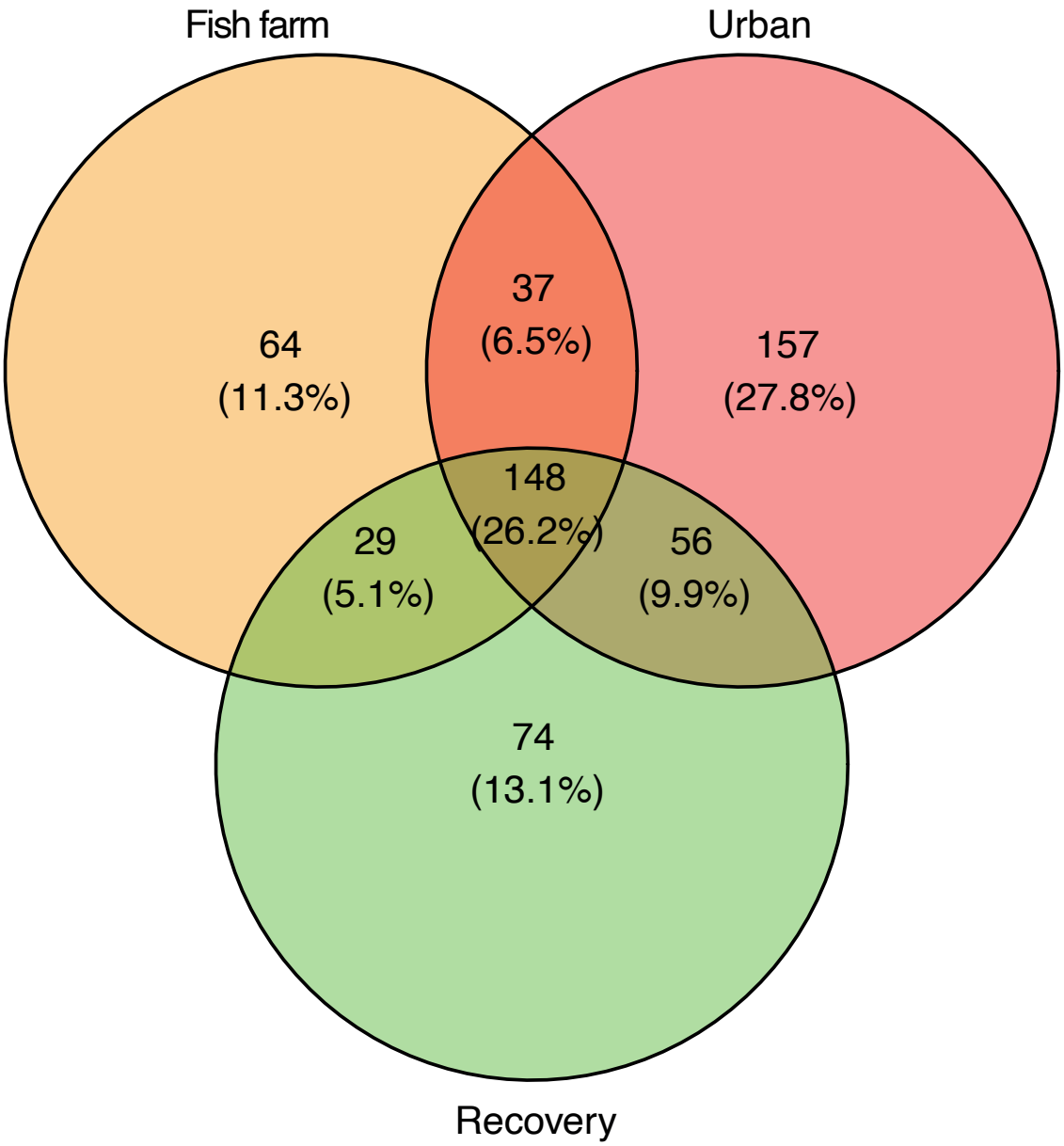
Supplementary Material SM2-21: ANCON-BC test for PHPB biomarkers (families) between season (A) and substrates (B).



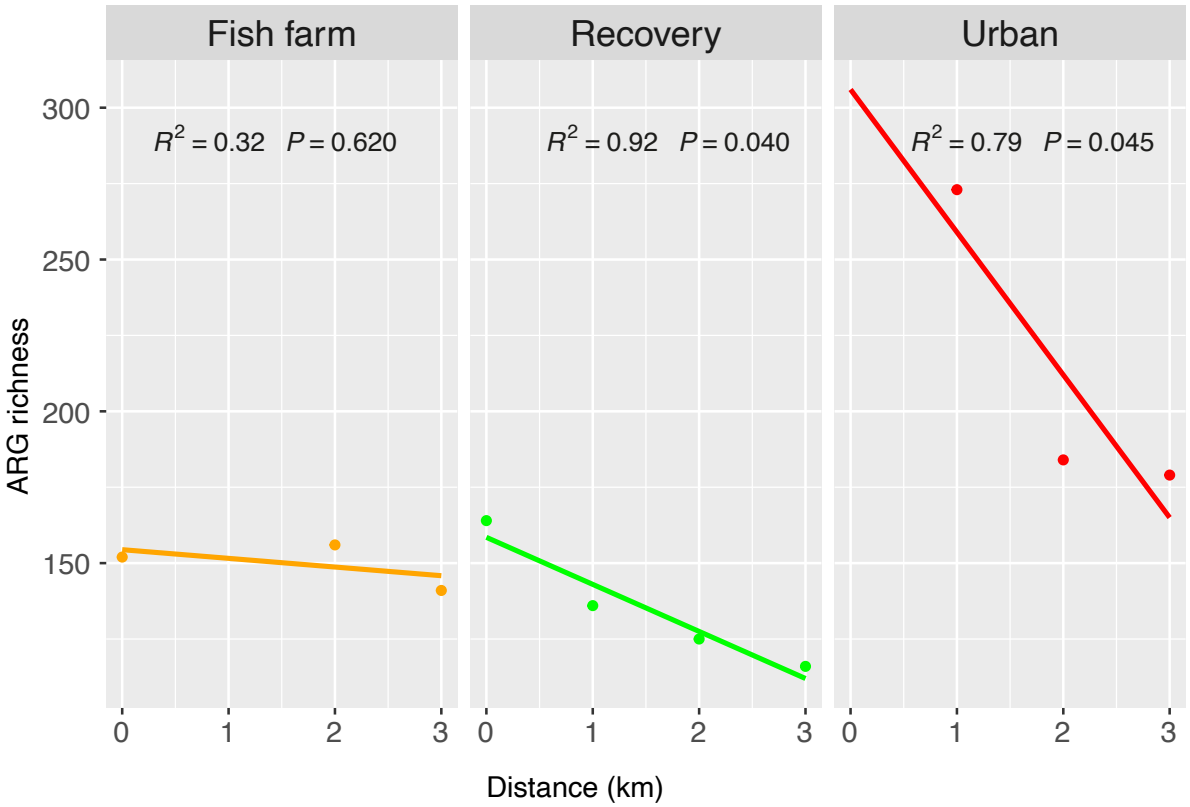
Supplementary Material SM2-22: ANCON-BC test for PHPB biomarkers (families) between transects in dry season (A) and rainy season (B).



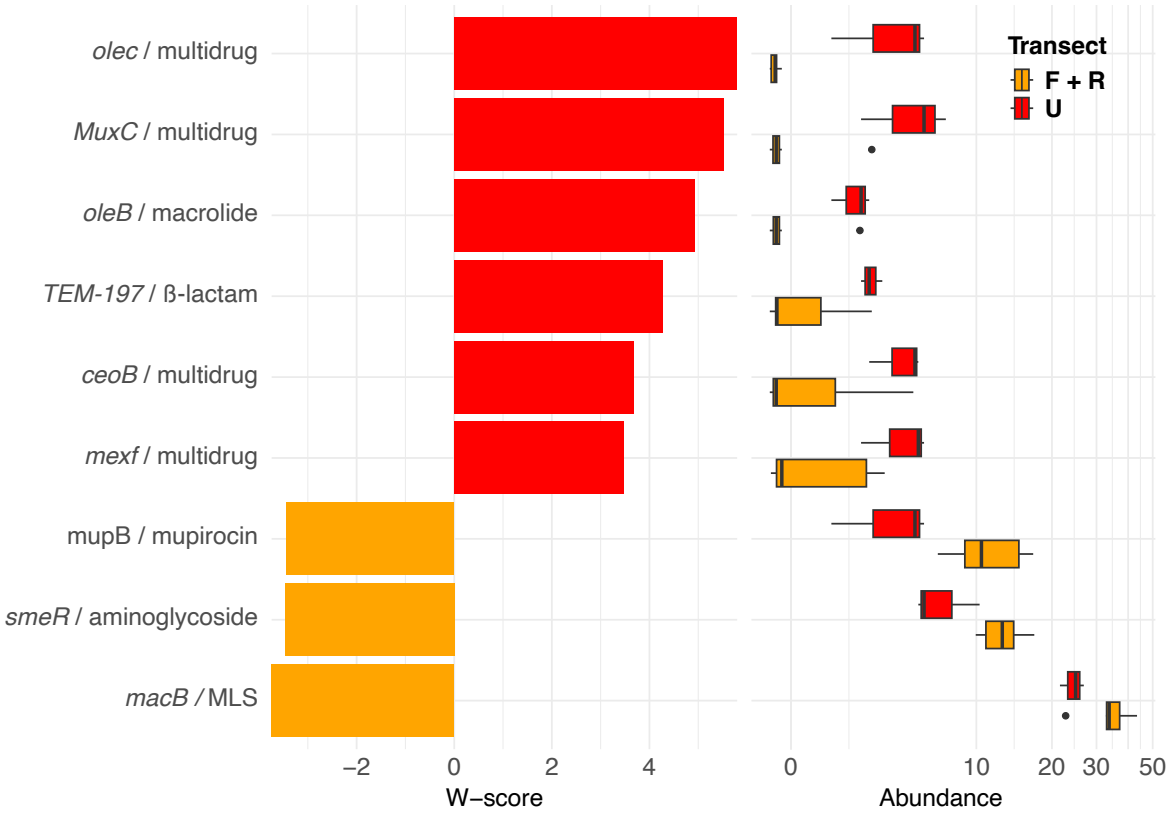
Supplementary Material SM2-23: Correlation (A) and multiple regression analysis (B) between PHPB richness, diversity and the environmental parameters (Positive relation: '+'; Negative relation: '-'). Only significant correlations (p-value < 0.05) are shown in the figure.



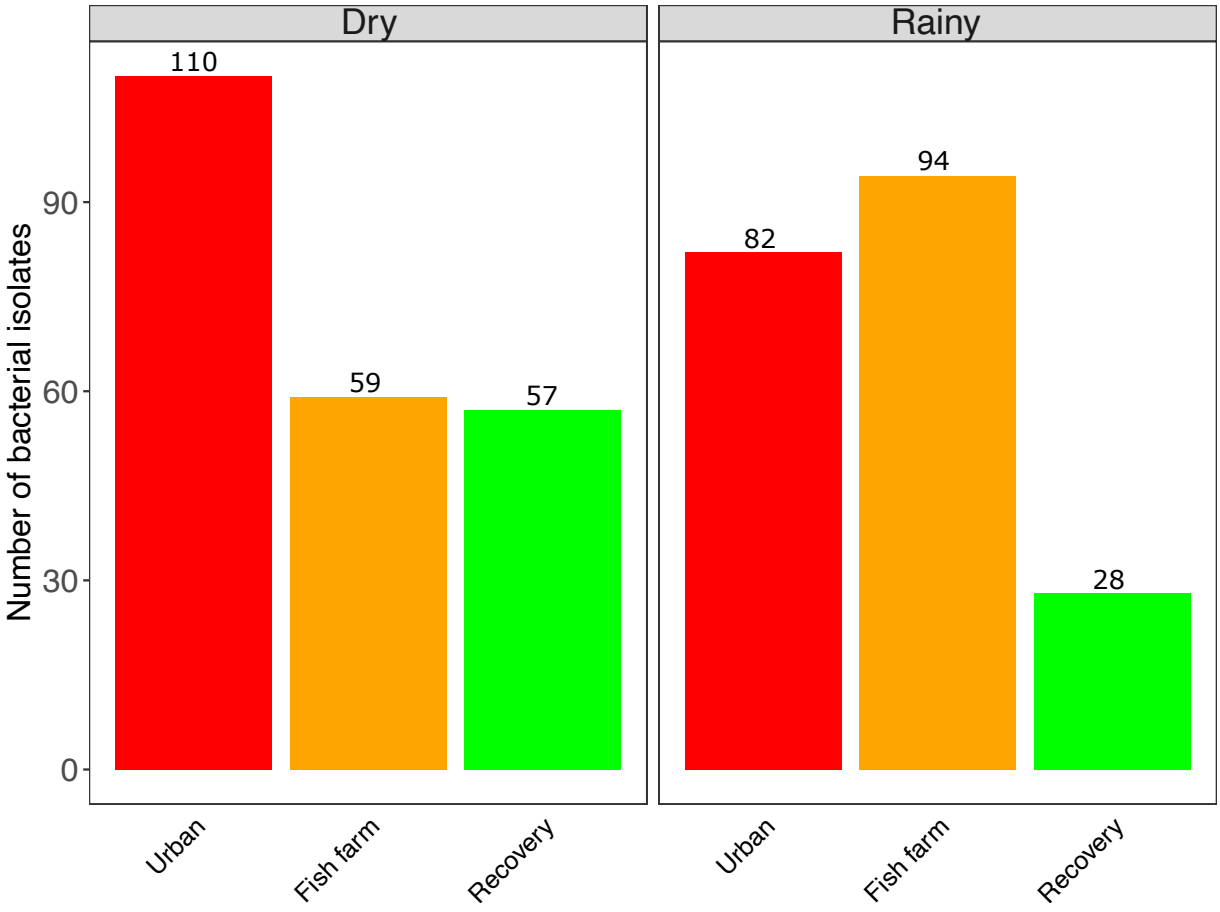
Supplementary Material SM2-24: Venn diagram of ARGs between transects



Supplementary Material SM2-25: ARGs richness between transects according to distance to shore (km).



Supplementary Material SM2-26: ANCON-BC test for ARGs biomarkers (ARGs and ARG class) between Urban transect versus Fish farm and Recovery transects.



Supplementary Material SM2-27: Total number of bacterial isolates between transects in each season.

Supplementary Material SM2-28: Detail of the identified bacterial isolates

Isolate_ID	Taxonomy	Substrate	Transect	Station	Season	Gram
STA F4S1.1	*Acinetobacter baumannii*	Sediment	Farm	1	Dry	-
SS F3W1.2	*Acinetobacter calcoaceticus*	Water	Farm	3	Dry	-
SS F4S1.1	*Acinetobacter calcoaceticus*	Sediment	Farm	4	Dry	-
STA F1S1.2	*Bacillus amyloliquefaciens*	Sediment	Farm	1	Dry	+
MAC F3W1.1	*Bacillus amyloliquefaciens*	Water	Farm	3	Dry	+
MA F3S1.1	*Bacillus amyloliquefaciens*	Sediment	Farm	1	Dry	+
MA F1W1.1	*Bacillus amyloliquefaciens*	Water	Farm	1	Dry	+
MAC F1W1.2	*Bacillus amyloliquefaciens*	Water	Farm	1	Dry	+
AERO F4S1.1.1	*Bacillus thuringiensis*	Sediment	Farm	4	Dry	+
SS F4W1.1	*Bacillus thuringiensis*	Water	Farm	4	Dry	+
SS F4S1.3	*Bacillus Toyonensis*	Sediment	Farm	4	Dry	+
SS F3W1.3	*Citrobacter freundii*	Water	Farm	3	Dry	-
SS F3W1.4	*Enterobacter cloacae*	Water	Farm	3	Dry	-
MAC F1W1.1	*Escherichia coli*	Water	Farm	1	Dry	-
AERO F3S1.3	*Klebsiella pneumoniae*	Sediment	Farm	3	Dry	-
AERO F2S1.2.1	*Klebsiella pneumoniae*	Sediment	Farm	2	Dry	-
SS F1W1.1	*Pseudomonas stutzeri*	Water	Farm	1	Dry	-
MAC F4S1.2	*Pseudomonas stutzeri*	Sediment	Farm	4	Dry	-
MAC F3W1.2	*Weissella paramesenteroides*	Water	Farm	3	Dry	+
F3.S.Mac2	*Acinetobacter haemolyticus*	Sediment	Farm	3	Rainy	-
F3.W.Ma2	*Bacillus horikoshii*	Water	Farm	3	Rainy	+
F4.S.Ma3	*Bacillus vietnamensis*	Sediment	Farm	4	Rainy	+
F3.S.Mac4	*Enterobacter cloacae*	Sediment	Farm	3	Rainy	-
F3.W.Mac3	*Escherichia coli*	Water	Farm	3	Rainy	-
F1.S.Mac1	*Escherichia coli*	Sediment	Farm	1	Rainy	-
F1.W.Ae2	*Klebsiella pneumoniae*	Water	Farm	1	Rainy	-
F2.W.Mac2	*Klebsiella pneumoniae*	Water	Farm	2	Rainy	-
F3.S.Mac3	*Klebsiella pneumoniae*	Sediment	Farm	3	Rainy	-

Supplementary Material SM2-28 (continued).

Isolate_ID	Taxonomy	Substrate	Transect	Station	Season	Gram
F1.W.Mac2	*Pseudomonas aeruginosa*	Water	Farm	1	Rainy	-
F3.W.Mac1	*Pseudomonas aeruginosa*	Water	Farm	3	Rainy	-
F4.W.Ae2	*Pseudomonas aeruginosa*	Water	Farm	4	Rainy	-
F4.W.Mac2	*Pseudomonas aeruginosa*	Water	Farm	4	Rainy	-
F4.S.Mac1	*Pseudomonas mendocina*	Sediment	Farm	4	Rainy	-
F2.W.Ae2	*Shewanella putrefaciens*	Water	Farm	2	Rainy	-
MA P1W1.1	*Bacillus amyloliquefaciens*	Water	Recovery	1	Dry	+
MA P2S1.1	*Bacillus amyloliquefaciens*	Sediment	Recovery	1	Dry	+
SS P4W1.1	*Bacillus velezensis*	Water	Recovery	4	Dry	+
AERO P2W1.1	*Citrobacter freundii*	Water	Recovery	2	Dry	-
SS P3W1.1	*Klebsiella pneumoniae*	Water	Recovery	3	Dry	-
TCBS P2S1.3.2	*Micrococcus luteus*	Sediment	Recovery	2	Dry	+
TCBS P3S1.3	*Staphylococcus haemolyticus*	Sediment	Recovery	3	Dry	+
MAC P1W1.3	*Stenotrophomonas maltophilia*	Water	Recovery	1	Dry	-
SS P3S1.1	*Vibrio alginolyticus*	Sediment	Recovery	3	Dry	-
SS P3W1.2	*Vibrio alginolyticus*	Water	Recovery	3	Dry	-
SS P2S1.1	*Vibrio parahaemolyticus*	Sediment	Recovery	2	Dry	-
R3.W.Mac1	*Acinetobacter baumannii*	Water	Recovery	3	Rainy	-
R1.W.Mac2	*Acinetobacter nosocomialis*	Water	Recovery	1	Rainy	-
R1.S.Mac2	*Acinetobacter pittii*	Sediment	Recovery	1	Rainy	-
R2.S.Mac1	*Acinetobacter pittii*	Sediment	Recovery	2	Rainy	-
R3.S.Mac4	*Acinetobacter pittii*	Sediment	Recovery	3	Rainy	-
R1.S.Ma1	*Bacillus alcalophilus*	Sediment	Recovery	1	Rainy	+
R2.S.Ma3	*Bacillus altitudinis*	Sediment	Recovery	2	Rainy	+
R2.S.Ae3	*Bacillus cereus*	Sediment	Recovery	2	Rainy	+
R3.S.Ma5	*Bacillus marisflavi*	Sediment	Recovery	3	Rainy	+
R1.S.Ma4	*Bacillus megaterium*	Sediment	Recovery	1	Rainy	+
R2.W.Ae3.1	*Citrobacter freundii*	Water	Recovery	2	Rainy	-

Supplementary Material SM2-28 (continued).

Isolate_ID	Taxonomy	Substrate	Transect Station	SeasonGram
R3.W.Ma2	*Clostridium baratii*	Water	Recovery3	Rainy +
R2.W.Ma1	*Exiguobacterium* sp[3]	Water	Recovery2	Rainy +
R2.W.Ma3	*Exiguobacterium* sp[3]	Water	Recovery2	Rainy +
R3.W.Mac3	*Klebsiella pneumoniae*	Water	Recovery3	Rainy -
R2.W.Mac3.1	*Klebsiella variicola*	Water	Recovery2	Rainy -
R2.W.Mac1.1	*Kluyvera ascorbata*	Water	Recovery2	Rainy -
R3.S.Ma2	*Kocuria marina*	Sediment	Recovery3	Rainy +
R1.W.Ae1.1	*Micrococcus flavus*	Water	Recovery1	Rainy +
R1.S.Ma3	*Micrococcus luteus*	Sediment	Recovery1	Rainy +
R1.W.Mac1	*Pseudomonas aeruginosa*	Water	Recovery1	Rainy -
R2.W.Mac2	*Pseudomonas aeruginosa*	Water	Recovery2	Rainy -
R3.W.Mac2	*Pseudomonas aeruginosa*	Water	Recovery3	Rainy -
R4.W.Ae1	*Pseudomonas aeruginosa*	Water	Recovery4	Rainy -
R1.S.Mac1	*Pseudomonas aeruginosa*	Sediment	Recovery1	Rainy -
R2.W.Mac1.2	*Pseudomonas nitroreducens*	Water	Recovery2	Rainy -
SS U3S1.1	*Acinetobacter calcoaceticus*	Sediment	Urban 3	Dry -
MAC U2S1.4	*Bacillus amyloliquefaciens*	Sediment	Urban 2	Dry +
MAC U3S1.2	*Bacillus amyloliquefaciens*	Sediment	Urban 3	Dry +
MAC U1S1.1.2	*Bacillus amyloliquefaciens*	Sediment	Urban 1	Dry +
MA U1S1.4	*Bacillus amyloliquefaciens*	Sediment	Urban 1	Dry +
MA U3W1.1	*Bacillus amyloliquefaciens*	Water	Urban 3	Dry +
MA U1S1.6	*Bacillus amyloliquefaciens*	Sediment	Urban 1	Dry +
MA U3W1.2	*Bacillus amyloliquefaciens*	Water	Urban 3	Dry +
MA U2W1.4	*Bacillus amyloliquefaciens*	Water	Urban 2	Dry +
MA U1S1.2.3	*Bacillus atrophaeus*	Sediment	Urban 1	Dry +
MA U2S1.4.2	*Bacillus cereus*	Sediment	Urban 2	Dry +
MA U2S1.5	*Bacillus marisflavi*	Sediment	Urban 2	Dry +
MA U2W1.3	*Bacillus marisflavi*	Water	Urban 2	Dry +

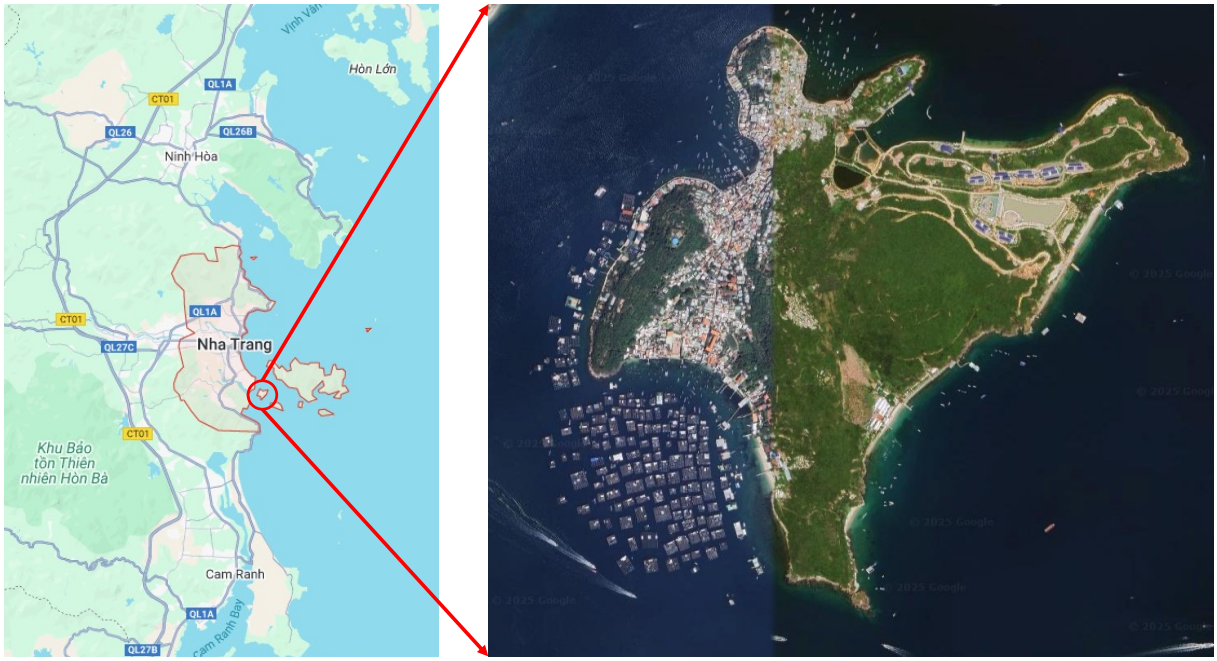
Supplementary Material SM2-28 (continued).

Isolate_ID	Taxonomy	Substrate	Transect	Station	Season	Gram
MA U2S1.2	*Bacillus pumilus*	Sediment	Urban	2	Dry	+
MA U1W1.3	*Bacillus subtilis*	Water	Urban	1	Dry	+
STA U3S1.5	*Bacillus velezensis*	Sediment	Urban	3	Dry	+
STA U1S1.3	*Bacillus velezensis*	Sediment	Urban	1	Dry	+
MA U3S1.1	*Bacillus velezensis*	Sediment	Urban	3	Dry	+
MA U2W1.1	*Bacillus velezensis*	Water	Urban	2	Dry	+
MA U2S1.1	*Bacillus vietnamensis*	Sediment	Urban	2	Dry	+
MAC U3W1.1	*Citrobacter brakkii*	Water	Urban	3	Dry	-
SS U4S1.1	*Enterococcus casseliflavus*	Sediment	Urban	4	Dry	-
MA U1S1.2.2	*Escherichia coli*	Sediment	Urban	1	Dry	-
MA U2S1.3	*Staphylococcus hominis*	Sediment	Urban	2	Dry	+
MA U4S1.1	*Staphylococcus warneri*	Sediment	Urban	4	Dry	+
SS U3S1.2	*Stenotrophomonas maltophilia*	Sediment	Urban	3	Dry	-
AERO U2S1.1	*Streptococcus pneumoniae*	Sediment	Urban	2	Dry	+
U2.W.Ae2.1	*Aeromonas caviae*	Water	Urban	2	Rainy	-
U3.S.Mac4	*Aeromonas caviae*	Sediment	Urban	3	Rainy	-
U1.S.Ae1	*Aeromonas hydrophila*	Sediment	Urban	1	Rainy	-
U4.W.Ae2	*Aeromonas hydrophila*	Water	Urban	4	Rainy	-
U4.S.Ae2	*Aeromonas hydrophila*	Sediment	Urban	4	Rainy	-
U1.S.Ma1	*Bacillus amyloliquefaciens*	Sediment	Urban	1	Rainy	+
U3.S.Ma3	*Bacillus cereus*	Sediment	Urban	3	Rainy	+
U1.S.Ma2	*Bacillus firmus*	Sediment	Urban	1	Rainy	+
U2.W.Ma1	*Bacillus halosaccharovorans*	Water	Urban	2	Rainy	+
U1.S.Ma4.2	*Bacillus horikoshii*	Sediment	Urban	1	Rainy	+
U1.S.Ma6	*Bacillus horikoshii*	Sediment	Urban	1	Rainy	+
U3.S.Ae2	*Bacillus horikoshii*	Sediment	Urban	3	Rainy	+
U3.S.Ma2	*Bacillus licheniformis*	Sediment	Urban	3	Rainy	+
U2.S.Ma5	*Bacillus megaterium*	Sediment	Urban	2	Rainy	+

Supplementary Material SM2-28 (continued).

Isolate_ID	Taxonomy	Substrate	Transect	Station	SeasonGram
U1.S.Ae2	*Citrobacter freundii*	Sediment	Urban	1	Rainy -
U2.S.Ae3.3	*Citrobacter freundii*	Sediment	Urban	2	Rainy -
U3.S.Ae3	*Citrobacter freundii*	Sediment	Urban	3	Rainy -
U2.W.Mac1	*Enterobacter asburiae*	Water	Urban	2	Rainy -
U1.W.Ma2	*Enterobacter bugandensis*	Water	Urban	1	Rainy -
U2.S.Ae1	*Enterobacter bugandensis*	Sediment	Urban	2	Rainy -
U1.W.Mac2	*Escherichia coli*	Water	Urban	1	Rainy -
U2.W.Ma3	*Escherichia coli*	Water	Urban	2	Rainy -
U1.S.Mac2	*Escherichia coli*	Sediment	Urban	1	Rainy -
U2.S.Mac2	*Escherichia coli*	Sediment	Urban	2	Rainy -
U4.S.Mac4	*Escherichia coli*	Sediment	Urban	4	Rainy -
U3.S.Ma4	*Gordonia rubripertincta*	Sediment	Urban	3	Rainy +
U3.W.Mac3	*Klebsiella pneumoniae*	Water	Urban	3	Rainy -
U4.W.Ae1	*Klebsiella pneumoniae*	Water	Urban	4	Rainy -
U2.S.Mac1	*Klebsiella pneumoniae*	Sediment	Urban	2	Rainy -
U3.S.Mac1	*Klebsiella pneumoniae*	Sediment	Urban	3	Rainy -
U1.W.Ae2	*Klebsiella variicola*	Water	Urban	1	Rainy -
U4.S.Ma2	*Microbacterium paraoxydans*	Sediment	Urban	4	Rainy +
U4.S.Ma1	*Micrococcus luteus*	Sediment	Urban	4	Rainy +
U3.W.Mac2.1	*Pantoea stewartii*	Water	Urban	3	Rainy -
U4.S.Mac1	*Pseudomonas aeruginosa*	Sediment	Urban	4	Rainy -
U1.W.Mac5	*Pseudomonas citronellolis*	Water	Urban	1	Rainy -
U1.S.Mac3	*Pseudomonas citronellolis*	Sediment	Urban	1	Rainy -
U4.S.Mac5.2	*Pseudomonas citronellolis*	Sediment	Urban	4	Rainy -
U2.S.Mac4	*Pseudomonas putida*	Sediment	Urban	2	Rainy -
U3.W.Mac1	*Serratia marcescens*	Water	Urban	3	Rainy -
U3.W.Mac2.2	*Serratia marcescens*	Water	Urban	3	Rainy -

Supplementary Materials for Chapter 3



Supplementary Material SM3-1: Sampling sites of Hon Mieu aquaculture floating cage farms, Nha Trang, Vietnam

Supplementary Material SM3-2: Details of the collected samples

Samples	Date	Location	Biosample	Type	Genus	Species	Taxa
HM-W-1	Nov_2022	Water	Environment	Water	NA	NA	NA
HM-W-2	Nov_2022	Water	Environment	Water	NA	NA	NA
HM-W-3	Nov_2022	Water	Environment	Water	NA	NA	NA
Fi-10-3	Jul_2023	Farm_10	Animals	Gut	*Siganus*	*canaliculatus*	*Siganus_canaliculatus*
Fi-10-4	Jul_2023	Farm_10	Animals	Gut	*Siganus*	*canaliculatus*	*Siganus_canaliculatus*
Fi-10-5	Jul_2023	Farm_10	Animals	Gut	*Siganus*	*canaliculatus*	*Siganus_canaliculatus*
Fi-10-6	Dec_2023	Farm_10	Animals	Gut	*Siganus*	*canaliculatus*	*Siganus_canaliculatus*
Fi-17-8	Jul_2023	Farm_17	Animals	Gut	*Rachycentron*	*canadum*	*Rachycentron_canadum*
Fi-18-1	Jul_2023	Farm_18	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-18-2	Jul_2023	Farm_18	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-18-3	Jul_2023	Farm_18	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-19-1	Dec_2023	Farm_19	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-20-1	Dec_2023	Farm_20	Animals	Gut	*Trachinotus*	*blochii*	*Trachinotus_blochii*
Fi-20-2	Dec_2023	Farm_20	Animals	Gut	*Trachinotus*	*blochii*	*Trachinotus_blochii*
Fi-3-6	Jul_2023	Farm_03	Animals	Gut	*Cyprinus*	*carpio*	*Cyprinus_carpio*
Fi-3-7	Jul_2023	Farm_03	Animals	Gut	*Cyprinus*	*carpio*	*Cyprinus_carpio*
Fi-4-1	Jul_2023	Farm_04	Animals	Gut	*Trachinotus*	*blochii*	*Trachinotus_blochii*
Fi-4-3	Jul_2023	Farm_04	Animals	Gut	*Trachinotus*	*blochii*	*Trachinotus_blochii*
Fi-4-4	Dec_2023	Farm_04	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-5-3	Jul_2023	Farm_05	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-5-4	Dec_2023	Farm_05	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-5-5	Dec_2023	Farm_05	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-6-4	Jul_2023	Farm_06	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-6-5	Jul_2023	Farm_06	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-6-6	Jul_2023	Farm_06	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-6-7	Dec_2023	Farm_06	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-6-8	Dec_2023	Farm_06	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-6-9	Dec_2023	Farm_06	Animals	Gut	*Platax*	*teira*	*Platax_teira*

Supplementary Material SM3-2 (continued)

Samples	Date	Location	Biosample	Type	Genus	Species	Taxa
Fi-10-1	Nov_2022	Farm_10	Animals	Gut	*Siganus*	*canaliculatus*	*Siganus_canaliculatus*
Fi-10-2	Nov_2022	Farm_10	Animals	Gut	*Siganus*	*canaliculatus*	*Siganus_canaliculatus*
Fi-3-1	Nov_2022	Farm_03	Animals	Gut	*Cyprinus*	*carpio*	*Cyprinus_carpio*
Fi-3-2	Nov_2022	Farm_03	Animals	Gut	*Cyprinus*	*carpio*	*Cyprinus_carpio*
Fi-3-3	Nov_2022	Farm_03	Animals	Gut	*Cyprinus*	*carpio*	*Cyprinus_carpio*
Fi-3-4	Nov_2022	Farm_03	Animals	Gut	*Cyprinus*	*carpio*	*Cyprinus_carpio*
Fi-3-5	Nov_2022	Farm_03	Animals	Gut	*Cyprinus*	*carpio*	*Cyprinus_carpio*
Fi-5-1	Nov_2022	Farm_05	Animals	Gut	*Rachycentron*	*canadum*	*Rachycentron_canadum*
Fi-5-2	Nov_2022	Farm_05	Animals	Gut	*Rachycentron*	*canadum*	*Rachycentron_canadum*
Fi-6-1	Nov_2022	Farm_06	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-6-2	Nov_2022	Farm_06	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-6-3	Nov_2022	Farm_06	Animals	Gut	*Platax*	*teira*	*Platax_teira*
Fi-7-1	Nov_2022	Farm_07	Animals	Gut	*Rachycentron*	*canadum*	*Rachycentron_canadum*
Fi-7-2	Nov_2022	Farm_07	Animals	Gut	*Rachycentron*	*canadum*	*Rachycentron_canadum*
Fi-7-3	Nov_2022	Farm_07	Animals	Gut	*Rachycentron*	*canadum*	*Rachycentron_canadum*
Fi-7-4	Nov_2022	Farm_07	Animals	Gut	*Rachycentron*	*canadum*	*Rachycentron_canadum*
Fi-7-5	Nov_2022	Farm_07	Animals	Gut	*Rachycentron*	*canadum*	*Rachycentron_canadum*
HM-W-4	Jul_2023	Water	Environment	Water	NA	NA	NA
HM-W-5	Jul_2023	Water	Environment	Water	NA	NA	NA
HM-W-6	Jul_2023	Water	Environment	Water	NA	NA	NA
HM-W-7	Dec_2023	Water	Environment	Water	NA	NA	NA
HM-W-8	Dec_2023	Water	Environment	Water	NA	NA	NA
HM-W-9	Dec_2023	Water	Environment	Water	NA	NA	NA
Hu-21-G	Jul_2023	Farm_02	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-21-S	Jul_2023	Farm_02	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-22-G	Jul_2023	Farm_02	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-22-S	Jul_2023	Farm_02	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-23-G	Jul_2023	Farm_03	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*

Supplementary Material SM3-2 (continued)

Samples	Date	Location	Biosample	Type	Genus	Species	Taxa
Hu-23-S	Jul_2023	Farm_03	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-25-G	Jul_2023	Farm_05	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-25-S	Jul_2023	Farm_05	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-26-G	Jul_2023	Farm_05	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-26-S	Jul_2023	Farm_05	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-27-G	Jul_2023	Farm_04	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-27-S	Jul_2023	Farm_04	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-28-G	Jul_2023	Farm_06	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-28-S	Jul_2023	Farm_06	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-29-G	Jul_2023	Farm_09	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-29-S	Jul_2023	Farm_09	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-30-G	Jul_2023	Farm_18	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-30-S	Jul_2023	Farm_18	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-31-G	Jul_2023	Farm_17	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-31-S	Jul_2023	Farm_17	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-32-G	Jul_2023	Farm_08	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-32-S	Jul_2023	Farm_08	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-43-G	Jul_2023	Farm_10	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-43-S	Jul_2023	Farm_10	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-44-G	Jul_2023	Farm_03	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-44-S	Jul_2023	Farm_03	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-45-G	Dec_2023	Farm_02	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-45-S	Dec_2023	Farm_02	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-46-G	Dec_2023	Farm_02	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-46-S	Dec_2023	Farm_02	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-47-G	Dec_2023	Farm_03	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-47-S	Dec_2023	Farm_03	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-48-G	Dec_2023	Farm_03	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*

Supplementary Material SM3-2 (continued)

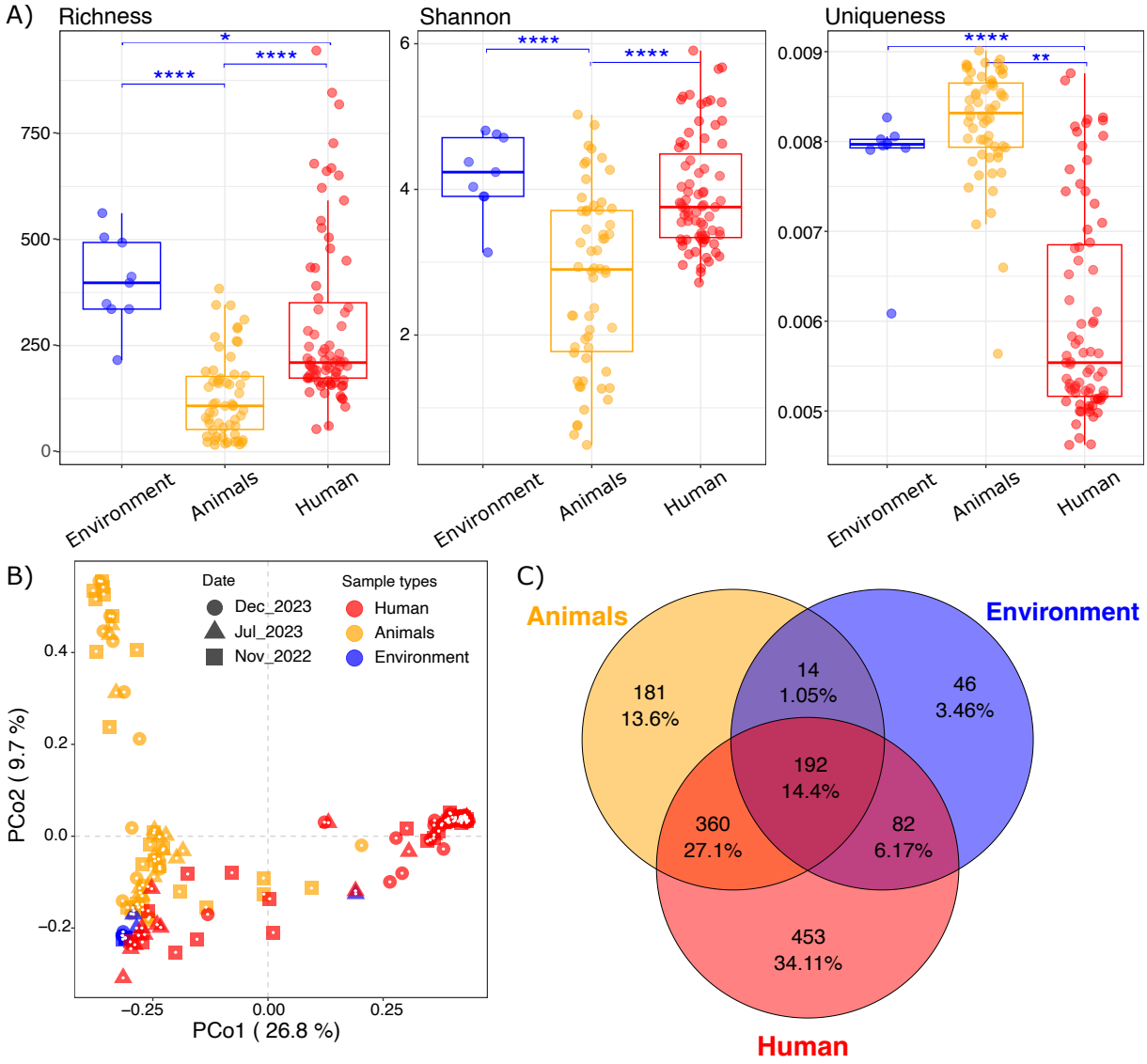
Samples	Date	Location	Biosample	Type	Genus	Species	Taxa
Hu-48-S	Dec_2023	Farm_03	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-49-G	Dec_2023	Farm_04	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-49-S	Dec_2023	Farm_04	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-50-G	Dec_2023	Farm_05	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-50-S	Dec_2023	Farm_05	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-51-G	Dec_2023	Farm_05	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-51-S	Dec_2023	Farm_05	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-52-G	Dec_2023	Farm_06	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-52-S	Dec_2023	Farm_06	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-53-G	Dec_2023	Farm_08	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-53-S	Dec_2023	Farm_08	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-54-G	Dec_2023	Farm_09	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-54-S	Dec_2023	Farm_09	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-55-G	Dec_2023	Farm_10	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-55-S	Dec_2023	Farm_10	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-56-G	Dec_2023	Farm_19	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-56-S	Dec_2023	Farm_19	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-57-G	Dec_2023	Farm_20	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-57-S	Dec_2023	Farm_20	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-10-G	Nov_2022	Farm_06	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-10-S	Nov_2022	Farm_06	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-11-G	Nov_2022	Farm_07	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-11-S	Nov_2022	Farm_07	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-12-G	Nov_2022	Farm_08	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-12-S	Nov_2022	Farm_08	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-13-G	Nov_2022	Farm_09	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-13-S	Nov_2022	Farm_09	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-14-G	Nov_2022	Farm_10	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*

Supplementary Material SM3-2 (continued)

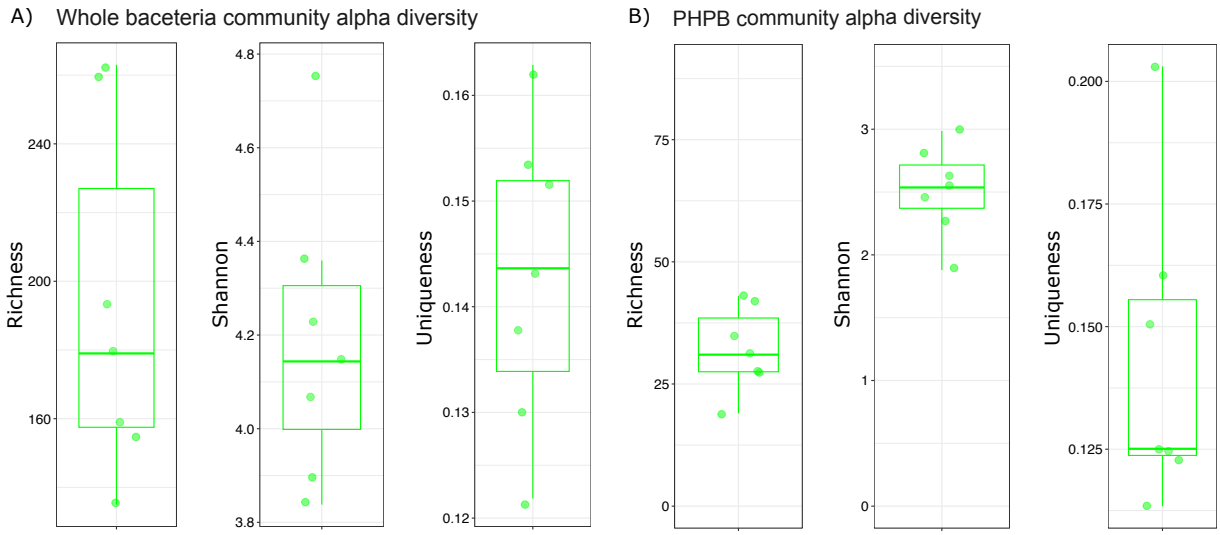
Samples	Date	Location	Biosample	Type	Genus	Species	Taxa
Hu-14-S	Nov_2022	Farm_10	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-3-G	Nov_2022	Farm_02	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-3-S	Nov_2022	Farm_02	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-4-G	Nov_2022	Farm_02	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-4-S	Nov_2022	Farm_02	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-5-G	Nov_2022	Farm_03	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-5-S	Nov_2022	Farm_03	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-6-G	Nov_2022	Farm_03	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-6-S	Nov_2022	Farm_03	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-7-G	Nov_2022	Farm_04	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-7-S	Nov_2022	Farm_04	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-8-G	Nov_2022	Farm_05	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-8-S	Nov_2022	Farm_05	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Hu-9-G	Nov_2022	Farm_05	Human	Gut	*Homo*	*sapiens*	*Homo_sapiens*
Hu-9-S	Nov_2022	Farm_05	Human	Skin	*Homo*	*sapiens*	*Homo_sapiens*
Lo-2-4	Jul_2023	Farm_02	Animals	Gut	*Panulirus*	*longipes*	*Panulirus_longipes*
Lo-2-5	Jul_2023	Farm_02	Animals	Gut	*Panulirus*	*longipes*	*Panulirus_longipes*
Lo-2-6	Dec_2023	Farm_02	Animals	Gut	*Panulirus*	*longipes*	*Panulirus_longipes*
Lo-2-7	Dec_2023	Farm_02	Animals	Gut	*Panulirus*	*longipes*	*Panulirus_longipes*
Lo-2-8	Dec_2023	Farm_02	Animals	Gut	*Panulirus*	*longipes*	*Panulirus_longipes*
Lo-2-9	Dec_2023	Farm_02	Animals	Gut	*Panulirus*	*longipes*	*Panulirus_longipes*
Lo-5-1	Jul_2023	Farm_05	Animals	Gut	*Panulirus*	*longipes*	*Panulirus_longipes*
Lo-5-3	Dec_2023	Farm_05	Animals	Gut	*Panulirus*	*longipes*	*Panulirus_longipes*
Lo-5-4	Dec_2023	Farm_05	Animals	Gut	*Panulirus*	*longipes*	*Panulirus_longipes*
Lo-5-5	Dec_2023	Farm_05	Animals	Gut	*Panulirus*	*longipes*	*Panulirus_longipes*
Lo-9-1	Dec_2023	Farm_09	Animals	Gut	*Panulirus*	*longipes*	*Panulirus_longipes*
Lo-9-2	Dec_2023	Farm_09	Animals	Gut	*Panulirus*	*longipes*	*Panulirus_longipes*
Lo-2-1	Nov_2022	Farm_02	Animals	Gut	*Panulirus*	*longipes*	*Panulirus_longipes*

Supplementary Material SM3-2 (continued)

Samples	Date	Location	Biosample	Type	Genus	Species	Taxa
Lo-2-2	Nov_2022	Farm_02	Animals	Gut	*Panulirus*	*longipes*	*Panulirus_longipes*
Lo-2-3	Nov_2022	Farm_02	Animals	Gut	*Panulirus*	*longipes*	*Panulirus_longipes*
Lo-4-1	Nov_2022	Farm_04	Animals	Gut	*Panulirus*	*ornatus*	*Panulirus_ornatus*
Lo-4-2	Nov_2022	Farm_04	Animals	Gut	*Panulirus*	*ornatus*	*Panulirus_ornatus*
Lo-8-1	Nov_2022	Farm_08	Animals	Gut	*Panulirus*	*ornatus*	*Panulirus_ornatus*
Lo-8-2	Nov_2022	Farm_08	Animals	Gut	*Panulirus*	*ornatus*	*Panulirus_ornatus*
Lo-8-3	Nov_2022	Farm_08	Animals	Gut	*Panulirus*	*ornatus*	*Panulirus_ornatus*
Lo-8-4	Nov_2022	Farm_08	Animals	Gut	*Panulirus*	*ornatus*	*Panulirus_ornatus*
Lo-8-5	Nov_2022	Farm_08	Animals	Gut	*Panulirus*	*ornatus*	*Panulirus_ornatus*


A)
Richness
Shannon
Uniqueness
Environment
Animals
Human
B)
Date
Dec_2023
Jul_2023
Nov_2022
Sample types
Human
Animals
Environment
PCo2 (9.7 %)
PCo1 (26.8 %)
C)
Animals
Environment
Human
181
13.6%
14
1.05%
46
3.46%
192
14.4%
360
27.1%
82
6.17%
453
34.11%
D)
Environment
Animal
Human
Control
Whole bacterial composition (%)
Phylum
Firmicutes
Bacteroidota
Proteobacteria
Cyanobacteria
Spirochaetota
Fusobacteriota
Actinobacteriota
SAR324 clade
Others

Supplementary Material SM3-3: Whole bacterial communities between compartments in floating cage farms. A: Indices of bacterial richness, diversity, and originality across floating cage farms compartments. B: Principal coordinate analysis of Bray–Curtis dissimilarities in the bacterial ASVs composition. C: Venn diagram of bacterial genera between compartments. D: Whole bacterial ASVs composition (Top 5 Phylum) of each compartment and control group.



Supplementary Material SM3-4: Alpha diversity of control samples in A: Whole bacteria community, and B: PHPB community.

Supplementary Material SM3-5: Permanova tests on composition of bacteria, PHPB, ARGs and phenotypic resistance

Data	**Factor**	R^2	**p-value**
Whole bacteria	Sample types	0.15866	0.000999
Whole bacteria	Season	0.02542	0.007992
PHPB	Sample types	0.17507	0.000999
PHPB	Season	0.02163	0.04795
Gram Negative isolate resistance	Sample types	0.06501	0.01099
Gram Negative isolate resistance	Season	0.37898	0.000999
Gram Positive isolate resistance	Sample types	0.09149	0.3027
Gram Positive isolate resistance	Season	0.12501	0.1199
ARGs	Sample types	0.08391	0.02298
ARGs	Season	0.03160	0.6973

Supplementary Material SM3-6: Kruskal – Wallis significance test and Dunn post-hoc test

Comparison	Observed	Shannon	LCBD
Whole bacteria			
Animals - Environment	8.38E-07	3.53E-01	1.95E-01
Animals - Human	9.94E-09	1.47E-01	4.69E-17
Environment - Human	3.15E-02	2.79E-02	5.44E-03
Dec_2023 - Jul_2023	3.53E-01	7.54E-01	8.86E-03
Dec_2023 - Nov_2022	1.47E-01	5.13E-01	6.67E-03
Jul_2023 - Nov_2022	2.79E-02	6.17E-01	7.70E-01
PHPB			
Animals - Environment	8.13E-01	1.16E-01	1.53E-01
Animals - Human	8.20E-17	7.70E-08	2.78E-12
Environment - Human	1.39E-05	7.10E-02	5.53E-02
Dec_2023 - Jul_2023	5.02E-01	7.65E-01	1.18E-01
Dec_2023 - Nov_2022	1.14E-03	7.80E-03	2.41E-01
Jul_2023 - Nov_2022	7.43E-03	3.13E-02	4.44E-01
MAR index			
Animals - Environment	0.27729	0.30341	1.00000
Animals - Human	0.20017	0.04023	0.98230
Environment - Human	0.10691	0.03341	0.82672
Dec_2023 - Jul_2023	5.26E-04	1.39E-06	0.88361
Dec_2023 - Nov_2022	2.98E-09	2.90E-08	0.12217
Jul_2023 - Nov_2022	2.16E-02	8.93E-01	0.21226
ARGs			
Animals - Control	3.38E-02	0.00093	0.06794
Animals - Environment	1.76E-01	0.94161	0.47675
Control - Environment	4.21E-01	0.00136	0.25394
Animals - Human	9.16E-07	0.00034	0.00004
Control - Human	7.15E-02	0.76166	0.16694

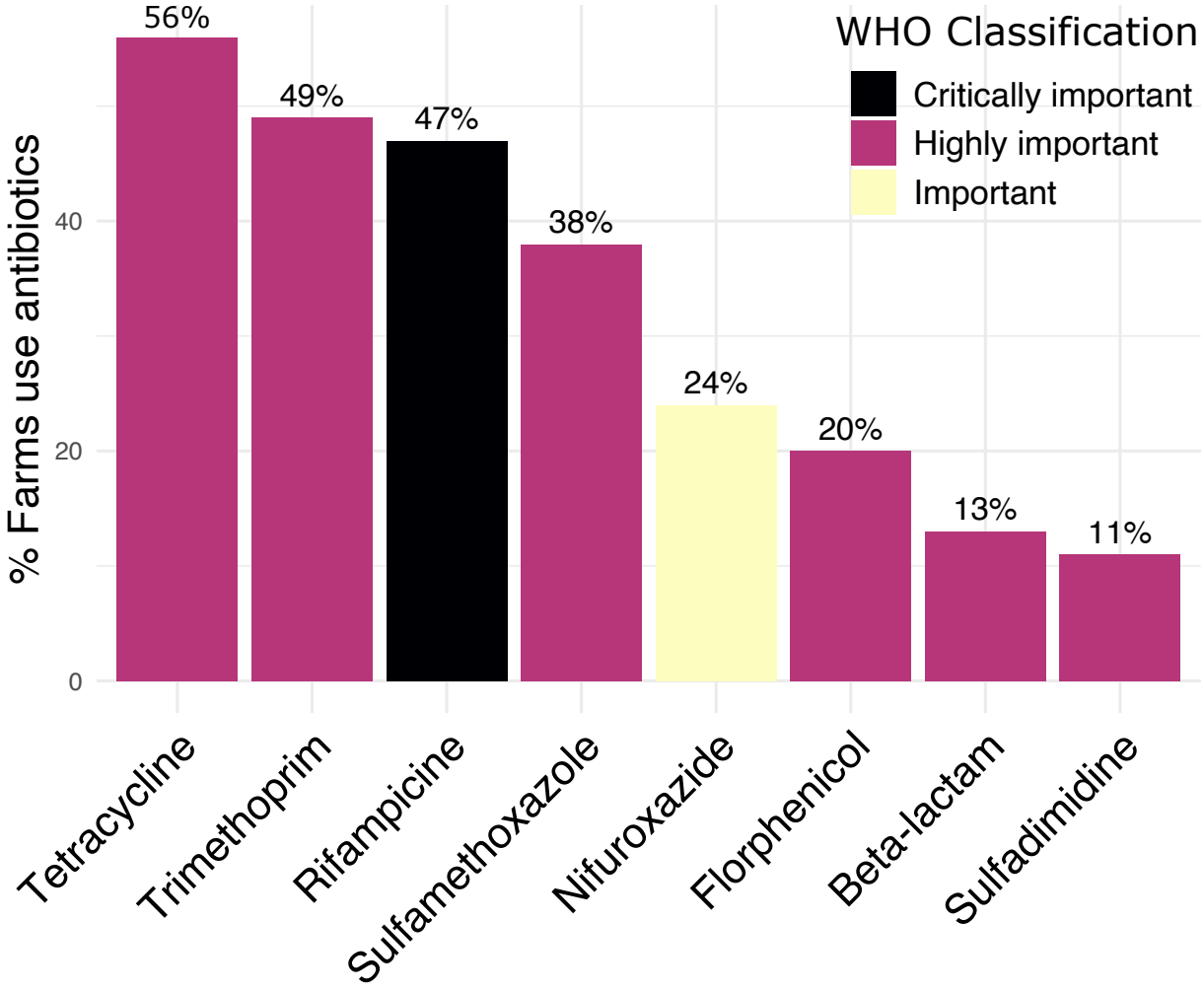
Supplementary Material SM3-6 (continued)

Comparison	Observed	Shannon	LCBD
Environment - Human	1.24E-02	0.00086	0.00736
Dec_21 - Dec_23	1.00000	0.08456	1.00000
Dec_21 - Jul_23	1.00000	0.35559	0.88662
Dec_23 - Jul_23	1.00000	0.35465	1.00000
Dec_21 - Nov_22	0.97910	0.16278	1.00000
Dec_23 - Nov_22	1.00000	0.56520	1.00000
Jul_23 - Nov_22	1.00000	0.64837	0.89602

Medically important antimicrobials						Not medically important
Authorized for use in humans only		Authorized for both humans and animals				Not authorized in humans
Class	Class	Categorization of categorization of antimicrobials antimicrobials				
		HPCIA	CIA	HIA	IA	
Aminoglycosides (plazomicin)	Lipopeptides	Cephalosporins (3rd, 4th generation)	Aminoglycosides	Amphenicols	Aminocyclitols	Aminocoumarins
Aminomethycyclines	Macrolides 18-membered ring (fidaxomicin)	Quinolones	Ansamycins	Cephalosporins (1st- and 2nd-generation) and cephamycins	Cyclic polypeptides	Arsenicals
Anti-pseudomonal penicillins (carboxypenicillin and ureidopenicillin)	Monobactams	Polymyxins	Macrolides (14-, 15-, 16-membered ring)	Lincosamides	Heterocyclic compounds	Bicyclomycins
Anti-pseudomonal penicillins with β-lactamase inhibitors	Oxazolidinones	Phosphonic acid derivatives		Nitroimidazoles	Hydroxyquinoline	Orthosomycins
				Tetracyclines		
Carbapenems with or without β-lactamase inhibitors	Riminofenazines			Penicillins (amidinopenicillins and aminopenicillins)	Pleuromutilins	Phosphoglycolipids
Cephalosporins (3rd-, 4th- and 5th-generation with β-lactamase inhibitors)	Sulfones			Penicillins (aminopenicillins with β-lactamase inhibitors)	Nitrofuran derivatives	Ionophores (including polyethers)
Cephalosporins (5th-generation)	Glycopeptides and lipoglycopeptides			Penicillins (anti-staphylococcal)		Quinoxalines
Cephalosporins (Siderophore)	Pseudomonic acids (mupirocin)			Penicillins (narrow spectrum)		Halogenated 8-hydroxyquinolines
Fluorocyclines	Phenol derivatives (clofoctol)			Streptogramins		
Glycylcyclines	8-hydroxy-5-nitroquinoline			Sulfonamides, dihydrofolate reductase inhibitors and combinations		
Drugs used solely to treat tuberculosis or other mycobacterial diseases				Fusidanes		

CIA: critically important antimicrobials; HIA: highly important antimicrobials; HPCIA: highest priority critically important antimicrobials; MIA: medically important antimicrobials.

Supplementary Material SM3-7: List of Medically Important Antimicrobials by WHO, 2024.



Supplementary Material SM3-8: List of widely used antibiotics in Nha Trang floating cage aquaculture and their AMR risk classification by WHO

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