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Antibiotic Resistance in Ligurian Fauna

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Chapter 1 – Introduction to Antimicrobial Resistance

1.1 Emergence and evolution of the phenomenon of antibiotic resistance (AMR)

One of the greatest accomplishments of current medicine is the discovery of antibiotics. The “antibiotic era” began with Alexander Fleming’s accidental discovery in 1928 of penicillin, which changed how we can treat bacterial infections to reduce mortality from diseases which were previously thought to be incurable — pneumonia, tuberculosis, syphilis and septicemia, now being lessened by antibiotics. Simultaneously Fleming himself issued early warnings about misuse and the dangers of selecting resistant strains. Some early resistance developed shortly after each new antibiotic was introduced (penicillin-resistant *Staphylococcus aureus* in the 1940s, followed by streptomycin, chloramphenicol, tetracycline, fluoroquinolone and carbapenem resistance). Methicillin-resistant *S. aureus* (MRSA) emerged shortly after methicillin became available. From the 1980s onward, MDR (Multidrug-Resistant), XDR (Extensively Drug-Resistant) and PDR (Pandrug-Resistant) strains have developed resistance to virtually all antimicrobials. Today, carbapenem-resistant *A. baumannii*, *P. aeruginosa*, *K. pneumoniae* and *Enterobacter* spp. are among the top threats. Antibiotic pressure kills susceptible bacteria and selects resistant ones that have a vertical and horizontal spread.

Agriculture and intensive farming have also helped, letting resistomes grow in wastewater and soil. The O’Neill Report from 2016 predicted 10 million AMR deaths per year by 2050, with ECDC (European Center for Diseases Control) now estimating more than 35,000 deaths in Europe annually. AMR poses a threat to surgeries, oncology, transplants and intensive care. As such, even simple infections can be fatal without effective antimicrobials (1, 6, 23).

1.2 Global and systemic issue

AMR is not only a biological challenge but is also a systemic and global challenge and has medical, social, economic and ecological dimensions. Resistant bacteria cross borders through travel, migration, trade and environmental pathways, thus AMR is a global public-good challenge that is analogous to climate change or biodiversity loss. Such a One Health vision is advocated by numerous international organizations (WHO, FAO, WOA). While the 2015 Global Action Plan urged countries to implement

AMR strategies, the implementation varies according to country. Countries with high income (e.g. Sweden, Netherlands, UK) have sophisticated surveillance systems, many LMICs (low- or middle-income economies), however, face counterfeit drugs, inadequate diagnostics, over-the-counter antibiotics and poor waste management. The Interagency Coordination Group of UN has also identified AMR as a failure of governance. Meanwhile, the antibiotic R&D pipeline is collapsing because of low profitability and regulatory barriers (economists are forecasting catastrophic costs by 2050). Surveillance has improved in Europe (EARS-Net, EFSA monitoring), but considerable disparities remain in the region between Northern and Southern/Eastern Europe. Environmental AMR surveillance remains the weakest component of national AMR plans, and demands multi-disciplinary expertise and more investment. Cultural forces also count for antibiotic misuse (self-medication in LMICs, defensive over-prescription in high-income countries). AMR prolongs hospital stay, increases the frequency of patients' deaths and medical costs, and undermines sophisticated procedures. It is a failure not only of biology but also human systems, like healthcare, agriculture, economics and education. A properly global response needs political dedication, collaboration across disciplines and a prolonged investment (2, 18, 41).

1.3 An environmental and not just a clinical problem

AMR had been largely considered a hospital issue for decades. But in the last two decades, the environment has become a major reservoir and accelerant of the resistance. The environmental resistome shows that resistance to clinical antibiotics predates by millions of years, while human antibiotic usage has greatly sped up its development. Co-selective pressures include heavy metals, pesticides, disinfectants and other pollutants. Antibiotics, resistant bacteria, mobile genetic elements (MGEs) are all present in hotspots in wastewater treatment plants, allowing for HGT (horizontal gene transfer). Antibiotic resistance genes (ARGs) such as blaCTX-M, blaNDM, mcr-1 and qnrS have been detected in treated effluents as well. Manure from antibiotic-treated livestock brings up resistant organisms and ARGs in soils that can persist for years. ARGs build up in aquatic ecosystems through urban runoff, aquaculture, and tourism in sediments. Wildlife becomes the sentinel and vector as it acquires resistance from contaminated water, soil, and food. Further, climate change, biodiversity loss, and ecological homogenization contribute to resistance dynamics. Environmental AMR is relatively under-represented in policy frameworks, with varying sampling and inefficient coordination of agencies. Environmental AMR is consistent with several UN SDGs (3, 6, 14, 15). Recognition of the environment as an active enabler rather than as a passive recipient is crucial for long term sustainable mitigation (11,29,32).

1.4 Thesis objectives and structure

This thesis aims to provide a local and global perspective on AMR, focusing on wildlife as a potential reservoir and environmental vector. It summarizes scientific evidence, policy frameworks, surveillance data, and One Health implications. The objectives include:

- Describing the historical and theoretical context of AMR
- Explaining physiological and genetic resistance mechanisms
- Highlighting wildlife and environmental dissemination routes
- Comparing national and Ligurian AMR situations
- Discussing future strategies and containment approaches

The study is organized into six chapters, covering:

1. Introduction to AMR
2. Mechanisms of resistance
3. Environmental AMR and wildlife
4. AMR in Italy and Liguria
5. Regulatory and surveillance frameworks
6. Final synthesis and future perspectives (18, 36)

Chapter 2 - Mechanisms of antibiotic resistance

2.1 Introduction

Antimicrobial resistance (AMR) is a crucial threat to contemporary medicine, veterinary medicine and public health as a whole. This also encompasses microbiological, clinical, environmental and socio-economic aspects. The discovery of antibiotics at the start of the 20th century transformed medicine by treating once-lethal infections efficiently and controlling them. In a short period of time, though, the emergence of resistant strains of bacteria showed that, as their most potent medicines failed (23,31,74), microorganisms possessed the capacity for evolution in this manner. Antimicrobial resistance, a term of art about what it is when a microbe – bacteria, viruses, fungi, or parasites – can persist and expand against levels of certain antimicrobial agents normally found to prevent or kill susceptible groups, is at the root of the issue.

2.1.1 Historical and Conceptual Background

This history of AMR has led to the development and use of antibiotics. Strains of *Staphylococcus aureus* that can form β -lactamase enzymes were already described when penicillin came to market in the 1940s. Over the years that followed, a new antibiotic group — tetracyclines, macrolides, aminoglycosides, fluoroquinolones and carbapenems — emerged along with their respective mechanisms of resistance (6, 23, 74). This dynamic reflects the coevolutionary arms race between humans and microorganisms. While antimicrobial development and innovation have slowed substantially since the 1980s, bacterial evolution has not decreased markedly — leading to the rise of multidrug-resistant (MDR), extensively drug-resistant (XDR), and even pan-resistant organisms (31, 73). These so-called “superbugs” have since undermined the effectiveness of many basic treatment strategies for human and animal populations (25, 31).

2.1.2 Mechanisms of Resistance and Genetic Adaptation

Molecular mechanisms involved in AMR are numerous and frequently connected.

They include:

- Drug enzymatic degradation or modification (e.g., β -lactamases, carbapenemases, aminoglycoside-modifying enzymes).
- Modification of ribosomal proteins or penicillin-binding protein (PBP) mutations at the target sites.
- Loss or modification of porin for decrease in permeability of drugs and reduction of antibiotic entry.
- Bacterial efflux pumps that pump drugs out of the bacterial cell.
- Biofilm formation — a protective mechanism physically and metabolically against antimicrobials (6, 88, 90).

The horizontal gene transfer (HGT) from unrelated bacterial species by means of plasmids, transposons, or integrons is particularly worrying. Such means allow rapid spread of resistance determinants across entire microbial populations and entire ecosystems. Resistance genes such as blaNDM, blaKPC, mcr, and tet(X) as provided by plasmids can then be disseminated between clinical, veterinary, and environmental pathogens while muddying the line separating these domains (7, 8, 10).

2.1.3 The Global Health Dimension

WHO and ECDC have designated certain bacteria as critical priority pathogens, calling for urgent attention. *Klebsiella pneumoniae*, *Escherichia coli*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* are the reported Gram-negative bacteria with high levels of resistance against last-resort antibiotics such as carbapenems and colistin (4, 54, 66). Gram-positive organisms such as *Staphylococcus aureus* (MRSA), *Enterococcus faecium* (VRE), and *Streptococcus pneumoniae* also greatly contribute to healthcare-associated infections and community-acquired diseases (31, 73). The therapeutic landscape around these infections is under greater pressure than ever. However, the pipeline of new antibiotics is still small, and several promising new compounds have not made it through their early stages in clinical trials.

2.1.4 AMR Drivers and Determinants

AMR largely spreads due to anthropogenic misuse of antimicrobials in different domains. In human medicine, selection of resistance is accelerated by overprescription, self-medication, and incomplete treatment courses. Antibiotics have commonly been used for therapeutic use, growth promotion and prophylaxis in high-pressure farming environments (24, 27, 37) in veterinary and agricultural environments. Additionally, antimicrobial residues, disinfectants, and contamination with heavy metals in the environment compound the problem by exerting selective pressure on environmental microbiota (29, 32). Other contributing factors are:

- Poor IPC (Infection Prevention and Control) practices in medical facilities.
- Limited access to diagnostic tools leading to empirical and in many cases unnecessary therapies.
- International travel and trade that allow resistant organisms to enter and leave their natural host.
- Climate change and urbanization that impact the existence, survival, distribution and spread of pathogens in natural populations of organisms (35, 41, 42).

Taken together, these drivers establish a network of global AMR characterized by feedback loops from human, animal and environmental systems (18, 73).

2.1.5 The One Health Approach and Environmental Context

AMR breaks ecological and species boundaries but respects the whole society. One Health acknowledges the interrelatedness between human, animal, and environmental health, recognizing that the use of antimicrobials in some domains inevitably influences others. The common antibiotics in human and animal excreta go into wastewater, soils, and aquatic systems and apply selective pressure to microbial populations. These environments can themselves be repositories of resistance genes that they can ultimately become active in clinical practice through water, food, or direct contact (18, 32, 79). In recent years, there is expanded research focusing on the environmental component of AMR by investigating how naturally rooted communities are supporting AMR and transmitting it. Metagenomic studies have revealed resistance genes in pristine environments like Arctic soils and Antarctic waters, suggesting that AMR is not just a by-product of modern medicine but a primitive, intrinsic microbial trait supercharged by anthropogenic processes. But, the enormous scale and speed at which the spread itself is now occurring are largely human induced. This is where veterinary medicine is in a pivotal role. Food safety, prevention, and control of zoonotic diseases, as well as for environmental sustainability, are at the core of problems with food-producing animals. Therefore, responsible use of antimicrobial agents in veterinary practice, including surveillance, diagnostics and regulatory frameworks, is important for long-term mitigation of AMR (20, 24, 79).

2.1.6 Toward Integrated Surveillance and Control

At global and regional levels, integrated surveillance systems have been instituted to address the complexity of AMR. These include WHO's GLASS, the European EARS-Net for human health, EFSA and EMA systems for the animal and food sectors, and environmental monitoring initiatives coordinated by UNEP and national institutes (1, 4, 54, 98). The intended use of such systems is to harmonize data collection, recognize new trends in resistance, and advance policy formation. However, there are large gaps, particularly in environmental and low-resource settings, where data are sparse and methodologies heterogeneous. Reinforcing laboratory networks, enhancing genomic sequencing capacity, and promoting open data sharing will be key next steps toward a truly global AMR surveillance architecture (32, 57, 99).

2.1.7 Conclusion

Starting from the introduction of that chapter, it's clear that AMR is more than a microbiological concern: it's an ecological, social and economic one. Its containment would need an interdisciplinary strategy which will involve microbiology, epidemiology, environmental science, veterinary medicine and public policy (18, 31, 73). This thesis will view AMR from a wider perspective concerning its clinical manifestations, environmental and geographical spread, with Italy and the Ligurian region in particular, as a case in point, the connection between marine ecosystems, wildlife, and human action as an example of One Health complexity (36, 67, 77).

2.2 Intrinsic and acquired resistance

There are two ways for bacteria to develop resistance to antibiotics - through intrinsic resistance and acquired resistance. Each bacterial subtype is different from each other in genetics, mechanistic explanations and potential clinical/ecological effects.

Intrinsic resistance represents an inherent property of a bacteria. It is present in the chromosomes and found in all species with or without being exposed to the respective antimicrobials. In addition, it results from structural resistance (on the outer membrane of Gram-negative organisms), absence of the molecular target of the antibiotic, or occurrence of constitutive efflux pumps (31, 45). Common examples include:

- *Pseudomonas aeruginosa* inherently resists various β -lactams and aminoglycosides;
- *Enterococcus* spp., by definition a cephalosporin-resistant organism;
- *Mycoplasma* spp., deficient in a cell wall and thus resistant to β -lactams (31, 76).

Intrinsic resistance is an environmental contributor to certain microorganisms, that is, a factor that makes the treatment of some infections particularly difficult since diagnosis.

Acquired resistance, in contrast, results when a species that was sensitive to the first infection acquires new genetic determinants that make the system resistant. This can take place:

- by spontaneous mutation of endogenous genes (e.g. target alterations in genes such as *gyrA* for the fluoroquinolones).
- by HGT (horizontal gene transfer), conjugation/transformation or transduction (23, 32, 88).

Mobile Genetic Elements Involved in Acquired Resistance are:

- Plasmids: separate extra-chromosomal elements that can express multiple genes associated with resistance (e.g. ESBL, carbapenemase);
- Transposons: DNA sequences that can “jump” from plasmids to chromosomes;
- Integrans: genetic systems which can acquire genomic cassettes integrated into gene sequences, typically consisting of AMR genes (32, 70). Examples of acquired genes:

- *bla*CTX-M, *bla*KPC, *bla*NDM (β -lactamase);
- *mecA* (methicillin resistance);
- *vanA* (vancomycin resistance);
- *tetA*, *ermB*, *sul1* (tetracyclines, macrolides, sulfonamides) (6, 7, 88).

These genes are able to travel rapidly between new strains and bacterial species, contributing to the global spread of resistance. The accumulation of intrinsic or acquired resistances in a strain can generate many

of the very troublesome microorganisms (e.g. *Pseudomonas* XDR, *Acinetobacter* MDR). The distinction between intrinsic and acquired resistance has important implications for antibiotic therapy, susceptibility test interpretation and prevention and surveillance policies (31, 73).

2.2.1 Spontaneous mutations and adaptation

One of the key ways by which bacteria can become resistant to antibiotics is the mechanism of spontaneous mutations. Such mutations are random alterations in the genetic material that may occur during the process of DNA replication, and which may provide a selective advantage in the presence of the antibiotic.

These mutations can affect:

- The genes encoding the antibiotic targets (e.g. mutations in the *gyrA* and *parC* genes for fluoroquinolones);
- The regulatory genes of efflux pumps or transport systems;
- The genes that modulate membrane or porin permeability (23, 31, 90).

One such example is resistance to fluoroquinolones, typically with point mutations in the *gyrA* (encoding DNA gyrase subunit A) and *parC* (topoisomerase IV) genes thus causing a decrease in these molecules' affinity to the drug. Mutations of porin genes (*ompF*, *ompC*) may decrease antibiotic entry, while hyperactivity of efflux pump (e.g., AcrAB-TolC) promotes the release of the molecule from the cell (88, 90).

One of the drawbacks of spontaneous mutations is that they are not always good for the bacterium. Often they involve a biological cost (fitness cost), which can happen during conditions where the antibiotic is not used. In case of such environment where selective pressure is constant, such as hospitals or farms or contaminated soil, mutant strains are selected and they can eventually become established and dominant (23, 31).

The mechanism by which mutations conferring resistance can also be combined with other mechanisms, such as the adoption of plasmids or the introduction of transposons that contain AMR genes, leading to an even more challenging treatment of bacterial strains. One of the greatest concerns is that mutations, after suboptimal therapy such as subtherapeutic dosing, early cessation of therapy or exposure to antibiotics outside of the clinic, may occur and become fixed. The adaptability of bacterial species and rapid replication and high cell capacity make spontaneous mutations a very potent evolutionary force when using AMR as a basis for infection (31, 73).

2.2.2 Biochemical resistance mechanisms

Antibiotic-resistant bacteria use various biochemical mechanisms that inhibit antimicrobials to protect themselves against antimicrobial treatment. Such ways have developed over the years in response to the selective pressure exerted by the excessive use of antibiotics in humans, veterinary and agriculture. The key biochemical pathways of resistance are classified as four broad classes (23, 31):

1 - Antibiotic inactivation.

Bacteria can produce enzymes that can cleave the antibiotic chemically or make it useless. The most famous instance is β -lactamases, enzymes that hydrolyze the β -lactam ring of penicillins, cephalosporins, and carbapenems.

These include:

- TEM, SHV, CTX-M (extended-spectrum β -lactamases – ESBL)
- KPC, NDM, VIM, OXA-48 (carbapenemases) (6, 7, 34).

Similar mechanisms are revealed for aminoglycosides (modifying enzymes like acetyltransferase, phosphotransferase) and for chloramphenicol (chloramphenicol acetyltransferase).

2 - Molecular target modification.

Resistance is acquired by a change of the antibiotic binding site in some bacteria. For example:

- Change to 23S rRNA, such as in the presence of mutations conferring macrolide resistance (erm genes);
- Alteration to rpoB gene, reducing the efficacy of rifampicin;
- Replacement of the D-Ala-D-Ala site with D-Ala-D-Lac confers resistance to vancomycin in vancomycin-resistant enterococci (VRE - vanA, vanB) (31, 45, 74).

3 - Reduction of permeability.

Gram-negative bacteria alter the porins of the outer membrane, which allows for less antibiotic entry (e.g. loss of OmpF in *E. coli*). It is well recognized that this mechanism underlies resistance to β -lactams and quinolones (31, 88, 90).

4 - Active efflux (the expulsion of the drug).

The bacteria can actively pump out the antibiotic from within them through efflux pumps. These pumps, which are encoded by certain genes (e.g., *acrAB-tolC*, *mexAB-oprM*), are especially important for Gram-negative bacteria. They may provide resistance to more than one antibiotic simultaneously (multidrug efflux systems) (88, 90).

These mechanisms are not exclusive: one bacterial strain can simultaneously employ multiple strategies to defend itself against one or more antibiotic actions. Resistance is complex and multifactorial and as such can be hard to combat, particularly in settings where bacteria survive side by side and exchange genetic material (23, 31, 73).

2.2.3 Horizontal gene transfer (HGT)

Transmission of resistant genes among bacteria, regardless if they are of different species, are among the most crucial determinants in the evolution of antibiotic resistance. This phenomenon is termed horizontal gene transfer (HGT) and mediates the spread of resistance among bacterial complex communities as well as in different environments and non-clinical areas (23, 32). HGT consists in three major mechanisms:

Conjugation: the most efficient and common which is direct transfer of DNA from donor bacterial to the recipient cell by conjugative plasmids. In any event, plasmids can carry multiple resistance genes inducing a multi-resistant recipient strain.

Transduction: It occurs via the intermediation of viruses that are bacteriophages (specific to bacteria), which can transmit segments of bacterial DNA from one strain to another during the lytic or lysogenic cycle.

Transformation: Is the uptake by a bacterium of free DNA present in the environment, produced by lysis of cells. This act is naturally proficient in a few species, including *Streptococcus pneumoniae* and *Acinetobacter baumannii* (23, 31).

Mobile genes are incorporated with these:

- Plasmids: autonomous replicons, frequently transmissible, with genes for resistance to β -lactams, aminoglycosides, fluoroquinolones, tetracyclines among others;
- Transposons: DNA sequences that can move in the genome or between plasmids and chromosomes, typically expressing the AMR genes;
- Integrans: genetic platforms capable of capturing and integrating cassettes of genes, several encoding antibiotic resistance. Class 1 integrons are extensively employed in MDR strains clinically (32, 70).

Furthermore, HGT plays a key role in the spread of AMR in non-clinical settings. The conditions for contact and gene transfer between different strains include wastewater, agricultural soils, sewage treatment plants, and biofilm system environments. As such, environmental or wildlife strains may carry clinically AMR-related genes while having never undergone antimicrobial therapy themselves. Hence, the monitoring of environment and wildlife in the context of AMR surveillance systems is essential to understanding and controlling the global circulation of resistant genes (11, 32, 71).

2.3 Classes of antibiotics and associated resistances

Various classes of antibiotics function via particular mechanisms on the structures or critical activities of the bacterial cell. They correspond to different modes of resistance developed and transmitted by bacteria in response to the selective pressure produced by the use of these drugs. These relationships are fundamental not only to the establishment of treatments, but also the interpretation of antibiograms and for surveillance work in the clinical, veterinary and environmental contexts (23, 31, 65).

- **β -lactams**

Examples: penicillins, cephalosporins, carbapenems

Mechanism: inhibition of bacterial wall synthesis (PBPs)

Resistances:

- β -lactamases (TEM, SHV, CTX-M, KPC, NDM)
- mutations in PBPs (e.g. *mecA* in MRSA)
- Reduced permeability (porins)
- Active efflux (6, 7, 74).

- **Aminoglycosides**

Examples: gentamicin, amikacin

Mechanism: inhibition of protein synthesis (30S)

Resistances:

- Modifying enzymes (acetyl-, phospho-, nucleotidyl-transferase)
- Ribosomal mutations
- Reduced uptake (23, 31).

- **Macrolides, lincosamides, streptogramins (MLS)**

Examples: erythromycin, clindamycin

Mechanism: inhibition of protein synthesis (50S)

Resistances:

- 23S rRNA methylation (erm)
- Efflux pumps (mef)
- Binding site mutations (31, 74).

- **Tetracyclines**

Examples: doxycycline, oxytetracycline

Mechanism: inhibition of protein synthesis (30S)

Resistances:

- Efflux pumps (tetA, tetB)
- Ribosomal protective proteins (tetM, tetO) (8, 31).

- **Fluoroquinolones**

Examples: ciprofloxacin, levofloxacin

Mechanism: DNA gyrase inhibition and topoisomerase IV

Resistances:

- Mutations in gyrA and parC
- Qnr protection proteins
- Active efflux (23, 31, 90).

- **Sulfonamides and trimethoprim**

Examples: sulfamethoxazole + trimethoprim

Mechanism: inhibition of folic acid synthesis

Resistances:

- Alternative genes (sul1, sul2, dfrA) (31, 70).

- **Colistin (polymyxins)**

Mechanism: destabilization of the outer membrane

Resistances:

- pmrAB mutations
- mcr-1, mcr-2 genes (plasmids) (8, 10, 70).

- **Glycopeptides**

Examples: vancomycin

Mechanism: D-Ala-D-Ala bonding (wall)

Resistances:

- Target modification (vanA, vanB) (31, 45).

This classification provides insight into the interactions between antibiotics, mechanisms of action and resistance genes, which have direct relevance to the therapeutic approach, infection control and design of new drugs (23, 73).

2.4 Emblematic case studies

Antimicrobial resistance (AMR) has gone from an incipient medical concern to a universal public health crisis, transcending species and environmental barriers. Exploring emblematic cases provides guidance regarding the molecular, epidemiological, and ecological mechanisms underlying this phenomenon and demonstrates how AMR can spread rapidly across geographic regions, hosts, and ecosystems. These cases demonstrate milestone events in the global course of resistance development, which has clinical and environmental implications (31, 34, 73).

2.4.1 The New Delhi Metallo- β -lactamase-1 (NDM-1)

The discovery of NDM-1 plasmid was the first step towards worldwide global AMR surveillance. Originally isolated from *Klebsiella pneumoniae* and *Escherichia coli* isolates from a Swedish patient of Indian origin in 2008, the NDM-1 gene (blaNDM-1) granted resistance to almost every single β -lactam antibiotic, including carbapenems – the "last line of defense for the majority of the most severe infections". Molecular studies indicated that NDM-1 is located in very mobile bacterial plasmids and is therefore easily available for horizontal gene transfer between non-related members of different bacterial species (7, 34). Environmental aspects of NDM-1 dissemination are particularly alarming. Later studies discovered the NDM-1 gene (blaNDM-1) in drinking water and hospital effluents as well as environmental samples in India, Pakistan, and later in Europe, Africa, and North America. This showed the importance of wastewater and contaminated aquatic environments as significant reservoirs for resistance genes. As such, NDM-1 represents a paradigm for One Health integration, with human medicine (32, 34, 71) connected to environmental microbiology and international travel contributing to the spread of AMR. Clinically, NDM-1 positive Enterobacterales are associated to high morbidity and mortality, and treatment options are limited to toxic or more ineffective agents, such as colistin and tigecycline. Rapid global spread of the gene highlights the need for surveillance systems that can detect new carbapenemase producer individuals both clinically and environmentally (4, 31, 66).

2.4.2 The Colistin Resistance Gene *mcr-1*

The mobilization of the colistin resistance gene (*mcr-1*) in 2015 placed critical strain on global antimicrobial stewardship. *Mcr-1*, first discovered in *E. coli* from pigs, raw meat, and humans in China, grants transferable resistance to colistin, a polymyxin antibiotic that was reintroduced as a last resort against multidrug-resistant Gram-negative bacteria. As distinct from chromosomal resistance mechanisms, *mcr-1* is plasmid-mediated, and its interspecies dissemination is rapid (8, 10). The importance of *mcr-1* stems from its relationship to agriculture and human health. Colistin was widely applied in livestock production, particularly as a growth promoter or prophylactic agent, resulting in high selective pressure. Detection of *mcr-1* in animal, food, and clinical isolates across multiple continents within months from its detection showed the global coupling of resistance ecology (20, 24, 70). Later variants, *mcr-2* to *mcr-10*, have been identified, featuring a wide variety of genetic backgrounds and their modes of transmission. In Europe, the reduction of colistin use in veterinary medicine according to EFSA and EMA recommendations has resulted in a slow decline in animal isolates and the effectiveness of integrated policies across sectors (4, 54, 77). From a molecular perspective, the *mcr-1* gene encodes a phosphoethanolamine transferase that modifies the lipid A component of lipopolysaccharide, reducing colistin binding affinity. This biochemical change highlights the potential implications of small enzymatic modifications on clinical therapy and public health (8, 10, 70).

2.4.3 The OXA-48 Carbapenemases

Another major class of resistance determinants is represented by OXA-48-type β -lactamases, first described in *Klebsiella pneumoniae* in Turkey in 2001. These enzymes hydrolyze penicillins and carbapenems but spare extended-spectrum cephalosporins, often leading to diagnostic challenges. The cryptic phenotype of OXA-48 producers has contributed to their silent spread across hospitals in the Mediterranean, Middle East, and Europe (6, 31).

Epidemiological investigations have traced OXA-48 plasmids to environmental and animal sources, supporting their inclusion in One Health surveillance strategies. In Italy, OXA-48-like carbapenemases (notably OXA-181 and OXA-232) have been increasingly detected in clinical isolates, reflecting regional dissemination patterns. Their coexistence with ESBLs and efflux pump mechanisms enhances the multidrug-resistant phenotype and complicates infection control (4, 54, 82).

2.4.4 Methicillin-Resistant *Staphylococcus aureus* (MRSA) Beyond Hospitals

MRSA has been a recognized healthcare-associated pathogen at the population level, but emerging through community and livestock-associated lineages provides evidence of the adaptive potential of bacterial pathogens. Livestock-associated MRSA (LA-MRSA), especially the clonal complex CC398, was discovered from intensive pig and veal farming in Europe before finding its way to farm workers, meat products, and companion animals. This cross-species transmission illustrates the blurring of the line between veterinary and human medicine; hence the need for comprehensive biosafety practices (37, 79). Besides, MRSA has evolved for environmental persistence through biofilm formation in which it can survive on different surfaces and medical devices. That, in combination with resistance against different classes of antibiotics, enables MRSA to survive in hospitals and community environments where large-scale infection control programs exist (31, 73, 80).

2.4.5 Lessons Learned and Future Perspectives

The typical cases of NDM-1, *mcr-1*, OXA-48, and MRSA show a lot about AMR emergence.

- Genetic mobility: plasmids, integrons, and transposons provide carriers for spread.
- Environmental interconnectivity: aquatic and soil ecosystems constitute long-term reservoirs of resistance determinants.
- Anthropogenic drivers: antibiotic overuse in humans, animals, and agriculture sustains selection pressure.
- Gaps in diagnosis and surveillance: when surveillance is slow, silent spread can exist before control measures are put in place (18, 31, 32).

Future AMR crises can be avoided with the One Health paradigm. Surveillance networks need to integrate clinical, veterinary, and environmental data, augmented by genomic technology that can follow mobile elements and resistance gene flow. Moreover, antimicrobial stewardship programmes not only apply in hospitals but also in farms, wastewater treatment plants, and food processing sites because they are connected to microbial ecosystems (18, 32, 79). On a worldwide scale, these case studies also emphasize the significance of international co-operation. Travel, trade, and the shipment of food commodities helped NDM-1 and *mcr-1* spread rapidly across the world and confirmed that containment of resistance cannot be based on national borders. Policies like the WHO Global Action Plan (2015) and

the EU One Health AMR Action Plan set out frameworks for joint monitoring and intervention but long-term political and financial commitment is crucial (1, 57, 59). Ultimately these illustrative instances demonstrate resistance is not an end in itself, but an evolutionary process – continuously conditioned by human conduct. Genomic surveillance, rational antibiotic use, and environmental risk assessment will be the key to tackling AMR's impact on public health, veterinary medicine, and ecosystem integrity as the future strategies (31, 32, 73).

2.5 Geographical and Environmental Spread

2.5.1 Introduction

Geological and ecological dispersal of antimicrobial resistance (AMR) is a multifaceted effect, resulting from human activities, environmental interaction, and microbial change. While the early recognition of AMR occurred in a hospital population, it was realized that resistance genes are transported across a broad continuum linking humans, animals, and the environment. This worldwide network driven by inappropriate use of antibiotics, poor sanitation, and ecological relationships has transformed AMR into an essentially planetary health challenge (18, 32, 36). Environmental compartments such as soil, surface water, sediments, and wildlife, that together comprise the "environmental resistome," are important reservoirs for resistance genes. Inside this resistome, human pollutants exert selective pressure leading to persistence and horizontal gene transfer (HGT) of the resistance determinants, even without direct antibiotic exposure. An integrated One Health approach, which addresses the biological and environmental aspects, is needed to elucidate the spread of AMR geographically (11, 32, 71).

2.5.2 Global Patterns and Regional Inequalities

The global burden of AMR is heavily geographically heterogeneous, reflecting differences in the health system, antibiotic stewardship, regulatory environment, and the socio-economic situation. In the Global Burden of Disease study (2022), sub-Saharan Africa and South Asia have the highest prevalence of resistant infections with >50% of global AMR-associated deaths. On the other hand, nations in Northern Europe and Oceania have a lower resistance due to tighter surveillance and stronger scientifically based antimicrobial guidelines (26, 31, 82). The WHO, in 2015, published the Global Antimicrobial Resistance and Use Surveillance System (GLASS), which became the gold standard for checking AMR measures. The report also notes rising resistance in the key pathogens *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. Especially concerning are carbapenem-resistant Enterobacterales (CRE) with dramatic growth in Asia and the Mediterranean and colistin-resistant isolates detected on every continent (1, 4, 54). Numerous interrelated factors underpin this uneven geography of resistance:

- Antibiotic overuse and self-medication among the human public, especially when antimicrobials are sold as products of self-medication rather than as prescription.
- Livestock that are industrialized by high-density use of antibiotics for growth or prophylaxis.
- Weak water treatment and waste effluent sanitation facilities.
- Climate and hydrological factors, which affect microbial survival and spread (24, 29, 35).

These drivers set up a vicious cycle: antibiotic residues and antibiotic resistant bacteria enter natural systems where they survive and change, returning again to human and animal populations through water, food, and direct contact (18, 32, 79).

2.5.3 Environmental Pathways and Reservoirs

The environment itself is one of both resistance gene recipients and amplifiers. In humans and animals, antibiotics are often excreted unmetabolized and enter wastewater treatment plants (WWTPs), which are major meeting areas for resistant microorganisms. Treatment processes reduce bacterial loads, but rarely eliminate resistance genes completely. In contrast, WWTPs serve as hotspots for genetic exchange, with microbial communities interacting where circumstances are more suited for horizontal gene transfer (29, 32, 71). Resistance determinants from these systems propagate into surface waters, rivers and coastal ecosystems and remain in sediments and biofilms. The presence of bla_{NDM-1}, mcr-1 and sul1 in downstream river sediments has been persistently observed in studies in Asia, Europe and the Americas. In addition to this, antibiotic residues themselves—mainly macrolides, fluoroquinolones and sulfonamides—have been determined at concentrations that act as selective forces against environmental organisms (29, 62, 83). Another important reservoir is in the soil. Agricultural soils amended with manure, slurry or sewage sludge containing high amounts of resistant bacteria and mobile genetic elements are commonly encountered in the soil of agriculture. Heavy metals, disinfectants, and antibiotics co-occur, thus, their co-select for resistance through shared efflux mechanisms or integrons. From this perspective, the resistome goes beyond antibiotics to include a wider range of chemical stressors (32, 39, 70). Biofilms are a key mechanism of persistence. Within biofilms, bacteria show increased resistance to antibiotics and environmental fluctuations, while by being close to one another, plasmid exchange happens. Biofilm sites in hospital pipelines, WWTP reactors and natural substrates can be considered micro-niches of resistance evolution, where new combinations of genes are introduced into the environment and spread eventually to bigger populations (31, 32, 83).

2.5.4 Wildlife and Ecological Interfaces

Wildlife species in the ecosystem are sentinels and vectors of the environmental AMR transmission. Birds, mammals, reptiles, and fish pick up resistant bacteria from tainted water, soil, or food and they can themselves spread it over vast distances. For instance, cross-continent migrating birds have proven to transmit ESBL-producing *E. coli* and fluoroquinolone-resistant Enterobacterales; so flyers can function as a bridge to other ecosystems in a distant domain (12, 48, 64). Marine organisms such as mussels and fish bioaccumulate resistant microorganisms and antibiotic residues and are useful proxies for monitoring the coastal pollution. Shellfish caught in polluted sites harbor AMR determinants equivalent to those of the water sources in local urban sewers, suggestive of the environment's co-extension (22, 39, 77). Wild mammals such as foxes, wild boars, and rodents were also implicated as host-biodiversity carriers of these clinically important resistance genes that may be classed as anthropogenic or agricultural contaminants. This support in the hypothesis suggests that wildlife occupy a unique ecological junction within terrestrial, aquatic, and anthropogenic habitats. So this surveillance of free-ranging animals is very much a prerequisite to understanding the geographical movements of resistance genes in the extrastructural and extramural environment in which the organisms are not being controlled by humans (14, 40, 81).

2.5.5 The Role of Agriculture and Food Chains

The agricultural sector is a growing major driver in AMR dissemination because it is heavily relying on antibiotics in animal use and aquaculture. A ubiquitous pattern observed in some low- and middle-income countries (20, 24, 27) remains the traditional practice of using antibiotics, not only therapeutically but also as growth promoters. Animal waste, which is often dumped onto fields as fertilizer, introduces antibiotic residues and resistant-microbial material to soil ecosystems. Runoff produced on farms then carries these contaminants into the watercourses, facilitating the spread of the resistance to downstream communities and wildlife (29, 39, 70). The problem is a similar one when it comes to aquaculture systems. In areas with no vaccines or biosecurity, intensive fish farming is frequently fueled by an excess of antibiotics that guard against bacterial infection. Accumulation of residue in sediments facilitates the persistence of multidrug-resistant bacteria such as *Aeromonas* and *Vibrio* species. Additionally, medicated feed and prophylactic treatments facilitate gene transfer between commensal and pathogenic microbes, effectively bridging aquatic and terrestrial AMR reservoirs (32, 65, 83). The human food chain

represents another transmission route for humans. Resistant bacteria originating in animals or the environment can colonize or contaminate food products during slaughtering, processing, or distribution. Several outbreaks of foodborne *Salmonella* and *Campylobacter* strains resistant to fluoroquinolones or third-generation cephalosporins have been linked to poultry and dairy products, illustrating how AMR can bypass medical settings altogether (65, 89, 92).

2.5.6 Regional Focus: Europe and Italy

AMR prevalence in Europe is a north–south gradient, with resistance being higher in Southern and Eastern countries. Rates of carbapenem-resistant *K. pneumoniae* and ESBL-producing *E. coli* are high in Italy, Greece, and Romania, according to ECDC data. These tendencies include higher antibiotic consumption and variable adherence to infection control measures (4, 54, 82). Across Europe EFSA, EMA and ECDC work together in the Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) to monitor trends in various areas. Results reveal that veterinary sales of antimicrobials are linked with patterns of resistance in zoonotic and commensal bacteria from animals and humans (4, 5, 54). Environmental AMR surveillance in Italy is coordinated by the Istituto Superiore di Sanità (ISS) with cooperation from ISPRA and regional authorities. The study identified a number of resistance genes in municipal wastewater, river sediments and coastal marine environments, including *bla*CTX-M, *bla*KPC and *mcr* variants. This finding confirms that treated effluents continue to be a primary route for the spread of AMR even with advancing purification technologies (29, 32, 72). In the Ligurian region, characterized by high population density along a narrow coastal strip, the interaction between human activity and marine habitat is a decisive driver for AMR transmission. This is confirmed by identification of ESBL- and carbapenemase-producing Enterobacterales on marine mussels (*Mytilus galloprovincialis*), along the Ligurian coastline, linking coastal pollution to urban effluent discharge. Hence, these sentinel organisms represent an early warning system for environmental AMR monitoring in the Mediterranean basin (22, 67, 77).

2.5.7 Emerging Surveillance Tools and Analytical Approaches

Considering the complex character of AMR ecology, modern surveillance technologies are required. The conventional culture-based approaches are not exhaustive because they do not consider the whole spectrum of environmental resistance determinants while being useful for clinical diagnosis. Metagenomics, shotgun sequencing and quantitative PCR (qPCR) have enabled researchers to directly profile resistomes on environmental samples without media cultivation. As examples, these tools show the presence of “silent” resistance genes and new variants not yet discovered in clinical isolates (32, 71, 91). Bioinformatics pipelines integrating genomic, spatial and epidemiologic data have also created opportunities for predictive modeling. This has given rise to **GIS** and machine learning algorithms to create AMR hotspots mapping, predictive dissemination routes, and high-risk interface mapping of human and environmental systems (32, 72, 83). On the policy side of the equation, incorporation of environmental AMR surveillance mechanisms into One Health national action plans (e.g. Italy’s PNCAR 2022–2025) is a step in the right direction. This integration ensures that emergence of resistance in water, soil or wildlife, and identification can occur early, prior to development into clinical threats (3, 43, 57).

2.5.8 Conclusions

Antimicrobial resistance (AMR) is among the most serious and evolving global health problems, resulting from an interplay between selective pressure on antibiotics, adaptation by microbes, and spread in the environment. The literature review and cases reviewed in this chapter highlight the realization that resistance determinants are now beyond clinical boundaries and embedded into ecological traits for application within human, animal, and environmental systems (18, 31, 36). The geographical and environmental context in which we describe AMR can demonstrate that the proliferation of resistant bacteria and resistance genes is not random but more closely related to human population density, healthcare activity, wastewater effluent discharges, and agricultural strain. The combination of multidrug-resistant bacteria in high anthropogenic activity regional areas (i.e. coastal and industrial regions) has caused contamination of effluents produced by hospitals, farms and urban sewers, which now cluster under a cohesive bacterial ecosystem. The interrelationship is exemplified by the dissemination of ESBLs, carbapenemases and plasmid mediated resistance genes (*mcr-1*, *blaCTX-M*, *blaKPC*) thus further establishing that AMR is a trans-ecosystemic phenomenon (4, 8, 29). A good

example of this area is the Ligurian region. Liaison of this situation with the high composition of the coastal area's population, with its increasing aging population as well as its higher level of maritime and industrial activities, the region is conducive to the transmission of resistance factors between clinical and environmental reservoir. Regional surveillance and environmental surveillance projects (such as Mare in Rete), also have demonstrated the presence of clinically applicable AMR genes in seawater, mussels and sediments implying cross-talk of AMR between human population and ecosystem. This underscores the pressing requirement for integrated and complementary One Health surveillance systems to detect, monitor and prevent AMR in all compartments (22, 67, 77). In this respect, human mobile systems, animal mobility, and the movement of goods are also significant contributors to the geographical spread of resistant strain organisms. Maritime transport, movement of wildlife and food trade networks are probably sources of the dissemination of resistance genes. The globalization of AMR thus necessitates local and global coordinated responses that mix public health interventions with environmental supervision and an ethical stewardship of antibiotics (18, 36, 73). This chapter will further enhance the interdisciplinary efforts of microbiology, molecular ecology, and geospatial epidemiology. Metagenomic and geostatistical approaches to map the spatial distribution of resistance genes have the potential to reveal hidden ways of transmission and to inform more effective methods of mitigation. Simultaneously, data from clinical, veterinary, and environmental sources need to be combined into explanatory modeling to move from descriptive and rough estimates of the spread of AMR toward predictive models of AMR (32, 71, 83). Conclusions From the geographical and ecologically perspective in AMR, the phenomenon is not just due to clinic errors in the use of antibiotics; it is a consequence of the ecological disequilibrium. The results here suggest that AMR must be addressed with a multinational but on a local level One Health approach targeting environmental resistance: intersectoral partnership and preventive intervention in lieu of passive activity. Liguria, with its high concentrations of population, active coastline and complex system of multi-stakeholder research, is a model area for integrating scientific evidence of AMR with public policy for a sustainable response to the disease (36, 43, 67).

Chapter 3 – Environmental and Wildlife AMR

3.1 Introduction

Antimicrobial resistance (AMR) surveillance and characterization of wildlife and environmental matrices is a key frontier area within the global One Health framework. At an age where the epidemiological and ecological relationships between human, animal and environmental health are becoming even more evident, regional studies help in understanding the mechanisms for the emergence and spread of resistance at the local level. In highly varied parts of the world such as Liguria, where humans, coastal ecosystems and wildlife all meet across a geographically narrow yet biologically rich space, these interactions become particularly salient (18, 32, 36). Human, domestic animal, wildlife, and environmental reservoirs have been continuously sharing microorganisms and genetic material; traditional epidemiological barriers have become blurred. Wastewater, agricultural runoff, and untreated effluents form “bridging zones” where resistant bacteria and resistance genes circulate fluidly across compartments. Such ecological interdependence emphasizes the importance of comprehensive surveillance that can identify clinical and non-clinical sources of resistance (29, 32, 93). At a global level, a number of institutions have highlighted the need for environmental and wildlife surveillance. The World Health Organization (WHO), World Organisation for Animal Health (WOAH), as well as Food and Agriculture Organization (FAO) advocate a One Health-based monitoring system across all areas of the economy. One of the pillars of the WHO Global Action Plan for AMR (2015) and the Tripartite AMR Framework is the need to embed environmental data within public health risk assessment (1, 2, 98). In the same way the European Centre for Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA) manage harmonized measures: EARS-Net and EFSA-AMR monitoring integrate human and animal surveillance under the One Health framework (4, 54, 57). Nevertheless, the environmental and wildlife aspects of AMR surveillance are less integrated than human and veterinary surveillance despite these advances. Natural ecosystem data are often fragmented, generated through isolated academic projects rather than extensive monitoring networks (32, 72). But wildlife can also be sentinels and vectors – a reflection of the levels of contamination in habitats – and they can pass resistant bacteria over large regions of space. For example, migratory birds can carry resistant organisms over continents, and terrestrial mammals and aquatic species track local exposure to anthropogenic contaminants (12, 46, 48). Within the Italian context, the National Action Plan on Antimicrobial Resistance (PNCAR 2023–2025) clearly recognizes the environmental and wildlife components of AMR

surveillance and is expected to foster inter-institutional collaboration among the Istituti Zooprofilattici Sperimentali, universities and regional health authorities (3, 43, 67). These facilities are central to implementing One Health approaches that incorporate research, diagnostics, and environmental monitoring. Environmental microbiology combined with epidemiological data from human and animal sectors provides the means to pinpoint cross-sectoral transmission routes and emerging hot spots (32, 36, 72). The northwestern part of Italy — Liguria — serves as a particularly interesting model for such studies. Its small geographical contours — bounded by the Apennines and the Ligurian Sea — form a patchwork of urban, agricultural, and natural worlds nestled nearby. This configuration enhances human, domestic animal, and wildlife interactions. The frequent environmental pollution of the coast, discharges of wastewater from all sources and crowded ports and tourism activities also contribute to the constant microbial exchange of terrestrial and marine ecosystems (22, 44, 77). Moreover, the fact that Liguria acts as both a migratory corridor for birds and as a habitat for terrestrial and marine fauna (cetaceans, seabirds) creates unparalleled opportunities to measure AMR spread through ecological gradients (14, 67, 81). Studies reported in regional and national projects, such as Mare in Rete and other Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta studies, have shown multidrug-resistant bacteria, and resistance genes in different matrices — mussels, seawater, and wild mammals. This finding supports international evidence that coastal environments are convergence zones where human pollutants interact with native microbial communities (22, 63, 77). Monitoring these environments is therefore important for public health, marine ecosystem conservation, and food safety. The experimental studies presented through this chapter will further this research and will also address the distribution and diversity of antibiotic resistant bacteria and resistance genes in wildlife and environmental samples collected in Liguria. The study aims to establish patterns of resistance circulation and possible environmental reservoirs and pathways of interspecies transmission using microbiological, molecular and ecological methods. In addition to its regional relevance, this work also aims to advance a broader understanding of AMR ecology along Mediterranean and European geographies. High anthropogenic pressure, dense coastal populations and intensive maritime trade in the Mediterranean basin make the basin a hotspot for AMR emergence worldwide (29, 36, 83). The semi-enclosed marine environment promotes the build-up of pollutants, and the soil microbial population and transfer of genes are shaped by climatic factors (i.e., high temperatures and seasonal variability in rainfall). Finally this chapter supports the argument for focusing on AMR research on a multidisciplinary basis, within an ecosystem-driven framework—one that would reach well beyond academic boundaries, and would treat the One Health paradigm as a central scientific priority and policy imperative (18, 32, 79).

3.2 Environment as a Reservoir of AMR

The environment is a principal underreported factor in antimicrobial resistance (AMR), though its role is overlooked. Previously regarded merely as the site of passive accumulation of antibiotic residues and antibiotic resistant bacteria, it has come to be characterized as a dynamic and active reservoir where resistance genes are captured, transmitted, and evolved. The environmental resistome consists of the entire set of resistance genes implicated in microbial communities and is one of the earliest genetic reservoirs of antibiotic resistance, with origins many millions of years before the clinical use of antibiotics (11, 32, 91). Human pressures have altered this equilibrium and the environment as it has always existed—where environmental ecosystems have become powerful amplifiers and dispersal factors of resistance determinants, spanning biology and geography, moving from natural resistomes to anthropogenic amplification. Antibiotic resistance genes (ARGs) are considered natural defense mechanisms in soil and aquatic bacteria producing antimicrobial compounds which are able to take place in pristine environments. This early coevolution established a dynamic equilibrium where selective pressures are localized and gene exchange is relatively confined to specific niches (11, 23). Industrial manufacture and widespread use of antibiotics in human and veterinary practice have disrupted this equilibrium. Environmental circulation of antibiotic residues from wastewater sources, agricultural effluents, aquaculture effluents, and solid waste has placed selective pressure by widespread exposure to them. Sub-inhibitory concentrations alter microbial ecology through horizontal gene transfer (HGT) and maintenance of resistance genes (29, 32, 62). In both soils and water, bacteria frequently share plasmids, transposons and integrons carrying ARGs, forming a genetic network between environmental and clinical microbiomes. As a result, resistance genes from either soil actinomycetes or saprophytes are transmitted to pathogenic bacteria (32, 70, 91).

The introduction of antibiotics and resistant bacteria into the environment occurs via multiple interconnected pathways. WWTPs collect effluents from hospitals, homes, and factories and discharge a combination of antibiotics, disinfectants and resistant microorganisms as mixtures in wastewater treatment plants (WWTPs). Even high standard treatments do not completely eradicate these pollutants; ARGs and residual antibiotics persist and contaminate treated effluents and sludges, often discharged into rivers or used in agriculture, long-term drivers of soil and water resistance (29, 39, 93). There is also a heavy linkage with the agri industry. Fertilizing manure, slurry, and sewage sludge feed terrestrial

ecology, which contains antibiotics and resistant flora. These compounds can persist in soils for months, influencing microbial community structure and enabling gene transfer. Pesticides, heavy metals and biocides exert co-selective pressures—non-antibiotic stressors that enhance the preservation of multidrug resistance by selecting mobile genetic elements with metal and antibiotic resistance genes (32, 39, 70). Aquaculture systems, particularly antibiotics used in the industry of fish and shellfish farming, enable the spread of AMR. Residual bacteria and resistant strain releases from aquaculture ponds into adjacent marine habitats, and their colonizing communities come to co-mingle with native microbial communities. Coastal zones, estuaries and sediments form ecological sinks that trap forms of resistance genes, sustaining their long-endurability (32, 65, 83).

The microbial community in the environment is a dynamic system that can react to anthropogenic stress. Biofilms, defined as bioorganism-based structures harboring microbial communities within extracellular polymeric matter, can accommodate for transfer of genes and resistance to environmental stress. Bacteria survive in biofilms in the presence of elevated antibiotic doses and can exchange plasmids and integrons via conjugation, which underlies ARGs to persist in the absence of direct selective pressure (31, 32, 83). HGT—through conjugation, transformation and transduction—remains the dominant mode for transmittal of resistance genes in the environment. Mobile genetic elements, especially class 1 integrons and insertion sequences, induce genomic integration and ARGs rearrangement. Metagenomic studies have demonstrated considerable overlap between environmental and clinical resistomes, suggesting that the environment in a large part constitutes a reservoir of resistance mechanisms as well as an incubator of novel combinations (32, 62, 71). This further is amplified by co-selection. Exposure to one class of contaminant can maintain resistance to others when determinants to antibiotics, metals, and biocides coexist on the same plasmids or transposons. Copper or zinc, both commonly employed in farming and animal feeding, can also select for multidrug resistant bacteria, even in the absence of antibiotics (39, 70, 96).

The dynamics of AMR selection and maintenance are unique to certain environmental compartments. Long term hosts of ARGs for long period of exposure to are animals are soil organisms with animal manure or wastewater. Soil bacteria *Pseudomonas*, *Bacillus* and *Stenotrophomonas*, for example, may be carrying resistance genes that are clinically relevant and are transferrable to human pathogens (29, 32, 39). Freshwater systems such as rivers, lakes and groundwater act as links between terrestrial and aquatic habitats and act as entry points for the movement of antibiotic residues and ARGs away from urban and agricultural environments and overland to the coastland. Sediments especially are stable environments

for microbial growth and HGT. As seen with the discovery of blaNDM, blaKPC and mcr genes in river sediments across Europe and Asia (29, 34, 70) the anthropogenic contamination is spreading on a global scale. Marine and coastal environments are now acknowledged as important AMR hotspots. The sea receives resistant microorganisms and ARGs from marine pollutants, shipping and aquaculture. Marine sediments, shellfish and biofilms can also be recognized as environmental AMR proxies and offer insights into the role terrestrial pollution plays on marine microbial habitats. In the Mediterranean basin, semi-enclosed marine regimes and vigorous human activity favors the growth of ARGs and the retention of disease resistant bacteria in the coastal environment (22, 36, 77).

The One Health approach necessitates a combined surveillance of human, animal and environmental systems, acknowledging the bidirectional transmission of resistance. Environmental monitoring pinpoints new threats and may support actions in preventing health problems such as better wastewater treatment, control regulation regarding agriculture, and pollution containment (18, 32, 98). Nevertheless, there are still very few practical applications of environmental AMR surveillance. Although monitoring programmes such as the Global Antimicrobial Resistance and Use Surveillance System (GLASS) exist, the majority of national and international surveillance still concentrates on clinical data. Environmental parameters such as antibiotic residues, ARG abundance, and microbial community profiles need to be included in order to enable risk assessment to take a comprehensive approach (1, 32, 72). Achieving this goal will require cooperation between health authorities, environmental agencies and scientific institutions. The ‘environment’ is not an ancillary building block of AMR crisis; it is the interface point of genetic, ecological and human influences. As well as being a sink and reservoir for resistance genes, the environment acts as a bridge between clinical and natural ecosystems. An integrated approach that includes microbiology, ecology, environmental chemistry and public health will be needed to interpret it. This allows a deeper exploration of the correlation between the environmental resistome and the development of a sustainable AMR mitigation strategy to protect ecosystems and human health (32, 36, 98).

3.3 European examples: comparing experiences

A number of other European countries have initiated environmental AMR surveillance, which provides some valuable practical aspects for the Italian context. The RESET (Resistance in Environmental Samples of Enterobacteriaceae from Terrestrial Animals) project in Germany explored the spread of resistant Enterobacteriaceae in the context of environmental and wildlife samples. ANSES integrated programmes have integrated environmental monitoring with veterinary surveillance in France. In the Netherlands, MARAN (Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals) is a good example of One Health using a system for monitoring humans, animals, food and environment (37, 57, 79). At a European level, the Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) and the new European PARC (Partnership for the Assessment of Risks from Chemicals) programme have enhanced integrated research and collection of environmental data. These examples demonstrate the importance of institutional cooperation and investment in environmental surveillance in order to identify spread of AMR and to prepare suitable interventions (32, 57, 59).

3.4 Future perspectives and One Health approach

The latest report from the European Centre for Disease Prevention and Control (ECDC) on antimicrobial resistance in EU/EEA countries shows that bloodstream infections due to resistant bacteria are still common and, in some cases, rising. For instance, MRSA (methicillin-resistant *Staphylococcus aureus*) incidence has decreased but remains relevant; *E. coli* resistant to third-generation cephalosporins remains above 2030 targets, and *K. pneumoniae* resistant to carbapenems has increased substantially (4, 54, 82). One valuable case to get a sense of this global perspective on AMR warfare is Montenegro, visited by the ECDC in 2023 with a One Health mission. The country has well-established national strategic plans on paper but has issues such as limited cross-sectoral coordination, weak integration of human, veterinary, and environmental sectors, and limited microbiological surveillance capacity. Continued high community use of antibiotics, including critical drugs such as fluoroquinolones, and poor information systems are also barriers to implementation (54, 57, 82). The comparison indicates that although Italy has a more comprehensive surveillance system, in-depth knowledge of the environmental element and interoperability of disparate regional as well as national information infrastructures is still required.

Antibiotic resistance is advancing globally at an ever-quicken pace and its effective control will only be possible if we take a comprehensive, integrated approach to environmental, animal health, and human health aspects (18, 36, 79). In Italy, intersectoral technical tables have been established in favour of the National Reference Centre for Antibiotic Resistance (CRN-AER) and on projects led by Istituto Superiore di Sanità. Plans for the future encompass the creation of additional robust early warning tools, gene flux modelling in diverse ecological compartments, and the development of interventions in livestock farms, industrial plants, and urban discharges to mitigate resistance (3, 43, 67). It is also important to establish processes of scientific communication and training such that health professionals, farmers, and citizens know about the correct and ecologically sound way in which antibiotic use can be conducted. One Health applications are successively applied on the basis of appropriate unification of knowledge from different fields or from different disciplines to bridge the gaps in current knowledge (41, 73, 79).

3.5 International comparison: the case of Montenegro

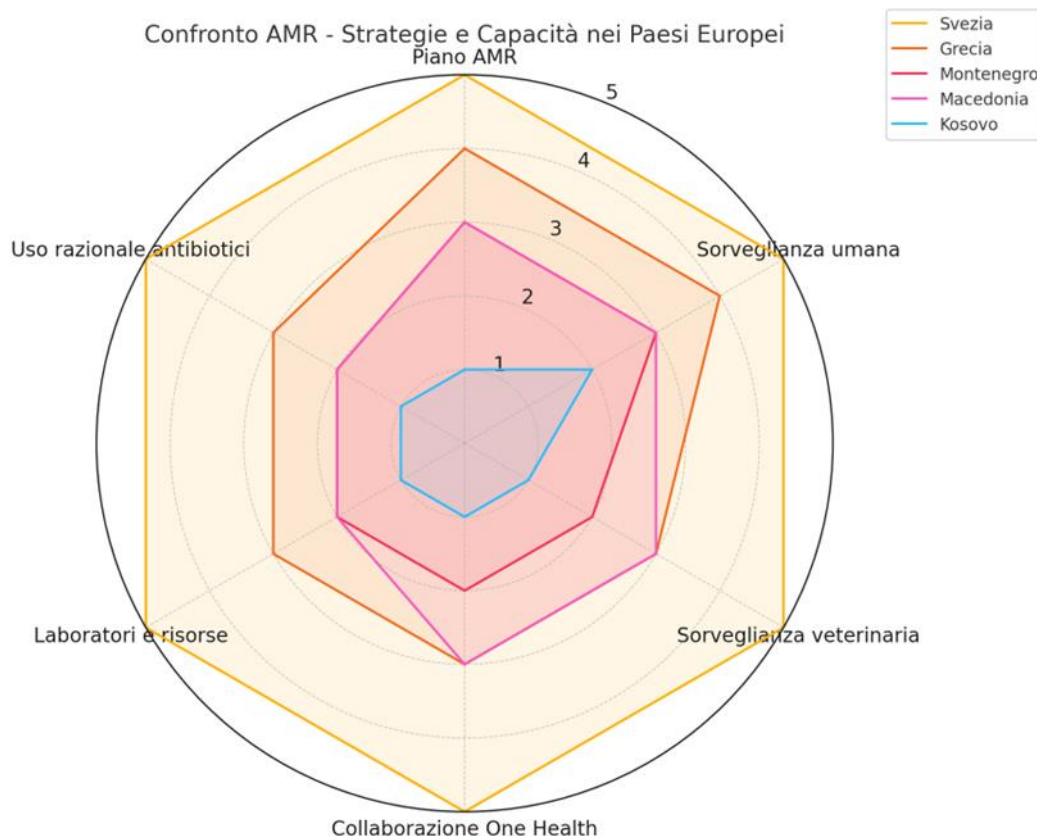


Figure 3.6 – Incidence of the main AMRs in Europe (2019–2023) and 2030 targets

The radar graph (not pictured here) summarizing AMR responses in several European and neighbouring states shows stark contrasts. For all markers, Sweden is on the highest scale for all indicators, indicating that AMR regulation, surveillance, and the rational use of antibiotics are mature areas. Greece also has intermediate–high degrees of implementation but serious obstacles in terms of One Health cooperation and antibiotic prescribing remain, with the need for good practice in appropriate antibiotic prescription. However, Montenegro and North Macedonia are placed in intermediate positions with parts of the structure set out, but room to improve to achieve full veterinary surveillance, laboratory capacity, and inter-sectoral collaboration. Kosovo ranks the weakest of countries examined, requiring an urgently implemented AMR plan, enhanced surveillance, and higher training and laboratory/tools development levels (54, 57, 82). Kosovo has participated in the AMR One Health initiative organized by the EU in 2023. At that time, the country was beginning to shape its response to AMR, which saw some institutional steps being implemented but was also afflicted by many structural problems. There was still a need for good microbiological surveillance systems, inadequate for both human and animal health; antimicrobial prescribing remained largely empirical; and resources and training of laboratories were very limited.

Collaboration in the Health sector was still weak, even though the authorities were motivated and asked for technical assistance to enhance governance frameworks (57, 82). Another illustrative case is Montenegro, one of the candidate countries for EU accession. The ECDC–WHO One Health visit in 2023 brought to light some promising signs but also major gaps in AMR control: there was a national AMR action plan, but not enough health facility coverage; monitoring was partial, fragmented, and uneven; and non-prescription antibiotic use was prevalent, especially in veterinary settings. Sectors were cooperating but there was little coordination at the ministerial level (54, 57). Sweden, in contrast, is often referenced as a model of best practice in antibiotic policy. According to joint ECDC–WHO–European Commission reports, Sweden has developed a comprehensive One Health solution, incorporating human, veterinary, and environmental health. Under strong political and public support, the STRAMA programme, born in the 1990s, promoted rational use of antibiotics, aided delivery of therapeutic guidelines and rapid diagnostics, and developed advanced national microbiological surveillance. Consequently, Sweden has some of the lowest resistance in Europe (24, 54, 79). North Macedonia is a transitional setting and has developed, formally and unevenly, a national AMR strategy implemented by administrative regions. It's marked by weak laboratory capacity, high empirical antibiotic prescription levels, and disjointed human, veterinary, and environmental sectors. Healthcare and veterinary personnel training has been acknowledged as a priority and authorities have asked for international aid towards reinforcing their One Health strategy (57, 82). Greece continues to be one of the harshest European contexts for AMR. Joint ECDC–WHO missions in 2023 revealed very high resistance rates, particularly to carbapenems and colistin, widespread inappropriate antibiotic use in hospitals and the community, inconsistent monitoring of microbiological data, and absence of coordination between human, veterinary, and environmental sectors. Public awareness and professional training were similarly found to be insufficient, in spite of the existence of a national AMR plan with only partial implementation (54, 66, 82). Taken as a whole, these international cases provide evidence that strong governance together with the One Health approach are critical to effective control of AMR. Whereas countries with longer-term stewardship programmes, combined surveillance, and government commitment have better performance, resource-limited and changing systems need longer-term international assistance to have similar results (18, 41, 73).

3.6 AMR in Long-Term Care Facilities (LTCF) in Europe

Long-Term Care Facilities (LTCFs) have a defining and, at times neglected role in the context of AMR epidemiology. These facilities—which include nursing homes, rehabilitation facilities, and chronic care hospitals—offer daily health care and help to residents with chronic illness, older age, and all the problems that come with disability. Their residents are particularly vulnerable: older persons, frequent hospitalisations, comorbidities, invasive devices, and extended exposure to antibiotics make them special precursors for colonization and the spread of multidrug-resistant organisms (MDROs). LTCFs are increasingly considered in Europe as "epidemiological bridges" between hospital, community and environment. They serve as a reservoir and amplifier of resistant pathogens, including ESBL-producing *E. coli*, *K. pneumoniae* harboring KPC, NDM and OXA-48, *P. aeruginosa*, *A. baumannii* and methicillin-resistant *S. aureus* (MRSA). ECDC reports suggest that AMR prevalence in LTCFs varies extensively between Member States because of differences in its stewardship, infection prevention and healthcare readiness. The HALT (Healthcare-Associated infections in Long-Term care facilities) surveys developed by ECDC since 2009 give the most evidence across countries. It was estimated that between 4% and 6% of LTCF's residents in Europe already bear at least one MDRO, with the highest prevalence found in Southern and Eastern Europe, including Italy, Greece, Romania, and Spain (54, 82). ESBL-producing *E. coli* continues to be the most common MDRO in European long-term care facilities, and colonisation rates were also noted to exceed 25% in some Italian and Greek institutions. In Northern Europe MRSA prevalence is comparatively low based on established infection control programs, however is still relevant in the Mediterranean countries. Recently, vancomycin-resistant VRE (*Enterococcus faecium*) and carbapenem-resistant Enterobacterales (CRE) have particularly raised concerns for MDROs in LTCFs due to their association with serious infections and high mortality (31, 66, 82).

3.6.1 Environmental interfaces and One Health considerations

LTCFs have an ecological reach beyond their walls. Wastewater from such facilities is enriched with antibiotics, disinfectants and resistant microorganisms that find their way into municipal treatment plants, adding to the environmental resistome. Clinically relevant ARGs such as blaKPC, blaNDM and mcr-1 have been identified in the wastewater from healthcare and LTC facilities in multiple European countries such as Italy, Germany and the Netherlands (29, 32, 93). These results underscore the necessity of incorporating LTCF wastewater into environmental monitoring initiatives, particularly in highly populated areas. From the perspective of One Health, LTCFs are micro-ecosystems where human, microbial and environmental interactions converge. There is a combination of frail human hosts and intensive use of antimicrobials along with environmental contamination that makes these organizations sentinel nodes for AMR emergence. Acknowledging LTCFs as one aspect of the wider AMR ecology facilitates development of initiatives that penetrate communities and environments beyond hospitals (18, 36, 79).

3.6.2 Surveillance and control in Europe

Most European programs focus on strengthening AMR surveillance and prevention in LTCFs. The ECDC HALT programme is still the principal cross-country system to monitor infection rates, antimicrobial prescriptions and MDRO prevalence with standardised estimates. Complementary projects, such as COMBACTE-MAGNET, EU-JAMRAI and EURGen-Net have produced critical genomic datasets on the mechanism of pathogen spread and the evolution of resistance in long-term care settings (57, 59, 82). Nationally, integrated stewardship programmes that connect hospitals, LTCFs and primary healthcare have been established in countries such as the Netherlands, Sweden and the UK. These include enforced infection control practices, guidelines on restricted antibiotic use, and routine microbiological review. In Italy, the PNCAR 2023–2025 specifically identifies LTCFs as priorities for AMR control, promoting surveillance via the Istituti Zooprofilattici Sperimentali and regional health authorities. In Emilia-Romagna, Lombardy and Liguria, pilot studies have demonstrated high ESBL-producing Enterobacterales and stressed the importance of systematic microbiological screening on admission and periodically thereafter (3, 43, 67).

3.6.3 Challenges and future directions

This is even as awareness is growing but there are some serious barriers:

- Under-resourced diagnostic mechanisms and reliance on empirical therapy.
- Absence of a skilled infection-control workforce and uneven implementation of standard precautions.
- Incomplete data collection, with only a few countries conducting continuous national LTCF surveillance.
- Financing and logistical limitations, particularly in privately owned and small facilities (41, 73, 82).

To fill these gaps, LTCF surveillance networks should be established on a national basis, to be integrated into the existing ECDC and WHO Europe infrastructures; strengthening environmental monitoring (surfaces, air, and wastewater) and antibiotic stewardship training for physicians, nurses, and caregivers. New technologies in molecular diagnostics and digital platforms for infection tracking could also help detection and prevention (66, 73, 91).

Long-Term Care Facilities represent one of the most critical — and often overlooked — fronts of the European AMR crisis. They connect hospitals, communities and the environment and act as continuous sources for resistance evolution and transmission. Combating AMR in LTCFs requires a comprehensive One Health approach that integrates clinical, environmental and policy-level interventions, not just traditional infection control. Enhancing surveillance, harmonising interventions among Member States and embedding environmental monitoring in LTCF regulations will be crucial for reducing the AMR burden and protecting some of Europe's most vulnerable populations (18, 31, 54).

3.7 Regulations and environmental surveillance in Italy

In this regard, Italy has gradually adopted the One Health approach to mitigate antimicrobial resistance (AMR). This paradigm recognizes that human, animal, and environmental health are interconnected. National institutions have recently adopted measures to develop environmental monitoring systems and to support the integration of monitoring with existing AMR surveillance networks. Nonetheless, challenges persist: standards, coordination, and data sharing need to be enhanced across different sectors.

National Framework: The cornerstone of Italy's AMR policy is the National Action Plan on Antimicrobial Resistance (Piano Nazionale di Contrasto dell'Antimicrobico-Resistenza – PNCAR), first published in 2017 and revised for 2023–2025. The PNCAR aligns with the Global Action Plan for AMR (WHO, 2015) and the EU One Health Action Plan against AMR. It sets strategic objectives in five pillars:

- Strengthening surveillance and monitoring systems.
- Promoting responsible and prudent use of antimicrobials in humans and animals.
- Increasing infection prevention and control.
- Raising awareness and education.
- Facilitating research and innovation from a One Health perspective.

For instance, the revised PNCAR 2023–2025 emphasizes environmental monitoring, which legally defines the role of the environment in AMR governance. The program includes monitoring environmental matrices (soil, water, wildlife, and surface waters) and antimicrobial residues, resistant bacteria, and resistance genes.

At the organizational level, policy and coordination of AMR are conducted by the Ministry of Health (Ministero della Salute), and the Istituto Superiore di Sanità (ISS) is established as the national reference for AMR surveillance with technical-scientific assistance. The Istituti Zooprofilattici Sperimentali (IIZZSA) network will be integral to veterinary and environmental surveillance, connecting research and field activities in all regions. This model facilitates territorial autonomy across all areas of Italy, aligning local ecological settings with national strategies.

Environmental Surveillance Systems: Environmental surveillance in Italy consists of various matrices measured via different systems of measurement that include the country's ecological and socio-economic diversity.

Wastewater Surveillance: Multiple regional programs, together with ISS and local Agenzie Regionali per la Protezione dell'Ambiente (ARPA), monitor urban wastewater and detect antibiotic residues, resistant bacteria, and resistance genes. Wastewater treatment plants (WWTPs) are important control sites for integrating clinical and community-derived resistomes. In pilot studies in Lombardia, Emilia-Romagna, and Liguria, various clinically relevant ARGs were isolated, such as blaCTX-M, blaKPC, and mcr-1, and the detected markers within various waters and effluents confirm the persistence of resistance determinants even after tertiary treatment.

Surface and Coastal Waters: The IZS network conducts microbiological and molecular analyses of rivers, lakes, and coastal regions in cooperation with ISPRA and ARPA agencies. Surveys monitoring the Ligurian and Tyrrhenian coastlines have identified the presence of multidrug-resistant Enterobacterales and Pseudomonas spp. in marine sediments and seawater, substantiating the environmental dissemination of resistance genes at anthropogenic coasts.

Wildlife and Fauna Monitoring: Working together with the IZS network, Italy is one of the few countries in Europe that has organized AMR monitoring in wildlife. Bioindicators for environmental pollution are observed in wildlife species, such as wild boars, seabirds, and small mammals. Wild animals have been found with ESBL-producing *E. coli* and carbapenemase-producing *K. pneumoniae*, emphasizing the sensitivity of natural ecosystems to human-induced environments.

For food and aquaculture systems, environmental surveillance also encompasses food matrices (e.g., fish, shellfish, and mollusks), thus linking information on marine pollution with food safety. Campaigns such as Mare in Rete, carried out by the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, have provided insight into mussels (*Mytilus galloprovincialis*) serving as sentinel hosts for coastal pollution and AMR dissemination.

It is relevant to consider the Italian environmental AMR surveillance within the larger European surveillance and integration with international and European frameworks in this matter. The European Antimicrobial Resistance Surveillance Network (EARS-Net), provided by ECDC and EFSA networks, offers a common operating framework for data collection and reporting among EU Member States. Italy contributes human, veterinary, and food service sector data to these platforms and is working on projects to incorporate environmental datasets. It also participates in joint reports of the JIACRA, led by the ECDC, EFSA, and EMA, linking antimicrobial use and resistance patterns in different areas. At the international level, Italy partners with WHO and WOAHA programs, adding to international databases of AMR and antimicrobial consumption and fulfilling GLASS requirements.

Challenges and Future Directions: Despite significant achievements, the full deployment of environmental AMR surveillance in Italy faces several challenges:

- **Fragmented Data:** Environmental data are collected by different entities (ISS, ISPRA, ARPA, IZS) using various sampling, laboratory, and reporting methods, making integration and comparison difficult.
- **Lack of Standard Protocols:** Unlike clinical AMR surveillance, environmental monitoring lacks harmonization on sampling frequency, target matrices, and molecular markers. Standard protocols need to be developed to ensure data comparison across geographical regions and over time.
- **Resource Scarcity:** Environmental surveillance depends on more complex equipment, molecular tools, and trained personnel. Costs and regional differences in laboratory staff may affect continuity and coverage.
- **Coordination Across Institutions:** Successful AMR governance is multi-disciplinary and requires that the ministry (Health, Environment, Agriculture), regional authorities, and research institutions work closely. In the era of regional and national One Health cooperation, stable One Health coordination platforms are essential for data exchange and policy harmonization.

Italy is, however, moving in this direction. The PNCAR 2023–2025 specifically requires the inclusion of environmental data in national reports and is fostering the use of metagenomic methods to identify resistomes. Early research consortia are exploring high-throughput sequencing and bioinformatics methods of mapping the environmental spread of AMR.

Conclusion: Italy's case offers both a window into the success of and the challenges to the adoption of One Health for monitoring AMR. The integration of environmental monitoring in the PNCAR and the active participation of the IZS network represent significant milestones. Full effectiveness will have to be achieved, however, by continued investment, methodological convergence, and intersectoral collaboration for the long term. By tightening up its environmental surveillance framework, Italy will be a model for other countries that want to realize the One Health principles and link environmental microbiology and public health policy.

3.8 Local considerations and perspectives

The Mare in Rete project, comprising ten Experimental Zooprophyllactic Institutes sampling mussels along the Italian coasts (Liguria included) from 2022 to 2023, studied the antibiotic resistance genes (ARGs) with metagenomics and advanced bioinformatics analysis. Samples were divided into seasonal pools and DNA was isolated and sequenced on Illumina NextSeq 550 platforms (22, 77). The raw data were analyzed with ARGs-OAP and verified against the SARG database. Overall ARG prevalence was low, but fluoroquinolone and tetracycline resistance genes were significantly represented. Basins showed differences: the Adriatic Sea showed a greater prevalence of “multidrug” classes compared with the Tyrrhenian, which exhibited a higher presence of genes for tetracyclines (22, 77). MRPP and PERMANOVA analyses suggested a small but significant difference ($p < 0.10$) between seas. ISA results detected a significant association between the “multidrug” class and Adriatic samples. NMDS analysis revealed a partial separation of resistomes in these seas, suggesting that the composition of resistant bacterial communities is more a function of basin of origin than seasons. No distinct differences in resistomes between seasons or seasonal–geographical interactions were found (22, 77, 83). Mussels are regarded a good bioindicators for marine AMR, with an understanding of selective pressure imposed by antibiotics and other contaminants in coastal basins. Their sessile lifestyle and filter-feeding behaviour renders them ideal sentinel animals for integrated coastal surveillance in Liguria and the greater Mediterranean (22, 63, 77).

Chapter 4 – AMR in Ligurian terrestrial wildlife

4.1 Introduction

Wildlife is now recognized as a key actor in the ecology and spread of antimicrobial resistance (AMR). Resistance to bacteria can spread from free-ranging species to humans or pathogens through environmental exposure, anthropogenic excreta, livestock and anthropogenic species. The animals, of course, may also be sentinels representing the degree of environmental pollution, or can facilitate the spreading of microbes of resistant characters throughout regions (46, 64, 81). *Escherichia coli* is one of the most important bacterial pathogens at the point with a notable index. Some *E. coli* pathotypes, especially Shiga toxin-producing *E. coli* (STEC), are known to lead to severe diseases in humans, ranging from mild diarrhea to hemolytic uremic syndrome (68, 69, 85). While ruminants are considered natural reservoirs, STEC has been more recently considered as an important mediating organism among wildlife, domesticated animals, and humans within the One Health context (47, 49, 50, 97). The purpose of this chapter is to present a study from Liguria (northwestern Italy) that was carried out to identify and characterize STEC isolates from wild ruminants. The investigation aimed to evaluate virulence gene presence and AMR (antimicrobial resistance) in the isolates. The findings provide knowledge of the occurrence and public health concerns of zoonotic *E. coli* in Ligurian wildlife (21, 63, 67).

4.2 Materials and Methods

Study area and sample collection

The study took place in the Ligurian region (northwest Italy), a narrow coastal strip bounded by the Apennine mountains and the Ligurian Sea. This area is typified by a patchwork of habitats—mountain forests, agricultural valleys, and peri-urban areas—with humans, wildlife, and livestock close together (44, 67). Samples were taken from wild ruminants that were legally hunted or found dead between 2021 and 2023. Species included roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), and chamois (*Rupicapra rupicapra*). Intestinal contents as well as fecal samples were aseptically collected and kept refrigerated and transported to the laboratories of the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (IZSPLV) (21, 63, 67).

Isolation of microbes and assays of molecules.

The samples were cultured as appropriate on selective media (MacConkey and TBX agar), with biochemical confirmation of *E. coli* colonies. The DNA was isolated from the presumptive isolates and analyzed by multiplex PCR for the major virulence genes:

- stx1 and stx2 (Shiga toxins),
- eae (intimin),
- ehxA (enterohemolysin).

Positive amplicons were confirmed via gel electrophoresis and sequencing (where subtype verification was essential) (47, 55, 80, 84).

Test for susceptibility to antimicrobials.

Antimicrobial resistance was assessed by disk diffusion method according to EUCAST guidelines. A panel of representative antibiotics was selected from major therapeutic classes:

- β -lactams (ampicillin, cefotaxime),
- tetracyclines,
- fluoroquinolones (ciprofloxacin),
- sulfonamides (trimethoprim/sulfamethoxazole),
- aminoglycosides (gentamicin).

Isolates were categorized as susceptible, intermediate or resistant. The control strain was *E. coli* ATCC 25922 (4, 31, 54).

Statistical analysis.

Prevalence and resistance frequencies were described by descriptive statistics. Species differences were assessed with chi-square or Fisher's exact tests, and significance was set at $p < 0.05$ (31, 82).

4.3 Results

Prevalence and distribution

Among approximately 110 samples analyzed from wild ruminants in Liguria, around 15% resulted in *E. coli* strains that had been identified with Shiga toxin genes (stx1 and/or stx2). STEC isolates were found in all three species studied in our analyses, although roe deer were found to have slightly higher rates than fallow deer and chamois. This prevalence is consistent with reports for wild cervids in other European regions (47, 49, 50, 81).

Species	No. of samples	STEC-positive (n)	Prevalence (%)
Roe deer (<i>C. capreolus</i>)	58	10	≈17
Fallow deer (<i>D. dama</i>)	32	4	≈13
Chamois (<i>R. rupicapra</i>)	20	2	≈10
Total	110	16	≈15

Table 4.1. Wild ruminant species sampled and detection of STEC.

The presence of virulence genes among isolates is summarized below.

Virulence genes	No. of isolates	Frequency (%)
<i>stx1</i> only	5	≈31
<i>stx2</i> only	7	≈44
<i>stx1</i> + <i>stx2</i>	2	≈12
<i>stx1/stx2</i> + <i>eae</i>	2	≈12
Total STEC isolates	16	100

Table 4.2. Virulence gene profiles of STEC isolates.

Most isolates carried *stx2*, frequently associated with higher pathogenic potential in humans (68, 69, 80). The *eae* gene, indicating the capacity for intestinal attachment, was detected in a limited number of cases, suggesting a relatively low virulence profile overall in this wildlife population (47, 55, 84, 97).

Antimicrobial resistance profiles

Phenotypic testing revealed that approximately 30% of the isolates showed resistance to at least one antimicrobial class. The most common resistances were to ampicillin and tetracycline, followed by sulfonamides. Multidrug resistance (resistance to ≥ 3 classes) was observed in two isolates (≈12%).

Antibiotic class	Marker antibiotic	% Resistant isolates
β -lactams	Ampicillin	31
Tetracyclines	Tetracycline	25
Fluoroquinolones	Ciprofloxacin	10
Sulfonamides	Trimethoprim/Sulfamethoxazole	15
Aminoglycosides	Gentamicin	6
Multidrug resistance (≥ 3 classes)	—	12

Table 4.3. Antimicrobial resistance patterns of STEC isolates.

The overall level of AMR was moderate but still had to be considered notable given the absence of direct antimicrobial pressure in wild populations (12, 40, 64, 81).

4.4 Discussion

The incidence of STEC in wild ruminants in Ligurian are suggestive of free-ranging ungulates as a potential reservoirs of zoonotic *E. coli* in regions where herd density is low (47, 49, 50, 81). The overall prevalence (a factor of around 15%) was similarly in the data for other European regions including the Alps and central Spain where the prevalence among wild deer could be in the order of 10–20% (47, 49, 81, 97). The large density of stx2-positive isolates confirms findings from earlier descriptions stressing that stx2 is more often a cause of severe human disease, whereas stx1-positive isolates are mostly present in animal reservoirs and with low virulence potential (68, 69, 80). Eae and ehxA genes detected were scarce, supporting the idea that most isolates are of moderate zoonotic risk (47, 55, 84). The relatively low (but detectable) resistance levels in wildlife suggest environmental exposure rather than selection for therapeutic use. *E. coli* strains that are resistant in wild animals can be potentially present because of environmental pollution from human sewage, agricultural runoff, or contact with livestock (29, 32, 39, 64). Identification of resistance to β -lactams and tetracyclines, two of the most utilized antibiotic classes in veterinary and agricultural medicine, reinforces this interpretation (24, 27, 37, 40). Consistent observations have been made among animals of the French, German, and Northern Italian variety, which

support the hypothesis that resistance genes are replicated over ecological compartments and independent of the use of antibiotics (12, 40, 64, 75, 87). Nevertheless untreated wild ruminants can serve as “sentinels” which replicate environmental contamination by resistant bacteria or residues of antimicrobials (46, 64, 81). The co-occurrence of virulence and resistance traits, even if low, is associated with the risk of horizontal gene transfer at the wildlife–livestock–human interface (18, 32, 91). Due to the large movement of wildlife over land and the close overlapping habitats in farming grounds, frequent surveillance is necessary to monitor the dynamics of the pathogens in the context of the wider One Health perspective (18, 36, 79).

4.5 Conclusions

This work demonstrates Shiga toxin–producing *E. coli* in Liguria wild ruminants as a representative of the wildlife and the AMR and zoonotic surveillance network (21, 63, 67). Stx-positive *E. coli* were about 15% common but had low to moderate resistance, most prevalent against the β -lactams and tetracyclines. Despite being characterised by a relatively mild virulence profile, the isolates were found in free-ranging populations and thus it appears that wildlife are an important point of reference and a vehicle for environmental AMR transmission (46, 64, 81). Incorporating wildlife data into regional AMR surveillance systems — such as those managed through Italy’s PNCAR framework — is a good idea, as it can provide us with early-warning signals on emerging zoonotic risks (3, 43, 67, 99). The genomic characterization of these strains, sources of environmental contamination, and interaction between wild, domestic, and human reservoirs in the One Health perspective would be necessary for further study (32, 71, 72, 91) in the context of AMR.

Chapter 5 – AMR in marine wildlife

5.1 Background and Rationale

The Mare in Rete project sought to explore the existence, diversity, and persistence of antimicrobial-resistant bacteria and resistance genes across Ligurian coastal ecosystems (22, 77). Conventional AMR surveillance in Italy has concentrated on clinical and terrestrial animal habitats, but the marine environment has been virtually unexplored; the marine environment remains a key part of the One Health continuum (3, 18, 32). Antibiotic residues are introduced into the sea by wastewater, river, and surface runoff, where they interact with native microbiota and biofilm communities (29, 39, 78, 83). This study aimed to assess whether the Ligurian Sea serves as a reservoir or amplification zone for clinically important AMR determinants especially *E. coli* and *Vibrio* that acted as sentinel species (61, 65, 83, 99).

5.2 Sampling and Analytical Approach

Seawater, sediment, and mussel (*Mytilus galloprovincialis*) samples were taken during 2022 and 2024 in 10 sites found on the Italian coast (22, 44, 77).

Microbiological studies involved:

- Enumeration and isolation of *E. coli* and *Vibrio* (ISO 16649-2; ISO 7899-2).
- Antimicrobial-susceptibility (AST) tests under disk diffusion (EUCAST 2023) with β -lactams, fluoroquinolones, tetracyclines, sulfonamides, and colistin (4, 54).
- Molecular detection of the major resistance genes (blaCTX-M, blaTEM, blaKPC, mcr-1, tet(A/B), sul1/2).
- Plasmid profiling and conjugation assays for transferable resistance.
- qPCR quantification of ARGs in environmental DNA extracts to estimate gene abundance (copies/mL or copies/g sediment) (32, 62, 71, 83).
- *E. coli* and *Vibrio* were chosen as indicator organisms because of their current use in food and water safety assessment and as vectors of clinically relevant ARGs (61, 65, 83, 99).

5.3 Materials and methods

5.3.1 Sampling

The project involved 10 Experimental Zooprophyllactic Institutes that sampled mussels in their respective areas of expertise from February 2022 to February 2023.

Specifically, the institutions involved were:

- Experimental Zooprophyllactic Institute of Piedmont, Liguria, and Valle d'Aosta
- Experimental Zooprophyllactic Institute of Umbria and Marche
- Experimental Zooprophyllactic Institute of Abruzzo and Molise
- Experimental Zooprophyllactic Institute of the Veneto
- Experimental Zooprophyllactic Institute of Lazio and Tuscany
- Experimental Zooprophyllactic Institute of Sicily (CRETAM)
- Experimental Zooprophyllactic Institute of Lombardy and Emilia-Romagna
- Experimental Zooprophyllactic Institute of Sardinia
- Experimental Zooprophyllactic Institute of Southern Italy
- Experimental Zooprophyllactic Institute of Puglia and Basilicata

Mussels were sampled monthly by each of the participating institutes, with the exception of the Istituto Zooprofilattico Sperimentale Lazio e Toscana, which only sent samples from the spring and summer seasons.

From each sample received, 10 individuals were pooled and frozen at -20°C for subsequent metagenomic analyses aimed at identifying and characterizing the resistome.

5.3.2 DNA Extraction and Sequencing

Mussel DNA was extracted according to Micic *et al.* (2002) adapted by further purifying it with a Qiagen column from the QIAamp Fast DNA Stool Mini Kit. During the extraction, the sample was kept on ice to avoid DNA degradation. The DNA quality was assayed by taking the absorbance values 260/280 and 260/230 in Nanodrop (Thermo Fisher Scientific) and visualizing the size of extracted fragments by a 1% agarose gel. The concentration of the extracted DNA was measured using the Qubit HS. The DNA concentration of the samples was normalized, and samples taken from the same area were then pooled into four pools, divided by season (Winter, Spring, Summer, and Autumn). The pools were sequenced by an Illumina NextSeq 550 sequencer. After confirming the quality of the extracted DNA, certain samples as shown in Table 1 were found to be unsuitable for sequencing.

IZS	Sampling season
Istituto Zooprofilattico Sperimentale Piemonte, Liguria e Valle d'Aosta	Summer
Istituto Zooprofilattico della Sicilia (CRETAM)	Fall
	Summer
Istituto Zooprofilattico Sperimentale Lombardia ed Emilia-Romagna	Summer
Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata	Spring
	Summer
	Fall
	Winter

Table 1: Samples found to be unsuitable for sequencing

5.3.3 Metagenomic Data Analysis

Combining the raw data, we obtained a single forward and a single reverse file, which were trimmed with Trimgalore 0.6.10 with default settings (minimum quality Phred score >20). Host removal was conducted from the trimmed reads, mapping them with Bowtie to the *Mytilus galloprovincialis* assembly (<https://www.ncbi.nlm.nih.gov/nuccore/JAWDJN000000000.1/>).

Finally, ARGs OAP (<https://github.com/alienzj/argsoap>) was used. This pipeline creates a structured analysis pipeline for the resistome of samples (by type, subtype, and gene), comparing the reads to the Structured ARGs database (SARG). This includes raw read output together with normalized read output of rRNA16s. and cells. SARG database (Xu, W., et al., 2024) segments antibiotic resistance genes into fifteen macrogroups, including the most prevalent antibiotic resistance classes and a "multidrug" group that confers more than one resistance. Each of these macrogroups is further subdivided by clustering of orthologous genes or single genes.

5.3.4 Statistical Data Analysis

The presence of identified antibiotic resistance genes and their relative abundances were analyzed by distinguishing the outputs based on the sea basin of origin (sea), sampling season, and combining the two variables (sea and season) using the following techniques:

- MRPP (Multi-Response Permutation Procedure): This is a nonparametric multivariate technique that requires no assumptions about data distribution or homogeneity of group dispersion. It tests whether there is a significant difference between two or more groups, using the distances between sampling units within each group. The test statistic (δ) is the mean distance between units within each group. Furthermore, MRPP also calculates the effect size, A , which ranges from -1 to 1: a low A value indicates a low separation between groups.

- PERMANOVA (PERmutational Multivariate ANalysis of Variance): is a nonparametric multivariate method used to compare groups of samples. This test assumes homogeneity of group variances (verified with Marti Anderson's PERMDISP2) and tests the null hypothesis that the centroids and dispersion of the groups are equivalent.

- SIMPER (Similarity Percentage): provides the contribution of each antibiotic class to the dissimilarity between groups. A high Similarity Percentage value indicates that a particular antibiotic class contributes more to differentiating the groups.

- ISA (Indicator Species Analysis): is a technique used to identify antibiotic resistance classes that are strongly associated with a group.

- NMDS (Non-metric Multidimension Scaling plot) is a graph that allows visualization of the level of similarity of the data.

The Bray-Curtis distance was used in all analyses.

5.4 Results

5.4.1 Sampling

Thirty samples were sequenced and analyzed, from nine different sampling locations (Tyrrhenian Sea and Adriatic Sea) collected during the four seasons of the year (winter, spring, summer, autumn). The number of samples was evenly distributed across the different locations and seasons.

Campione	Stagione	Località	Mare
23-69973	Primavera	Golfo di La Spezia	Mar Tirreno
23-69969	Inverno	Golfo di La Spezia	Mar Tirreno
23-69979	Autunno	Golfo di La Spezia	Mar Tirreno
23-70008	Primavera	Costa marchigiana	Mare Adriatico
23-70016	Inverno	Costa marchigiana	Mare Adriatico
23-70024	Autunno	Costa marchigiana	Mare Adriatico
23-70021	Estate	Costa marchigiana	Mare Adriatico
23-70071	Primavera	Abruzzo Bio Mare San Vito Chietino	Mare Adriatico
23-70031	Inverno	Abruzzo Bio Mare San Vito Chietino	Mare Adriatico
23-70077	Autunno	Abruzzo Bio Mare San Vito Chietino	Mare Adriatico
23-70075	Estate	Abruzzo Bio Mare San Vito Chietino	Mare Adriatico
23-70082	Primavera	Laguna Veneta	Mare Adriatico
23-70080	Inverno	Laguna Veneta	Mare Adriatico
23-70086	Autunno	Laguna Veneta	Mare Adriatico
23-70085	Estate	Laguna Veneta	Mare Adriatico
23-70092	Primavera	Litorale laziale	Mar Tirreno
23-70093	Estate	Litorale laziale	Mar Tirreno
23-70101	Primavera	Lago Faro	Mar Tirreno
23-70096	Inverno	Lago Faro	Mar Tirreno
23-70110	Primavera	Sacca di Goro	Mare Adriatico
23-70106	Inverno	Sacca di Goro	Mare Adriatico
23-70114	Autunno	Sacca di Goro	Mare Adriatico
23-70119	Primavera	Laguna di Tortoli	Mar Tirreno
23-70118	Inverno	Laguna di Tortoli	Mar Tirreno
23-70122	Autunno	Laguna di Tortoli	Mar Tirreno
23-70120	Estate	Laguna di Tortoli	Mar Tirreno
23-70125	Primavera	Golfo di Napoli	Mar Tirreno
23-70123	Inverno	Golfo di Napoli	Mar Tirreno
23-70127	Autunno	Golfo di Napoli	Mar Tirreno
23-70126	Estate	Golfo di Napoli	Mar Tirreno

Figure 5.4.1: List of analyzed samples with their corresponding metadata

5.4.2 Metagenomic Data Analysis

Although the ARGs OAP output provides results at three levels of different specificity, antibiotic resistance genes were analyzed at the resistance class level to aggregate similar results.

Few resistance matches were found, and no antibiotic resistance genes were identified in six samples (23-69979, 23-70082, 23-70092, 23-69973, 23-70080, 23-70075). The results are shown in figure below.

In this scenario of reduced antibiotic resistance gene prevalence in the analyzed samples, it emerges that fluoroquinolone and tetracycline resistance genes were the most prevalent. The Ligurian sample 23-69969 expressed antibiotic resistance genes for tetracycline and polymyxin in line with the other samples either from the Adriatic and the Tyrrhenian sea.

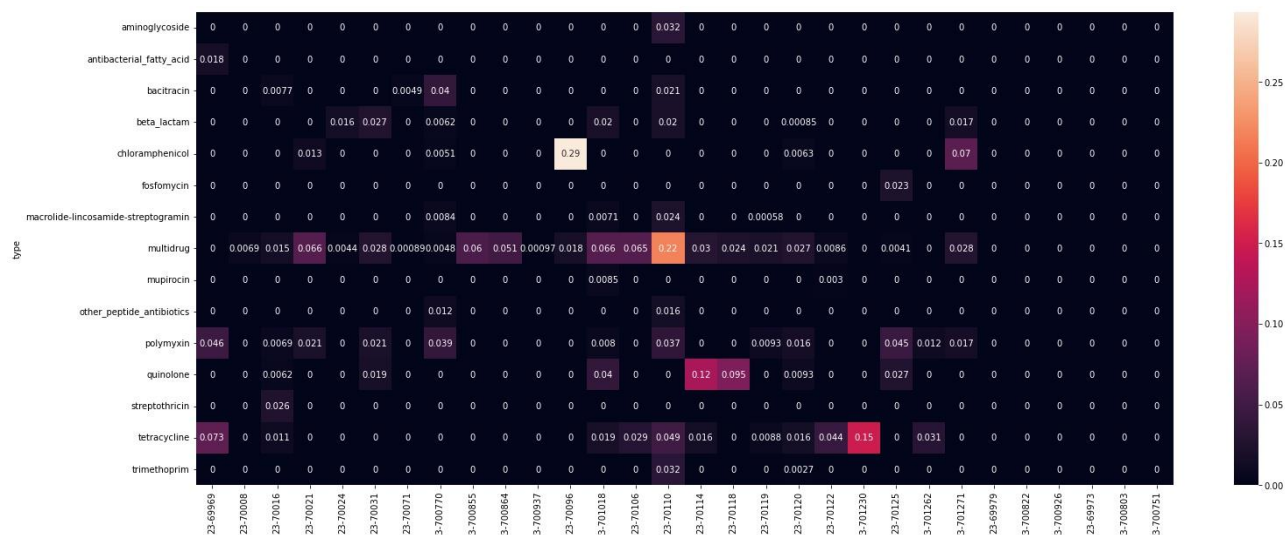


Figure 5.4.2: Heatmap of the number of ARGs normalized to 16S rRNA copies

5.4.3 Statistical Analysis

Statistical analyses differentiated the samples by season (Spring, Summer, Autumn, Winter), sea of origin (Tyrrhenian Sea and Adriatic Sea), and a variable integrating both season and origin. The sum of the resistances for each of these variables is shown in Figures a,b, and c, respectively.

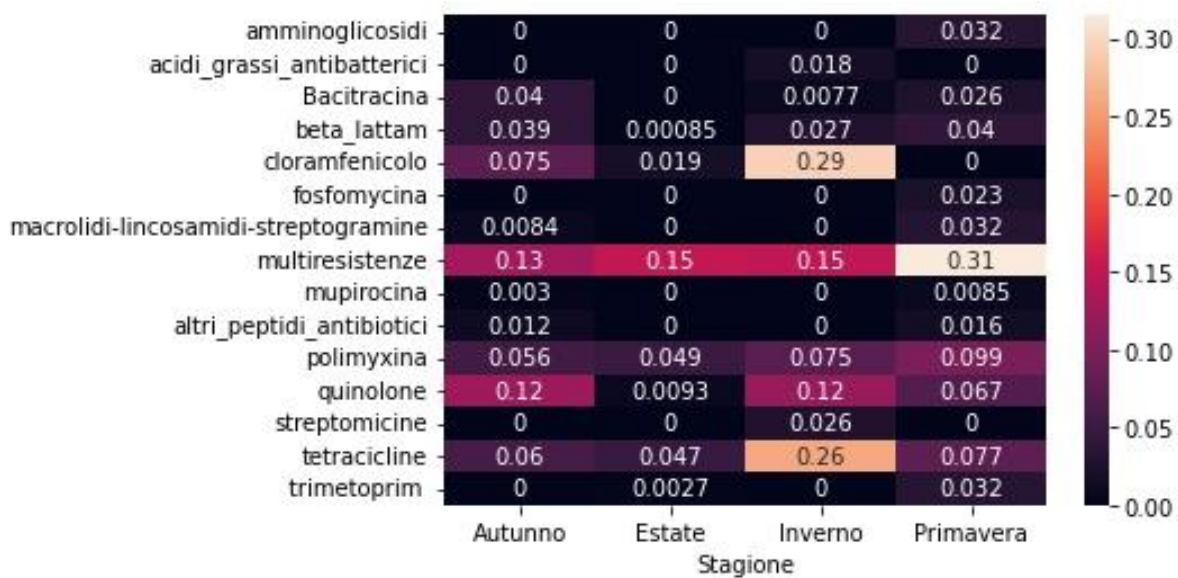


Figure 5.4.3 a: Heatmap of normalized ARG count, divided by Season

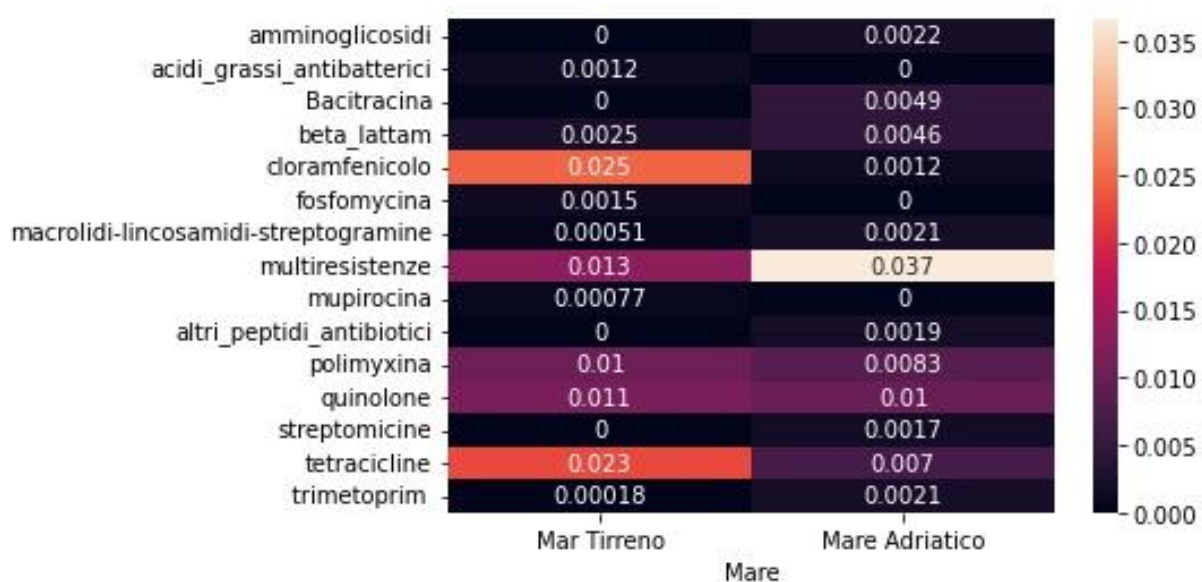


Figure 5.4.3 b: Heatmap of the normalized number of ARGs, divided by Sea of Origin

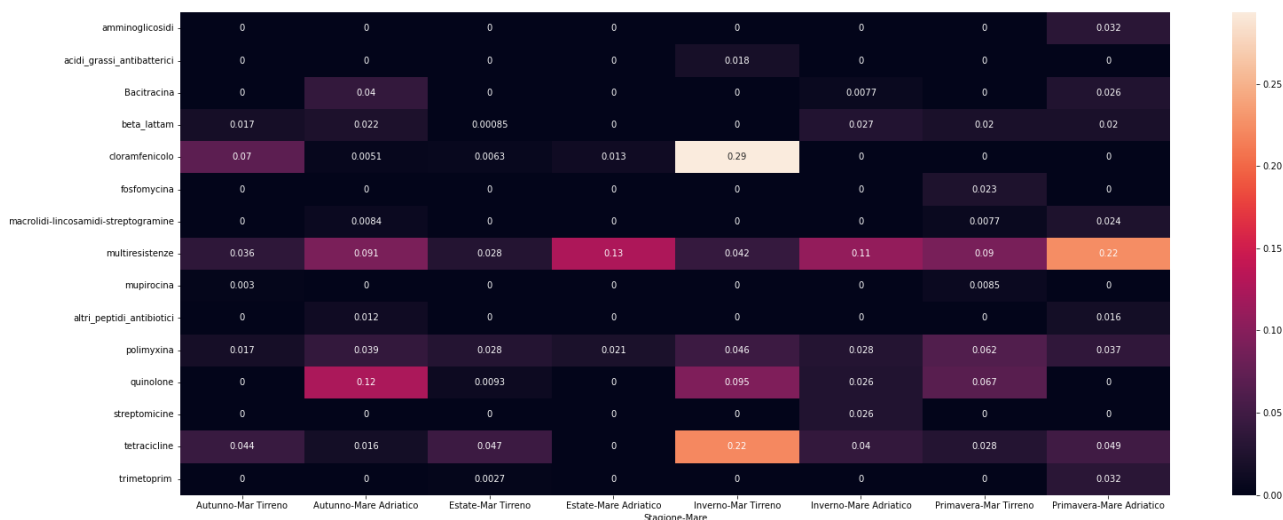


Figure 5.4.3 c: Heatmap of the normalized number of ARGs divided by Season and Sea

Regarding the analysis of the samples studied based on their origin (Tyrrhenian and Adriatic Seas), statistical analyses revealed the following:

The MRPP analysis indicated a significant difference (p-value 0.091), at an α level of 10%, between the Tyrrhenian and Adriatic Seas in the relative abundance of antibiotic resistance classes. However, the difference between the two groups was small ($\alpha=0.01549$), indicating that the groups exhibited an observed homogeneity equal to that which would be observed by chance.

The PERMANOVA analysis, conducted after verifying the homogeneity of variances (p-value >0.05), indicated a significant difference (p-value=0.095), at an α level of 10%, in the relative abundance of antibiotic resistance classes between the Tyrrhenian and Adriatic Seas.

Both non-parametric techniques used highlighted the presence of a significant difference (p-value<0.10) between the relative abundances of the antibiotic resistance classes in the samples from the two seas being compared.

Using the percentage similarity technique to interpret differences between groups, the antibiotic resistance classes that contributed most to differentiating the resistome of samples from the Tyrrhenian and Adriatic Seas were reported in Table 5.3.4. The term "Multidrug" was used to indicate the antibiotic resistance gene class that most differentiated the two groups, but its p-value was not significant at an α level of 5%. Tetracycline resistance genes were the only statistically significant resistance class that contributed to the difference between the two groups. The mean percentage contribution of all resistance classes is reported in Figure 5.4.3 d.

Classe antibiotica	Contributo medio (%)	p-value
multidrug	24.43	0.353
tetracycline	20.74	0.053
quinolone	13.81	0.527
chloramphenicol	12.36	0.252

Table 5.4.3. Average contribution of the most influential antibiotic classes with p-value.

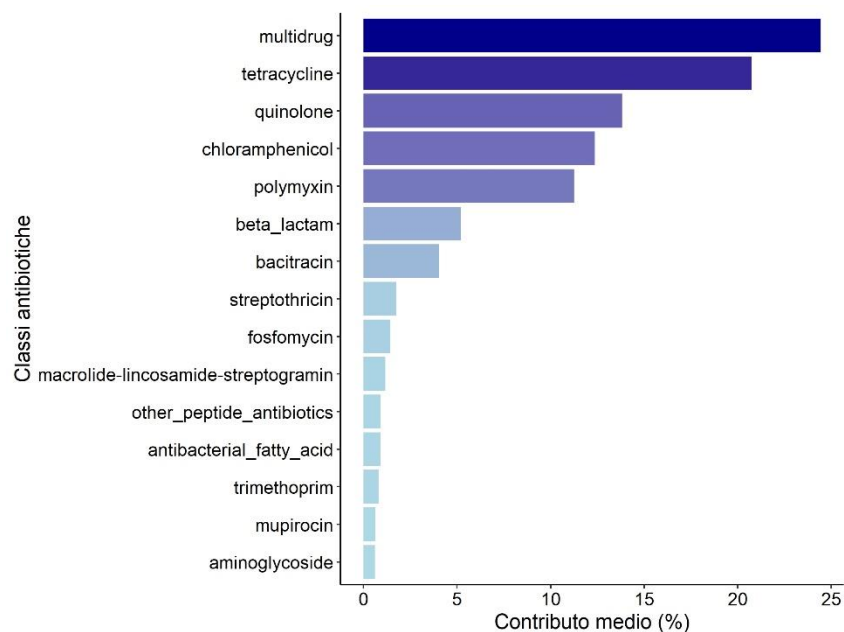


Figure 5.4.3 d: mean percentage contribution of all resistance classes

Finally, the ISA analysis indicated that the multidrug class (p-value = 0.045) was most strongly associated with the Adriatic Sea.

In the NMDS shown in Figure 5.4.4 e, the points represent the samples from the dataset and are colored according to their respective groups. The distances between the points are based on a dissimilarity matrix calculated using the Bray-Curtis distance. The figure shows that there was no clear separation between the samples belonging to the two seas, indicating their similarity.

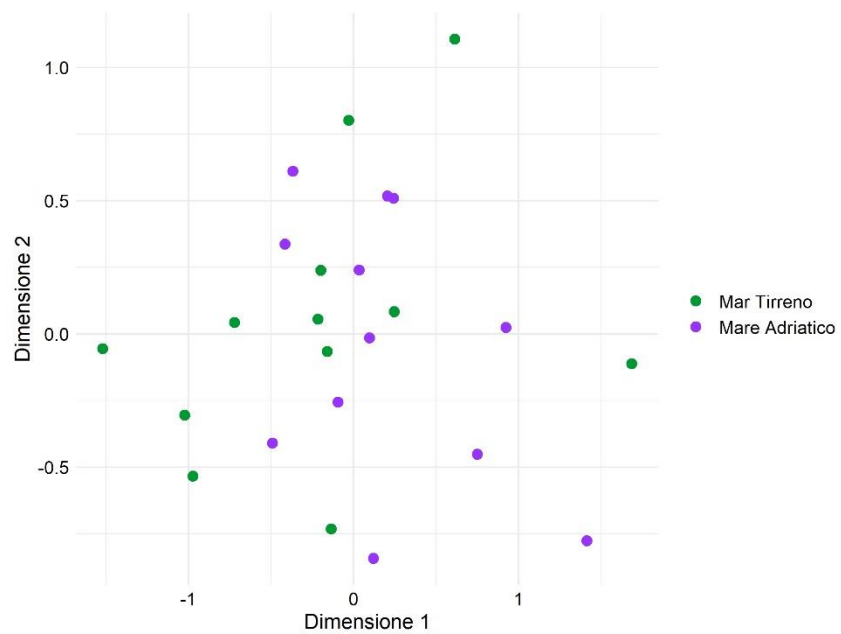


Figure 5.4.4 e: Grafico NMDS

In Figure 5.4.3.f, antibiotic classes have been added to the points representing the dataset samples colored by their sea basin of origin. The antibiotic classes closest to each other show similar behavior.

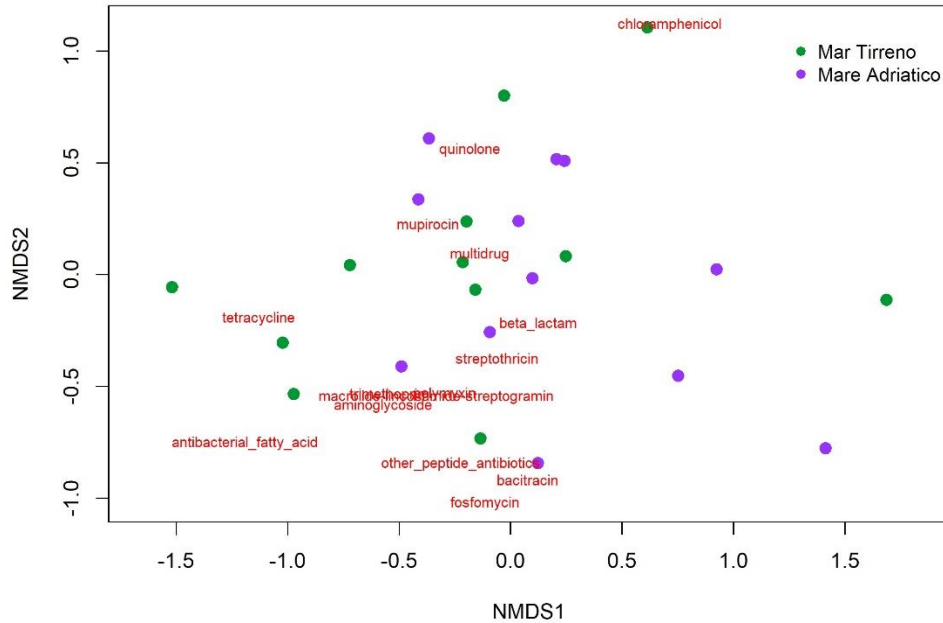


Figure 5.4.3 f: NMDS plot with antibiotic classes

The analysis of the differences identified by season (Winter, Spring, Summer, Autumn) revealed the following:

The MRPP analysis indicated that there was no significant difference ($p\text{-value} = 0.978$) between the four seasons in the relative abundance of antibiotic resistance classes. The difference between the four groups was, in fact, very small ($A = -0.03544$), indicating that the groups exhibited heterogeneity equal to that expected by chance.

The PERMANOVA analysis, conducted after verifying homogeneity of variances ($p\text{-value} > 0.05$), indicated no significant differences ($p\text{-value} = 0.976$) in the relative abundance of antibiotic resistance classes between the four seasons.

Both nonparametric techniques used indicated that there was no significant difference ($p\text{-value} > 0.10$) between the relative abundances of antibiotic resistance classes in the four seasons compared.

As regards the integrated analysis between the interaction between the variables Season and Origin (Sea), the following emerged:

The MRPP analysis indicated that there was no significant difference ($p\text{-value} = 0.434$) in the relative abundance of antibiotic resistance classes between the four seasons combined with the Tyrrhenian Sea

and the Adriatic Sea. The difference between the groups was, in fact, very small ($A = 0.004742$), indicating that the groups exhibited homogeneity equal to that expected by chance.

The PERMANOVA analysis provided a p-value of 0.711 for the model. Sea, season, and their interaction were not significant ($p = 0.124$, $p = 0.978$, and $p = 0.440$, respectively). This suggests that there is no significant difference in the relative abundance of antibiotic resistance classes in the resistome between samples collected in the four seasons (summer, autumn, winter, and spring) combined with their provenance.

Both non-parametric techniques used indicate that there is no significant difference ($p\text{-value} > 0.10$) between the relative abundances of the antibiotic resistance classes observed in the seas in the four seasons compared.

5.4.4 Implications for One Health Surveillance

These Mare in Rete findings broaden the view of AMR surveillance beyond clinical and terrestrial systems.

Coastal ecosystems mirror terrestrial dynamics of AMR and play an environmental “sink” role for anthropogenic resistance (29, 32, 78).

Seawater and bivalve surveillance can provide early-warning signals of sanitary and ecological deterioration (22, 61, 77, 98). Integration of this data (through ALiSa and PNCAR frameworks) that includes both human and veterinary data enables risk-based interventions in hotspot areas (3, 43, 54, 67).

Environmental microbiology laboratories located in IZS networks can operate as regional AMR observatories, unified in their analytical methods and data handling (22, 67, 77).

Liguria is positioned to function as a pilot region for Italy’s planned national environmental AMR surveillance plan (3, 43, 98, 99).

5.4.5. Conclusions

The Mare in Rete initiative verifies that antimicrobial resistance is a measurable and ecologically relevant issue in the marine ecosystems (22, 29, 32, 77). The persistence of resistant *E. coli* and *Vibrio*, and their association with chemical pollutants show a complex interplay of human activity, microbial adaptation, and ocean health (29, 70, 71, 78, 98). Follow-up research, along with genomic characterization and metagenomic mapping of ARGs, will be critical for benchmarking of marine AMR and assessing mitigating measures, including wastewater treatment improvements and drug and chemical emissions with increased regulations (32, 62, 71, 72, 98). In summary, the Ligurian experience shows that AMR surveillance could be effective only by combining coastal and terrestrial data can One Health truly protect public and environmental health (18, 32, 79, 98, 99).

Chapter 6 – General Discussion and Integrated One Health Perspective: The Ligurian Experience

6.1 Overview of the Ligurian AMR context

This chapter presents a logical and integrated examination for chapters 4 and 5 of results in the context of the overall One Health approach of the Ligurian region. Liguria is a distinctive ecological and socio-environmental region wherein mountain ecosystems, agricultural valleys, and coastal cities coexist in close proximity. This geographic variability encourages interspecies interactions between wildlife, domestic animals, and humans and thus the optimal conditions for the transmission of antimicrobial-resistant (AMR) bacteria through compartments (18, 32, 36, 79). Combined, the studies presented in this thesis exhibit the complexity of the AMR phenomenon in Liguria. The terrestrial and marine ecosystems discussed here disclose diverse but interrelational paths of bacterial persistence and resistance-persistency. Specifically, the emergence of Shiga toxin genes in *E. coli* strains in wild ruminants (Chapter 4) and the identification of resistant bacteria in aquatic matrices and marine organisms (Chapter 5) reflect a common environmental reservoir (21, 22, 63, 67, 77). Such results highlighted the integration of wildlife, food, and environmental surveillance as well as clinical and livestock surveillance components (3, 4, 32, 54, 99). The Ligurian experience also mirrors the national trends discussed in the Italian National Plan on Antimicrobial Resistance (PNCAR 2022–2025), which calls for the integration of non-traditional compartments (e.g. wildlife, environmental monitoring) into coordinated surveillance programs for AMR (3, 43, 67). Together, terrestrial and marine data yield a multidimensional picture of dissemination of AMR. A total of 15% of Ligurian wild ruminants harboured Shiga toxin-producing *E. coli* (STEC) strains, which were predominantly positive for the *stx2* gene with low antimicrobial resistance against β -lactams and tetracyclines (21, 63, 67). In comparison, marine matrices (as well as sentinel species stranded cetaceans) demonstrated resistance characteristics more in line with direct human influence, especially in reference to wastewater effluents and with coastal urbanization (22, 29, 32, 61, 77, 83). These findings together indicate that terrestrial settings serve as a second, though not primary, receptor that the resistant bacteria and the marine environment as a terminal collector where contamination has accumulated (29, 32, 78, 83). The identification of congruous resistance trends — namely to antibiotics of long duration such as ampicillin and tetracycline — indicates the connection of land and sea genetics pathways through watercourses and runoff (24, 27, 32, 39). Wildlife habitats and human zones of production (agriculture, hunting, and tourism) in Liguria overlap, which also fosters

inter-sectoral cross-pollination of microorganisms. As such, wild ruminants, birds, and marine life can act as sentinels of AMR trends in the region with their respective impacts on anthropogenic pressures and ecosystem health (46, 64, 81). This analysis provides the proof that environmental AMR spread is an evolutionary process that integrates soil, body of water, wildlife and humans (18, 32, 71, 72). The mild prevalence of resistant STEC among Ligurian wildlife even though no antibiotics have been released, suggests environmental human-derived contamination is of importance (21, 63, 67, 81). Residential wastewater and agricultural waste produced on the farm, combined with residual antimicrobial residues from livestock farms, can be sources of the release of resistant bacteria and antimicrobial residue to rivers and soils, which are in turn contact areas of wild fauna (24, 27, 29, 39). From the One Health perspective, these findings demonstrate that AMR is not limited to hospitals or farms, but transcends these ecological boundaries (18, 36, 79, 98, 99). While not the source of resistance itself, wildlife can serve as a biological measure of environmental quality and human influence (46, 64, 81). They are particularly helpful in areas like Liguria, where uneven, diverse land use and greater complexity of topography create a nature-human interface. For wildlife, the detection of moderate resistance-resistant *E. coli* stx-positive strains means that AMR is likely a reflection of (human), rather than an actual selection-pressure (21, 63, 67). This reinforces the need for cross-sectorial surveillance to detect environmental factors early in the life span before resistant strains reach clinical prevalence (3, 4, 32, 54, 99).

6.2 Regional monitoring and policy framework

Liguria achieved substantial improvement in the national AMR strategy, through regional cooperation from ALiSa (Azienda Ligure Sanitaria) and surveillance through the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (IZSPLV) (43, 67). However, existing monitoring systems mainly concentrate on healthcare and livestock with restricted linkage of wildlife and environmental information (3, 32, 54). Indeed, the findings reported by this thesis strongly suggest that wildlife and environmental monitoring should be formally incorporated into regional AMR surveillance networks. If Liguria had this inclusion it would serve the purpose of the European One Health Action Plan against AMR, which recognizes the mutual interdependence among human, animal and environmental health (58, 59, 99). Some recommended actions to accomplish this are: Promote systematic sampling programs for wild terrestrial and marine species as AMR sentinels (22, 46, 64, 77, 81). Improve data sharing among environmental, veterinary and public health authorities through interoperable databases and harmonized indicators (3, 32, 57, 98, 99). Improve laboratory capabilities for characterization of molecular AMR (e.g. resistome and virulome analysis) using metagenomics and whole-genome sequencing (62, 71, 72, 91). Encourage public awareness and stakeholder participation in responsible usage of antimicrobials and environmental stewardship (1, 18, 73, 79). Such initiatives would enhance detection capacity of emerging resistance trends, as well as identification of ecological hotspots for AMR dissemination across Liguria (3, 43, 67, 98, 99), with the region positioned as a pilot area for national environmental AMR surveillance.

6.3 Conclusions and future directions

The results of these studies prove that the environmental and ecological risk of AMR is just as valid as the medical one (18, 32, 36, 79, 98). The observation of resistant and possibly zoonotic *E. coli* in Ligurian wild ruminants suggests that wildlife also serves as a crucial node in the chain of AMR transmission (21, 63, 67, 81). Seen in the context of marine and foodborne data, these findings point to a requirement for inter-sectoral and unified surveillance (22, 61, 65, 77, 99). Further investigations should focus on the genomic characterization of environmental isolates as well as season-wise long-term monitoring and the integration of wildlife AMR data into national databases (62, 71, 72, 91, 99). Enlarging these efforts will enhance early identification of potential resistance determinants, support evidence based policymaking, and improve preparedness for potential zoonotic threats (25, 26, 51, 73, 86). Considering their high diversity and existing ecosystem and resourced research networks, the Ligurian region is well placed to

develop One Health-type surveillance systems (18, 32, 79, 98, 99). Grounded in this thesis, the ongoing collaboration between research organizations, health authorities and environmental agencies can help ensure AMR management progresses from sectoral control to integrated preventive measures (3, 43, 57, 67, 98).

6.4 Limitations and perspectives

While this thesis presents novel perspectives of AMR in Ligurian wildlife, some caveats must be addressed. The number of samples analyzed from wild ruminants and marine matrices, though representative, remains limited compared with the diversity of species and habitats in the region. Bacterial prevalence and resistance could also be influenced by temporal factors—in the event there are seasonal changes, rainfall patterns, and migration routes (32, 71, 72, 83). We also used a range of phenotypic and targeted molecular approaches. Future work using whole-genome sequencing (WGS) and metagenomics would provide further characterisation of gene transfer mechanisms and the potential clonal relationships between clinical and environmental isolates. Notwithstanding these limitations, the current study shows that wildlife have the capacity and importance to be included in AMR monitoring. It forms a solid foundation for prospective research aiming to trace environmental reservoirs and to develop preventive strategies that consider the entire ecosystem (18, 36, 79, 98, 99).

6.5 Final statement

In summary, this doctoral work is the first integrated assessment of AMR in Ligurian terrestrial and marine wildlife, and adds to the emerging literature on antimicrobial resistance in the One Health setting (21, 22, 63, 67, 77). These findings emphasize that protecting public health requires protecting environmental health as well (18, 32, 79, 98, 99). By emphasizing local data as well as tying it back to national and European priorities, One Health becomes not just an idealistic concept but a practical necessity towards sustainable disease control and ecosystem resilience (1, 3, 57, 59, 98, 99).

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