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Evaluation of Plastic Pollution and associated Multidrug Resistant Bacteria in a River of Central Italy

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Thesis Outline

This thesis is a publications-based dissertation.

The 3 publications are constituted by individual figures and tables numbering, supplementary data, and references list.

Chapter 3: Plastic and River role in the spread of Antimicrobial Resistance.

- *Microplastics and antibiotic-resistant bacteria contamination in a river of central Italy* (under review to Environmental Advances)
- *Plastisphere and River Systems as Potential Reservoir for Antibiotic Resistant Bacteria* (in preparation)

Chapter 4: Surveillance of Antimicrobial Resistance Genes and Class 1 integrase system.

- *Versatile and Portable Cas12a-mediated Detection of Antibiotic Resistance Markers* (under review to Spectrum Microbiology) deposited in Biorxiv https://doi.org/10.1101/2024.11.14.623642.

1 Introduction

The rapid rise of antimicrobial resistance (AMR) has emerged as one of the most critical threats to global public health of the 21st century, as recognized by the World Health Organization (WHO). This crisis is characterized by an increasing prevalence of multidrug-resistant (MDR) bacteria and the failure of current antibiotic treatments. (Tanwar J, 2014, Spellberg B, 2013)

In Europe, the situation has become alarming, with resistance to third-generation cephalosporins and carbapenems rising in opportunistic pathogens such as *Klebsiella pneumoniae* and *Escherichia coli*. Surveillance data from the European Centre for Disease Prevention and Control (ECDC) reports high resistance rates, particularly in Southern and Eastern Europe, where resistance to carbapenems, considered antibiotics of last resort, is increasingly widespread. (ECDC, 2022).

The AMR crisis extends beyond clinical settings and need to be recognized in a One Health approach which includes human, animal, and environmental health (Robinson TP, 2016). Human activities significantly contribute to the environmental spread of antibiotic resistance genes (ARGs). Rivers and plastic pollution play a crucial role, as water bodies serve as reservoirs and conduits for the spread of ARGs. Moreover, while the presence of pollutants can selectively enrich bacteria carrying ARGs. Plastics, a widespread form of pollution, provide an ideal surface for microbial biofilm formation, facilitating the horizontal gene transfer (HGT) of ARGs among bacterial communities (Guo X ,2020). HGT is recognized as the primary mechanism driving the rapid spread of resistance, allowing the transfer of resistance genes across species and genera (Sun D, 2018). This process is further facilitated by the presence of mobile genetic elements (MGEs) such as plasmids, transposons, and integrons, (particularly Class 1), which serve as key mechanism for the acquisition and expression of resistance genes (Partridge SR, 2018).

The freshwater ecosystems act as hotspot for the MDR bacteria, with the potential to act as a vector for ARGs and pathogens spread in the environment (Laganà P, 2019). This work aims to address gaps present in the literature, which prevalently focused on marine ecosystems. By investigating the role of rivers in the spread of AMR, this work highlights the urgent need for better understanding these environmental pathways.

1.1 AMR threat

The widespread dissemination of antibiotic resistance has caused the lack of efficiency of antibiotic treatments currently used in human medicine, with alarming consequences on the diffusion and control of bacterial infections (Lewis, 2013). The rise of antibiotic-resistant bacteria is classified by the WHO as one of the three biggest threats to public health in the 21st century. It is driven by the difficulty to discover novel antimicrobial agents and the increasing prevalence of MDR bacteria. The consequence is a growing number of treatment failures with an estimated 700,000 fatalities occurring globally each year (Spellberg B, 2013). Moreover, it has been predicted that antibiotic-resistant bacterial infections will be the cause of over 10 million annual fatalities by 2050. (O'Neill, 2014; Aslam B, 2018). A One Health approach is required to address the rising threat of antimicrobial resistance: this considers the health of humans, animals, and environment as connected and dependent on each other (Robinson TP, 2016). In fact, antibiotics are routinely used in veterinary settings, to prevent and treat diseases in animals, as well as to enhance growth and productivity. However, the nonhuman use of antibiotics has been linked to the emergence of resistance also in human populations (Robinson TP, 2016).

Additionally, antibiotic residues from both human and veterinary practices, as well as agriculture and aquaculture, may return to natural environments through wastewater treatment plant (WWTP) effluents (Zhang X, 2009; Kümmerer K, 2009). These are characterized by high microbial densities, abundant nutrients, and sub-inhibitory concentrations of antimicrobial compounds, creating ideal conditions for the selection and proliferation of antibiotic-resistant bacteria (ARB) and ARGs (Manaia C, 2018).

When WWTP effluents are discharged into freshwater ecosystems, ARGs are dispersed into the environment, facilitating the acquisition of antibiotic resistance traits by human pathogens, further enhancing the AMR crisis (Hernando-Amado S, 2019).

1.2 Critical resistant bacteria

The WHO has classified critical pathogens based on their widespread resistance and their ability to cause severe healthcare-associated infections. Among them are carbapenem-resistant *Enterobacteriaceae* (CRE), and ESBL-producing and third-generation cephalosporin-resistant *Enterobacteriaceae*. (WHO, 2024)

Extended-Spectrum Beta-Lactamases are enzymes that confer resistance to most beta-lactam antibiotics, including third-generation cephalosporins, and often coexist with other resistance mechanisms, causing multidrug resistance profiles (Husna A, 2010). Biochemically, ESBLs belong to the serine β-lactamases group and are classified as class A enzymes under the Ambler molecular classification. They are characterized by the ability to hydrolyze expanded-spectrum β-lactam antibiotics, like cephalosporins, and are inhibited by β-lactamase inhibitors, such as clavulanate (Bush K and Jacoby GA, 2010). The prevalence of ESBL-producing Gram-negative pathogens has become a significant problem, not only in healthcare but also in community settings (Pitout JD, 2005).

Carbapenem resistance is mediated by a group of enzymes known as carbapenemases, which confer resistance to almost all available beta-lactam antibiotics. Carbapenemases enzymes are a heterogeneous group able to hydrolyze most beta-lactams, including carbapenems that nowadays are considered antibiotics of last-resort (Nordmann P, 2011). These enzymes are often named based on the specific enzyme produced, leading to variants such as *Klebsiella pneumoniae* carbapenemases (KPC)-producing CRE, oxacillinase-48 (OXA-48)-producing CRE, and CRE producing metallo-beta-lactamases such as New Delhi metallo-beta-lactamase (NDM), Verona integron-encoded metallo-beta-lactamase (VIM), and IMP-type metallo-beta-lactamases (David S, 2019).

The surveillance data from the ECDC in 2018 highlighted the growing concern of antimicrobial resistance in *K. pneumoniae* across Europe, especially to third-generation cephalosporins. Countries in Southern (Greece and Italy) and Eastern Europe reported particularly high rates exceeding 50%. In addition, the same countries have shown similarly high resistance rates to carbapenems and other antimicrobial classes (ECDC, 2018).

In 2022, ECDC surveillance reported that resistance to third-generation cephalosporins (3GC) and carbapenems remained higher in *K. pneumoniae* than *E. coli*. From 2019 to 2022,

over 40% of European countries have consistently reported resistance rates of 50% or higher for 3GC-resistant *K. pneumoniae.* This trend remains particularly prevalent in southern and eastern Europe, where resistance rates for this microorganism-antimicrobial combination continue to rise. (ECDC, 2022). In addition, the latest WHO report released in 2024 confirms alarming trends in Europe, with resistance rates for third-generation cephalosporin-resistant *E. coli* ranging from 30% to 40%, and a prevalence of carbapenem-resistant *K. pneumoniae*, with resistance rates from 50% to 82% in some Countries. (WHO, 2024). The increasing prevalence of these critical resistant strains poses global public health concern due to the limited therapeutic options available for these infections. Rather than moving towards the target of a 5% reduction by 2030, the situation has worsened. This concerning trend underscores the urgent need to strengthen prevention and control measures in EU Member States, as emphasized in the EU Council recommendation (Council of the European Union, 2023).

1.3 *E. coli* in human and environmental health

The significance of *E. coli* as a pathogen extends across both human and environmental contexts, establishing it as a critical organism in antimicrobial resistance (AMR) surveillance. In humans, *E. coli* is a leading cause of severe infections, including urinary tract infections, sepsis, and gastrointestinal illnesses (Pitout JD, 2005). The emergence of ESBL-producing *E. coli* strains, which are resistant to critical antibiotics such as third-generation cephalosporins, has further complicated treatment options, posing significant challenges in both clinical and community settings (Bush & Jacoby, 2010).

In the environment, *E. coli* serves as a sentinel organism, indicating fecal contamination and signaling potential health risks. Its persistence and adaptability in ecosystems, including surface water, agricultural runoff, and wastewater, underscore its role in the environmental AMR spread (WHO, 2024).

Recognizing this, the WHO developed the Tricycle protocol in 2017. This surveillance framework employs *E. coli* as the central indicator organism for monitoring AMR across the human, food chain, and environmental sectors. Studies demonstrate wide variations in ESBL-*Ec* colonization rates across countries and over time (Woerther PL, 2013), links between antibiotic usage in the food chain and human morbidity (ECDC, 2019), and the reduction of ESBL-*Ec* prevalence following decreased antibiotic exposure in humans or

animals (Mughini-Gras L, 2019; Høg BB, 2019). By focusing on ESBL-producing *E. coli*, the Tricycle protocol highlights its critical role as a driver and marker of resistance (WHO, 2024).

1.4 Plastic pollution

The wide diffusion of ARB and ARGs in the environment is closely related to the anthropogenic pollution (Barros and Seena, 2021). Plastic among the pollutants is one of the growing global environmental problem due to its accumulation in the environment. It derives from synthetic polymers obtained from fossil fuels or renewable sources (Plasticseurope, 2019) combined with other added chemicals (Dekiff JH, 2014; Thompson RC, 2009), to provide desirable properties such as strength, flexibility, durability, and lighter weight (Andrady and Neal, 2009). Currently there are up to 30,000 different polymers registered for use in the European Union, including the commonly used polyethylene (PE), polypropylene (PP), polyethylene tere- phthalate (PET), polyvinyl chloride (PVC), and polystyrene (PS) (Halden R, 2010).

Over the last decade, the production of plastic increased reaching 348 million tons produced in 2017, and projection estimating a doubling value by 2025 (Geyer R, 2017; Plastics – the Facts, 2018). This exponential growth is attributed to the widespread use of polymers across a wide range of applications in our daily life. However, the inadequate management of plastic litter from the anthropogenic activities is the primary problem related to the release of plastic in the environment. Once in the environment, plastic persist for extended periods, undergoing degradation processes that cause the formation of small particles known as microplastics (MPs), with a size diameter ranging from few micrometers to 5mm (Horton A, 2017; Zhang K, 2021). MPs can originate from primary and secondary sources. Primary microplastics are synthetized as pellet or small particles and are widely used in a variety of manufacturing industries, such as therapeutics and cosmetic products (Kawaguchi H, 2000), while secondary microplastics result from the breakdown of larger plastic items due to environmental and physical factors, that make plastics susceptible to fragmentation (Andrady, 2017, Barnes DK, 2009, Hidalgo-Ruz et al., 2012). Secondary MPs exhibit a variety of shapes, depending on the origin and fragmentation process, including fragments, microbeads, sheets, films and fibers (Hidalgo-Ruz, 2012). Further fragmentation of MPs

lead to the formation of nano plastic particles, with a size lower then 1.6 μm (Galgani F, 2010).

1.5 MPs into freshwater

Microplastics, due to their lightweight nature, move across several ecosystems predominantly through freshwater systems, serving as both recipients and distributor of plastic pollution in the environment. Among these freshwater bodies, rivers play a key role, transporting approximately the 80% of the plastic currently found in oceans (Meijer LJJ, 2021). Rivers facilitate the transfer of MPs into various ecosystems encompassing wetlands, lakes, estuaries, coastal regions, and ultimately, oceans (Lebreton LCM, 2017; Meijer LJJ, 2021; Schmidt C, 2017). The urbanization process significantly influences the presence of MPs within riverine environments, primarily with land-based activities, which introduce these particles via runoff processes (Horton A, 2017). Studies conducted by Mani T, 2015 and Yonkos LT, 2014 consistently highlight urban areas as hotspots for MPs accumulation, reporting higher concentrations of MPs in proximity to urban areas compared to more remote sites. However, while urban areas are known hotspots for microplastic accumulation, Horton A, 2017 discovered elevated particle concentrations also in rural areas with minimal human-associated activity. Although the increasing detection of MPs pollution in freshwater environments, the current knowledge of its accumulation and impacts is significantly lower respect to the marine ecosystems (Thompson RC, 2009; Wagner M, 2014). This disparity emphasizes the need for further research focused on rivers within the context of MPs contamination in aquatic systems.

1.6 MPs Sorption ability

Anthropogenic activities, as those from industrial and agricultural sectors, contribute to the release of pollutants into aquatic ecosystems, through wastewater treatment plants, uncontrolled sewage discharges, and rivers. These sources release several pollutants including MPs, antibiotics, biocides, and heavy metals. (Wei and Yang, 2010; Gonçalves AC, 2014; Manyi-Loh C, 2018; Browne, M. A, 2011). The presence of antibiotics, highly susceptible to adsorption by MPs, not only accelerates the proliferation of multidrug-resistant bacterial strains but also facilitates the horizontal transfer of antibiotic resistance genes among both clinical and environmental communities.

These contaminants contribute to the dissemination of AMR by enriching resistance gene determinants through co-selection mechanisms, which occur into three different processes: co-resistance, cross-resistance and co-regulation. Co-resistance arises when genes for different resistances are physically linked on MGEs, causing their co-selection (Chapman JS, 2003). Cross-resistance occurs when a single mechanism, like a multi-drug efflux pump, provides resistance to multiple agents. Co-regulation involves the coordinated expression of distinct resistance mechanisms in response to a single stressor (Baker-Austin C, 2006). MPs enhance this phenomenon due to their capacity to adsorb these pollutants. This adsorption process, combined with the formation of microbial biofilms on plastic surfaces, creates an additional selection pressure that favor the enrichment of ARGs on microplastic surfaces (Caruso, 2019; Selvam S, 2021). Consequently, the interaction between MPs, pollutants, and microbial communities amplifies the risk of ARG dissemination in aquatic environments.

1.7 Plastisphere

Once in the environment, plastic rapidly becomes colonized by biofilm comprising a diverse microbial community, which includes both eukaryotic and prokaryotic species (Amaral-Zettler LA, 2021). The colonization of plastic surfaces is referred with the term of plastisphere (Zettler, 2013) to describe the microbial community attached to plastic differing from its surrounding environment (Du Y, 2022). It is estimated that globally 1,000–15,000 tons of microorganisms are part of plastic biofilms (Mincer TJ, 2016). Biofilm-forming microorganisms on plastic debris were first discovered in marine ecosystems (Carpenter and Smith, 1972) but knowledge of microbial diversity in freshwater plastispheres remains

limited, with the first high-throughput sequencing of freshwater plastisphere started only a decade ago (McCormick et al, 2014).

Bacterial biofilms are surface-associated microbial communities embedded within an exopolymeric substance (EPS) matrix, that act as protective and nutritive structure, facilitating metabolic cooperativity and increasing the gene transfer between cells (Costerton JW, 1995; Davey and O'Toole, 2000). Biofilm formation is a developmental cycle regulated by quorum sensing (QS), a cell-to-cell communication system that modulate their gene expression in response to changes in cell density, with signaling molecules called autoinducers (AIs) that coordinates the transition from planktonic to biofilm states (Irie Y, 2008). This process occurs in several distinct steps: 1) in the initial phase, free-living planktonic cells reversibly attach to the plastic surface and 2) then secondary colonizers promote irreversible attachment through the production of pili, adhesion proteins, and EPS, altering the substrate's properties and enhancing biofilm stability 3) once adhesion has been established, they begin to form microcolonies and enter the maturation step. 4) Once the biofilm has matured, cells can detach from the surface and move on to colonize new substrates (Flemming HC, 2016).

Different conditions, as nutrient availability, the microbial community composition and temperature can affect biofilm structure (Stoodley P, 1997). Additionally, physical properties of the polymer, such as hydrophobicity, particle shape, roughness, crystallinity, and surface charge, also play a role in the selection of primary colonizers, which as consequence influences the selection of further communities (Min K, 2020; Pinto M, 2019). For example, porosity and hydrophobicity make plastic debris able to adsorb organic pollutant and represent an ideal habitat for microorganisms. Studies reported that plastic could adsorb antibiotics, heavy metals and pesticides (Zhang H, 2018; Hirai H, 2011; Wang F, 2018). Biofilms are generally associated with an increase in AMR due to the proximity of individual bacteria, which facilitates HGT of MGEs carrying ARGs. (Arias-Andres M 2018). This process suggests that plastisphere could be a driver of the transmission of human pathogens and ARBs. (Parthasarathy A, 2019).

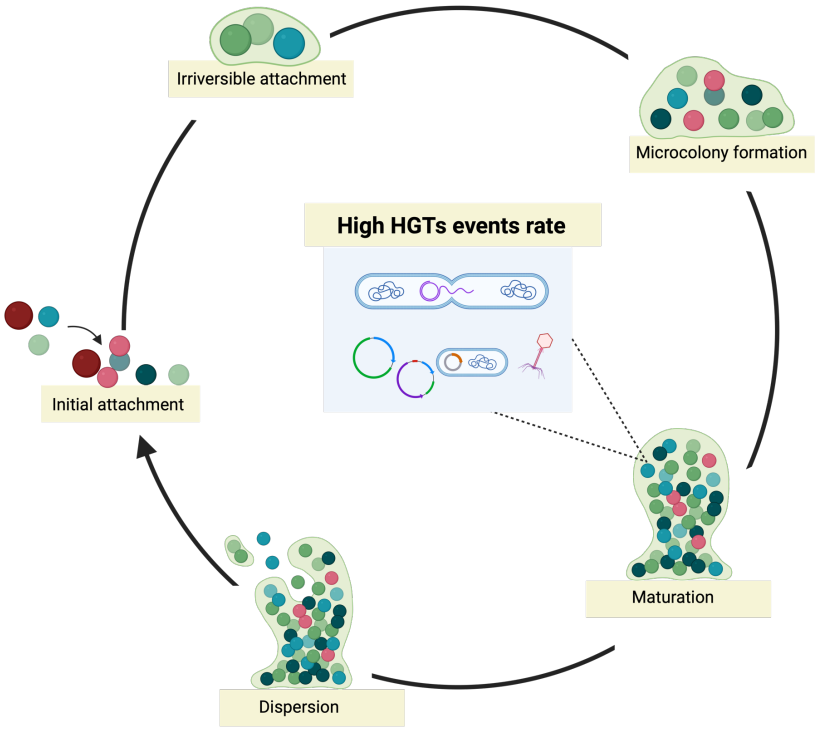

Figure 1. Biofilm development steps. 1) Planktonic cells reversibly attach to a substrate 2) The attachment becomes irreversible 3) micro-colonies are formed 4) the biofilm is remodeled and, the develops into a structured community 5) cells are released to colonize new surfaces or return to a planktonic state.

1.8 ARGs mobility

Antimicrobial resistance can be transmitted both through vertical inheritance and HGT. HGT is the primary driver of ARG dissemination, allowing the transfer of resistance genes within and between species. This process occurs through three main mechanisms: (1) transformation, where bacteria take free DNA from the surrounding environment, (2) transduction, where bacteriophages are involved in the movement of genetic material, and (3) conjugation, where genes are mobilized a direct contact between the donor and recipient cells, using structures such as pili (Wintersdorff V, 2016; Burmeister AR, 2015). These mechanisms are prevalent in biofilms, where the frequency of HGT is significantly higher compared to planktonic bacterial cells (Madsen JS, 2012).

This process is further facilitated by MGEs, such as plasmids and conjugative transposons, and by gene acquisition elements like integrons, which play a key role in the integration and expression of ARGs (Stalder T, 2012).
Although integrons themselves are not mobile, they are able to acquire and excise resistance genes through site-specific recombination (Hall RM and Collis CM,1995). Integrons are frequently found associated with other MGEs, enhancing their ability to spread ARGs across different bacterial populations (Partridge SR, 2018).

Among all the classes, Class I integrons are the most common type present in clinical isolates of the *Enterobacteriaceae*, acting as reservoirs and exchange platforms for resistance genes (DeLappe N., 2003). The structure is composed of three essential elements: the integrase gene (*intI*) that mediates the integration of gene cassettes, the recombination site (*attI*), and the promoter (*Pc*) that drives the expression of integrated genes (Barraud O and Ploy MC, 2015). The combination of different gene cassettes within it creates a highly adaptable genetic organization that enhances bacterial survival and resistance capabilities (Stalder T, 2012).
Importantly, Class 1 integrons are associated not only with antibiotic resistance but are also known to confer resistance to a wide range of compounds, including disinfectants, heavy metals, and environmental pollutants (Liebert CA, 1999; Partridge SR, 2001). This broad resistance profile suggests that Class 1 integrons are influenced by human activities, and for this, integrase 1 has been proposed as a potential marker for anthropogenic pollution. The

presence *intI1* is present in a wide variety of both pathogenic and commensal bacteria in humans, domestic animals and environmental settings (Goldstein, 2001; Stokes and Gillings M, 2011). Monitoring the *intI1* occurrence and distribution could therefore provide new knowledges about the AMR spread and the impact of human activity on microbial ecosystems.

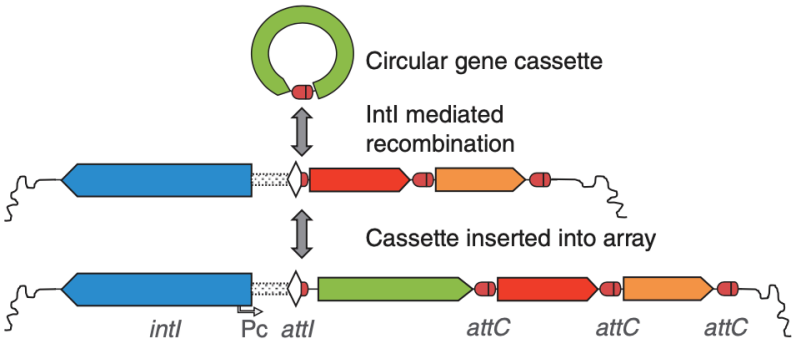

Figure 2 Class 1 integron-integrase system. The circular gene cassette is inserted via *intI1* integrase, which recombines at the attI site. Multiple gene cassettes are integrated into an array, with each cassette containing specific attC recombination sites (Gillings M, 2015).

2 Aim of the research

The aim of this research was to investigate the interplay between microbial communities that colonize plastic materials and those present in the river water, and to analyze the downstream consequences on the AMR spread within the natural environment. Focusing on Chienti, a river that flows northeast from the Appennino Umbro-Marchigiano mountains in central Italy to the Adriatic Sea, this study explores the role played by (i) plastic as ideal surfaces for microbial biofilm formation, providing a suitable habitat for multidrug-resistant (MDR) bacteria; (ii) river water as a dynamic reservoir of antibiotic resistance genes (ARGs).

The key points of this research were:

1. To study the Chienti river water, subjected to various anthropogenic pressures, as a carrier for both microplastics (MPs) and antimicrobial resistance (AMR). Specifically, in a pilot study carried out in the year 2022, the investigation involved the analysis of the MPs, and the characterization of critical antibiotic-resistant bacteria present along the Chienti River.
2. To perform a field experiment to investigate multi-drug resistant (MDR) bacteria found in the plastisphere and in river water, associated to third-generation cephalosporin (3GC)-resistant *Enterobacteriaceae*, at both phenotypic and genotypic levels. The focus of this study, carried out in the year 2023, was on the role played by Class 1 Integrons, which are frequently linked to MGEs, as drivers of the spread of ARGs.
3. To comprehensively study the composition of the plastisphere by metagenomic analysis exploring the microbial community, and its ARGs and MGEs.
4. To tackle the design, development and application of a dedicated CRISPR/Cas assay for a versatile ARG detection and monitoring system.

While marine environments have received great attention in studies focused on the impact of MPs and AMR, this work highlights the role of rivers as both reservoirs for MDR bacteria and conduits for the transfer of resistance genes across ecosystems. By examining the Chienti River, the study aims to provide new findings into the combined effects of plastic pollution and AMR within freshwater and emphasizes the need to develop effective surveillance strategies for detecting critical ARGs in such environments. The development of the CRISPR/Cas12a assay, is a notable example of technology transfer, where the know-how derived from basic research is turned into an opportunity to bridge the gap between

laboratory analysis and field applications, helping to improve timely interventions to mitigate the AMR in natural ecosystems.

3 Plastic and River role in the spread of Antimicrobial Resistance.

Plastic pollution and AMR represent two of the most pressing global environmental and public health challenges. MPs defined as plastic particles smaller than 5 mm, are widespread in freshwater and marine ecosystems and represent ideal surfaces for biofilm formation that potentially act as vehicles for pathogens and ARB. This chapter addresses the dual problem of plastic pollution and AMR, specifically focusing on the role of rivers as reservoirs and "drivers" for the spread of MDR bacteria and ARGs. Additionally, it investigates how plastic waste, into aquatic environments, contributes to this growing threat.

This research was focused on the Chienti River (Marche region, Italy) as a conduit of both AMR and pollution. The study characterizes the MPs present in the Chienti river and the antibiotic resistant enterobacteria isolated from river water, as part of a pilot experiment conducted in June 2022. This initial finding confirmed the presence of antibiotic-resistant bacteria and various types of microplastic polymers inside the river. In the next step, we performed a field experiment to study the combined threats, specifically the presence of MDR bacteria forming biofilms on plastic surfaces, called "plastisphere", in parallel with their characterization in river water.

The objective was to investigate the relationship between plastic pollution, AMR, and the river ecosystem, with a particular focus on 3GC resistant *Enterobacteriaceae,* characterizing their antibiotic resistance profile at phenotypic and genotypic levels. Furthermore, we explored the Class 1 Integron as a genetic element potentially involved in the spread of resistant determinants.

A significant challenge in environmental microbiology is that most microorganisms found in natural systems cannot be cultured under standard laboratory conditions, posing a problem for linking ARB and ARG data within environmental biomes (Noyes NR, 2016). To overcome this limitation, the data derived from both microbiology and molecular biology approaches have been complemented applying a shotgun metagenomic analysis on the total microbial communities, with the aim to characterize the overall features of the community, as well as its associated ARG and MGE.

3.1 Microplastics and Antibiotic-Resistant Contamination in a river of central Italy.

MPs, very well known for their durable and long-lasting properties, are now widespread contaminants in aquatic ecosystems. Concurrently, their capacity to transport harmful microorganisms, including ARB, amplifies the role of MPs as detrimental pollutants. Antimicrobial resistance has the potential to affect people at any stage of life, as well as the healthcare, veterinary, and agriculture sectors, and riverine systems are increasingly identified as potential reservoirs and facilitators for the spread of MDR bacteria.

The combination of these threats poses several public health risks. Due to their characteristics as durability, low density, and small size, MPs persist in freshwater environments for long periods without degrading, thereby facilitating the long-distance transport of the associated microorganisms. While significant attention has been given to the impacts of microplastics and AMR in marine environments, riverine ecosystems remain largely underexplored, despite their crucial role in receiving and transferring pollutants from terrestrial sources to marine ecosystems.

In this short communication, the Chienti River, located in central Italy, was investigated to identify and characterize the presence of both plastic debris and antibiotic resistant *Enterobacteriaceae*, with the aim to highlight a new potential pathway through which antibiotic resistance determinants can spread. This River, subjected to multiple anthropogenic pressures by agricultural runoff, urban waste, and industries along its course, represents an ideal case study to start investigating the interconnected threats of plastic pollution and AMR.

Specifically, in this work we have identified polymer composition of meso- and microplastics that have been concentrated adapting to the river water the Manta-net, which is a trawl commonly employed for collecting samples from the seawater surface. Additionally, we have characterized the antimicrobial resistances to 3GC associated with *Enterobacteriaceae* selectively isolated from river water.

By examining both the presence of MPs and the resistance profiles of isolated bacteria, this research provides initial findings on the potential risks posed by the co-presence of these global threats. These preliminary results underscore the urgent need to carry out deeper analyses to evaluate the extent and consequences of the combined effects of MPs and AMR, and possibly to develop strategies for risk mitigation.

Microplastics and antibiotic-resistant bacteria contamination in a river of central Italy

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Abstract

Plastic pollution and antimicrobial resistance (AMR) are two critical global environmental and health threats. In aquatic environments meso- and micro-plastics (MPs), particles smaller than 25 mm and 5 mm respectively, are widespread. In addition, antibiotic-resistant bacteria (ARB) are increasingly detected in water bodies, posing significant risks to the public health. If combined, these threats are amplified, as meso- and MPs provide the surface for biofilm formation, delivering ARB throughout the environment. In this study we evaluated the levels of meso- and microplastic pollution and the prevalence of antibiotic-resistant enterobacteria in the Chienti river (Marche Region, central Italy). Meso- and MPs were collected using a Manta-Net at two representative sites, and analyzed using Fourier Transform Infrared Spectroscopy (FTIR). Polyethylene (PE) was the most abundant polymer accounting for over 60% of the total detected plastic debris. In parallel, river water was filtered to isolate third-generation cephalosporin (3GC)-resistant *Enterobacteriaceae*, and their antibiotic susceptibility profile was investigated according to EUCAST guidelines. The bacterial species identified were *Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae,* Salmonella enterica, *Vibrio parahaemolyticus* and *Citrobacter koseri*. They showed a high percentage of resistances to levofloxacin (56%), sulfamethoxazole/trimethoprim (40%), and amoxicillin **(**52%) in addition to cefotaxime and ceftazidime (96%). Findings of this research demonstrate the co-existence of these threats within the Chienti river, suggesting the possibility of a phenomenon involved in the

amplification of AMR diffusion in the environment. However, further studies are needed to better understand their combined effects on public health.

Introduction

Plastic pollution and antimicrobial resistance (AMR) represent two of the most critical environmental and public health challenges of our time. Meso-plastics, defined with a size range between 5 and 25mm, and microplastics (MPs) defined as debris smaller than 5 mm, are now widely distributed across global environments (Kibria, 2024; Thompson et al., 2009). Both plastic types contribute to the total plastic accumulation in aquatic systems projected to reach 53 million tons annually by 2030 (Borrelle et al., 2020) . On the other hand, AMR is rapidly increasing, with an estimated 39 million deaths caused by antibiotic-resistant infections by 2050 (Collaborators et al., 2024) . Between the 2016-2020 the European Center for Disease Control (ECDC) reported that third-generation cephalosporin (3GC) resistant *Escherichia coli* infections were the main cause of AMR-related diseases, both in terms of case numbers and deaths (ECDC, 2022).

AMR bacteria are increasingly being detected also in natural environments, with freshwater systems becoming hotspots for multidrug-resistant (MDR) strains (Nnadozie and Odume, 2019). At the same time, freshwater ecosystems are involved in the transport of plastic debris to seas and oceans, where approximately 98% of primary MPs are from land-based sources (Boucher and Friot, 2017; Cincinelli et al., 2019; Lebreton et al., 2017). These pollutants are particularly concentrated in estuaries, highlighting the significance of rivers as a major source of microplastics being delivered to marine ecosystems (Gallagher et al., 2016; Sadri and Thompson, 2014). Despite this, less than 4% of global studies investigated their presence and effects in rivers and lakes (Lambert and Wagner, 2017), while most research on MPs has disproportionately focused on marine environments.

Recent studies suggest that meso- and MPs provide surfaces for biofilm formation and may act as vehicles for multidrug-resistant (MDR) bacteria, promoting their transport and spread through aquatic systems (Marques et al., 2023; Zhang et al., 2020). Considering these interconnections, this study aims to investigate both the levels of meso- and microplastic pollution and antibiotic resistance associated to third generation cephalosporin (3GC) resistant *Enterobacteriaceae* in a river of central Italy. The Chienti river is subject to pollution pressures from surrounding agricultural activities, urban runoff, and industrial

sources. Monitoring from the Agenzia Regionale per la Protezione Ambientale delle Marche (ARPAM) between 2018–2020 identified various pollutants present in the Chienti river, alongside significant microbiological contamination, including high levels of *Escherichia coli*, an indicator of fecal contamination and possible presence of pathogenic bacteria. The characterization of MPs and AMR profile of *Enterobacteriaceae* in the Chienti river represents an initial step to further understand their combined effects within this ecosystem.

Results and Discussion

The simultaneous presence of plastic debris and ARB in freshwater represents a serious combination that may promote the spread of antibiotic resistance. In this study we assess the level of plastic pollution and characterize the antimicrobial resistance of clinically significant bacteria in the Chienti river. Meso- and microplastic pollution was studied by quantifying the plastic particles dispersed in river water and by identifying the most abundant polymer types. We have adapted a seawater samplings method involving the use of the Manta-net (Fig.1A) to extract plastic debris from river water. Specifically, samplings with the Manta-net were performed in December 2022 and April 2023 at two sites of the Chienti river: Pontelatrave (near the source) and Montecosaro (approx. 8 km upstream the mouth). Filters from the last step of the extraction process contained meso- and microplastics different in size, shape, and color (Fig.1B). Using Fourier Transform Infrared (FTIR) spectroscopy, we could discriminate the polymers of the collected plastic particles.

A total of 99 microplastics were recovered and analyzed over 2 sampling campaigns: 56 were found at Pontelatrave and 43 were recovered at Montecosaro site. Specifically, at Pontelatrave, 29 fragments were recovered in December 2022 with a density of 0.060 fragments/m^3, and 27 fragments during the sampling in April 2023 corresponding to a density of 0.046 fragments/m^3. At Montecosaro, 22 MPs were detected in December 2022 resulting in a density of 0.049 fragments/m^3, while 21 were recovered in April 2023 corresponding to 0.051 fragments/m^3. Only one meso-plastic fragment was collected at Pontelatrave site in December 2022. The overall density findings are below values reported for other rivers, reflecting the reduced urban or industrial contributions in this area. For instance, the Ofanto River in Italy exhibits MPs concentrations ranging from 0.5 to 18 fragments/m^3 (Campanale et al., 2020). Similarly, the Po River, Italy's largest river, displayed MP concentrations ranging from 0.29 to 3.47 fragments/m^3 (Munari et al.,

2021). The MP densities in the Chienti are much lower than those observed in the Ticino River in northern Italy, where concentrations averaged 33±20 fragments/m³. Similarly to our results, the Ticino study reported the absence of an increasing trend of MP concentrations with river length (Winkler et al., 2022). In addition, when compared with Rhine River in Switzerland, our results align with MP concentrations ranged from 0.04 to 9.97 fragments/m³ (Mani and Burkhardt-Holm, 2020), with the Chienti MPs densities falling toward the lower end.

Chemical characterization shows that the polyethylene (PE) was the most abundant polymer detected, accounting for over 60%. The remaining were 11% polypropylene (PP), 10% polyester (PES) and almost 7% polyamide (PA). Only 2 fragments of polystyrene (PS) and one fragment of ethylene vinyl acetate (EVA) were detected at Montecosaro (Fig.1C). The predominance of PE, PP, PES, and PA correlates with the widespread use of this plastic products in our daily life (Rodrigues et al., 2019). Pontelatrave site, the closest to the river's source, showed a density concentration of 0.053±0.007 fragment/m^3 comparable to the Montecosaro site showing a concentration of 0.050±0.001 fragment/m^3. This is somehow surprising considering that the plastic materials usually accumulate following a gradient of concentration, which increases traveling toward the river mouth (Emmerik and Schwarz, 2020; Lebreton et al., 2017). A possible role in mitigating this type of pollution, could be played by the three artificial reservoirs located along the river Chienti.

Moreover, we studied the AMR profile of the bacteria isolated from the river water, focusing on 3GC-resistant *Enterobacteriaceae* and vancomycin-resistant enterococci. The water samples collected in June 2022 along the Chienti River, were analyzed in Coliform Chromogenic Agar (CCA) supplemented with 4 mg/L Cefotaxime (CTX), and in Slanetz and Bartley medium with 8 mg/L vancomycin; then isolated colonies were biochemically identified. No vancomycin-resistant fecal enterococci were isolated, while 34 oxidase-negative strains were detected on CCA. Specifically, the isolated enterobacteria were *E. coli*, E. cloacae, *K. pneumoniae,* S. enterica, *V. parahaemolyticus, and* C. koseri. Twenty-five strains were selected and characterized by antimicrobial susceptibility test (AST). The panel of antibiotics used includes ceftazidime (CAZ), gentamicin (CN), levofloxacin (LEV), sulfamethoxazole/trimethoprim (SXT), meropenem (MEM), amoxicillin/clavulanic acid (AMC), and tigecycline (TGC), according to EUCAST guidelines. *Overall, these* isolates exhibited high percentage of resistance with the 44% (11/25) being multidrug resistant

bacteria. Specifically, they showed resistances to: Cefotaxime**:** 100 %**,** Ceftazidime**:** 96 %, Levofloxacin**:** 56 %, Amoxicillin: 52 %, Sulfamethoxazole-Trimethoprim**:** 40 %. Three resistant strains to Gentamycin and one to Tigecycline, respectively, were detected**,** while no resistant strains to Meropenem were found (Fig.2).

The findings of this study demonstrate the copresence of plastic pollution and critical resistant bacteria in the Chienti River. Recent studies have demonstrated that the presence of microplastics in aquatic environments facilitates the spread of antibiotic resistance genes (ARGs), promoting biofilm formation and horizontal gene transfer (HGT) among bacterial communities (Ferheen et al., 2024; Yu et al., 2022). Anthropogenic activities impacting on the Chienti river, including deposition of plastic, potentially enhance the AMR spread in the environment.

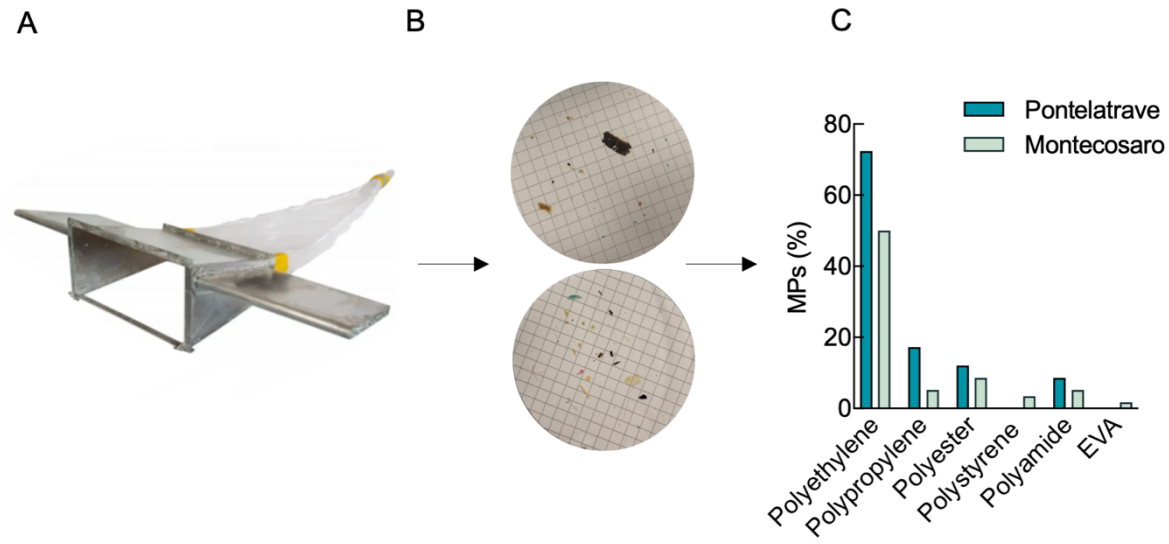

Fig.1 Detection of microplastics in the Chienti river. (A) Manta-net used for plastic debris sampling at two different sites: Pontelatrave and Montecosaro. **(B)** Filters containing meso- and microplastic after the extraction process. **(C)** Percentage of plastic polymers recovered during the two sampling campaigns. Ethylene vinyl acetate (EVA).

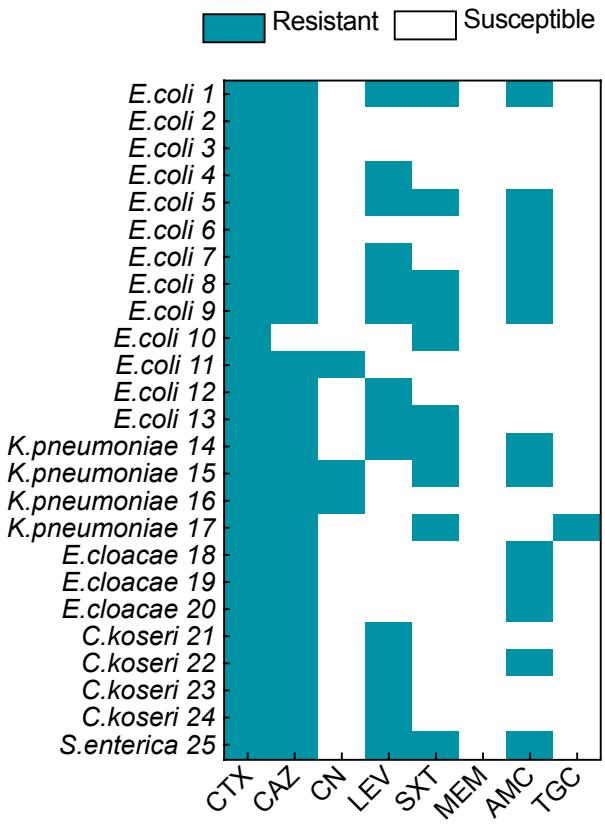

Fig.2. **AMR profile of Enterobacteria isolated from river water.** 3GC-Resistant *Enterobacteriaceae* from water isolates collected along the Chienti river were characterized based on the EUCAST guidelines.

Conclusion

The detection of microplastics in the river, alongside the isolation of 3GC-resistant *Enterobacteriaceae*, is strong evidence supporting a potential new pathway for the spread of critical bacteria in the environment. Due to their durability, poor degradability, low density, and small size, MPs remain for a long time in freshwater systems facilitating the transport of adherent ARB over long distances. The high abundance of organic matter in small river waters, together with the small size of MPs makes the latter difficult to collect, and may underestimate the MP densities, limiting sampling and monitoring activities. On the other hand, this study found an alarming 44% of MDR enterobacteria among all 3GC-resistant isolates, currently challenging infection treatments.

This work together with other reports, the extensive use of plastics and the growing challenge of antibiotic resistance highlight the urgent need to investigate the interactions between MPs and AMR occurring in freshwater environments. Such studies will be crucial to develop

effective strategies aimed to mitigating the environmental and public health consequences of these tightly related threats.

Materials and Methods

Meso- and micro-plastics were collected in the river water using a Manta-net (Hydro-Bios) with a mouth opening 30 x15 cm, a length of the net bag of 200 cm and mesh size of 300 microns, equipped with a mechanical flowmeter (Hydro Bios). Samples were collected in duplicate during a period of 20 min of water flow. Recorded water volumes were in the range of 300 to 600 m^3. The net was rinsed three times using river water, and the retained materials were collected into a glass jar and immediately transferred to the laboratory. The sample pretreatment involved a density separation step with saturated sodium chloride solution in water. The material floating on the surface was then recovered to perform organic matter digestion with 35% hydrogen peroxide solution in a 1:1 ratio (Rani et al., 2023) . The resulting solution was filtered on filter paper (Whatman 3MM) and the meso- plastics were identified by visual inspection, while putative microplastic particles were identified under the stereomicroscope (Olympus SZ61, Optika) based on the physical properties of plastic (color, size and shape). Particles were characterized by FT-IR ATR analysis, performed with a PerkinElmer FT-IR spectrometer Spectrum Two UATR, equipped with a ZnSe crystal. The measurements were conducted in the 400–4000 cm^{-1} range at a resolution of 2 cm^{-1} with 4 scans and processed using the PerkinElmer data manager (Spectrum).

River water was cultured to isolate cefotaxime-resistant coliforms and *E. coli* using the membrane filtration method. Fixed volumes (10 and 100 ml) of river water were filtered through 0.45 µm nitrocellulose membranes. The membranes were then transferred into coliform chromogenic agar medium (CCA) (Thermo Fischer Scientific) supplemented with cefotaxime (4 µg/ml). Plates were incubated at 37°C for 24 hours, after which colonies were counted and isolated. Cefotaxime-resistant isolates were stored in 20% glycerol stocks at -80°C for the following analysis. For the isolation of vancomycin-resistant faecal enterococci, a similar volume of river water was filtered, and the membranes were placed on Slanetz and Bartley (SB) medium (Thermo Fischer Scientific) supplemented with vancomycin (8 µg/ml). To confirm the identification, filters were transferred to the surface of Aesculin Azide Bile Agar (Thermo Fischer Scientific) plates. Isolates resistant to cefotaxime were biochemically identified using API 20 E™ strips (Biomerieux) for the detection of *Enterobacteriaceae.* Identification was performed using APIWEB software. The antibiotic susceptibility profiles

of cefotaxime-resistant *Enterobacteriaceae* strains were determined using the disk diffusion method. Mueller-Hinton agar plates were spread with the 0.5 McFarland bacterial suspension and the disks containing antibiotics were placed on its surface. Antibiotic tested were cefotaxime (CTX, 5 µg), ceftazidime (CAZ, 10 µg), gentamicin (CN, 10 µg), levofloxacin (LEV, 5 µg), cefoxitin (FOX, 30 µg), trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 µg), meropenem (MEM, 10 µg), amoxicillin/clavulanic acid (AMC, 20/10 µg), and tigecycline (TGC, 15 µg). After incubation at 37°C for 18±2 hours, the diameter of the inhibition zones was measured and interpreted according to EUCAST guidelines.

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Authors Contribution

S.A., R.S. and D.P.: Conceptualization, Methodology, Formal analysis, Validation, and Writing- Original draft preparation; R.S. and D.P.: Supervision; S.A.: Visualization; S.A., G.P. and F.S.: Investigation; S.A., R.S., S.G., G.P., F.S. and D.P.: Reviewing and Editing.

Declaration of competing interest

The authors declare that they do not have competing financial with the work reported in this short communication.

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3.2 Plastisphere and River Systems as Potential Reservoir for Antibiotic Resistant Bacteria

This work focused on the characterization of the microbial biofilm forming on plastic debris floating in the Chienti River, in parallel with the study of ARB found in water. The plastisphere, consisting of thin layers of biofilms formed on plastic debris, has emerged as a hotspot for microbial interactions and genetic exchange, including the horizontal transfer of ARGs mediated by MGEs such as integrons and conjugative transposons. This unique microenvironment facilitates the survival and transport of resistant bacteria, creating pathways for AMR to spread across interconnected ecosystems. In this study, caged plastic fragments were introduced into the river, allowing for the assessment of biofilm formation and microbial colonization over time. Coupled with water sampling and analysis, this research aimed to identify and characterize MDR bacteria, to investigate resistance mechanisms, and to analyze the microbial communities and ARGs associated with the plastisphere through metagenomic approaches.

Specifically, plastic fragments of polyethylene (PE) and polypropylene (PP) were deliberately submerged in the river for three weeks to allow bacterial colonization. River water and plastic samples were simultaneously collected at multiple sites along the river, focusing on the isolation of 3GC-resistant *Enterobacteriaceae* with the aim to characterize their complete antibiotic resistance profiles at both phenotypic and genotypic levels. The presence of Class 1 Integron, a genetic element potentially involved in the spread of resistances, was also investigated to characterize the ARGs present in the gene cassette.

As a last step, the entire plastisphere, collected and used to extract total DNA associated to plastic materials, was investigated using shotgun metagenomic analysis to explore the whole bacterial community (other than enterobacteria), with a focus on antibiotic resistance genes and mobile genetic elements.

Overall, these findings emphasize the urgent need to address research gaps in freshwater ecosystems to identify potential risks due to often underestimated public health threats.

Plastisphere and River Systems as Potential Reservoir for Antibiotic Resistant Bacteria

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Keywords: Plastisphere$_1$, Microplastic$_2$, Antimicrobial Resisistance$_3$, Biofilm$_4$, Class 1 Integron$_5$, River$_6$, Resistome$_7$, Enterobacteria$_8$.

Abstract

Antimicrobial resistance (AMR) is a critical global health threat, with freshwater ecosystems acting as a potential route for the spread of multidrug-resistant (MDR) bacteria and antibiotic resistance genes (ARGs). In this study, caged plastic fragments were deliberately introduced into three sites along a river of central Italy for three weeks, and in parallel river water samples have been collected and analyzed. Out of 267 bacterial isolates obtained from plates supplemented with cefotaxime (CTX), 50 CTX-resistant enterobacteria were selected for further phenotypic and genotypic analysis. The results indicated a high prevalence of MDR bacteria, with 84% of water-derived and 72% of plastic-derived isolates. Additionally, we found a high presence of ESBL producers, with 80% of plastic-associated strains and 56% of water-derived strains, with river water exhibiting a higher percentage of AmpC producers. Among ESBLs, five carbapenem-resistant enterobacteria (CRE) produced KPC (*Klebsiella pneumoniae* carbapenemases) enzymes, and two strains were positive for metallo-β-

lactamases (NDM and VIM). Three KPC-producers, identified as *Klebsiella pneumoniae*, showed the sequence type ST1519, detected for the first time in a natural environment in Italy. The detection of the 3GC-resistant enterobacteria in rivers, categorized as bacteria of public health importance, is alarming because these bacteria can potentially re-enter human commensals through environmental pathways. Shotgun metagenomic analysis provided a comprehensive snapshot of the plastisphere's microbial communities, revealing dominance by biofilm-forming families such as *Comamonadaceae*, *Sphaerotilaceae*, and *Flavobacteriaceae*, which play key roles in biofilm formation and stability. The resistome highlighted the key ARGs conferring resistance to clinically important antibiotics, such as beta-lactams, vancomycin, and tetracyclines, alongside mobile genetic elements (MGEs) such as integrons, which facilitate the horizontal transfer of resistance genes. Our study emphasizes the potential of rivers and plastic to act as reservoirs for critical bacteria, including 3GC-resistant enterobacteria and CRE, facilitating the spread of resistance determinants to clinically significant pathogens, currently challenging the public health care systems.

1.0 Introduction

Antimicrobial resistance (AMR) has become one of the most critical public health threats of the 21st century, requiring immediate global action, as emphasized by the World Health Organization (WHO) and the European Centre for Disease Prevention and Control (ECDC). Its rise is associated with elevated treatment failures and relapsing infections, higher morbidity and mortality rates as well as with increasing healthcare costs (Huemer et al., 2020; Ding et al., 2023). It is estimated that by 2050, antibiotic-resistant infections will be the cause of over 10 million annual deaths, with 700,000 deaths already occurring each year (O'Neill et al., 2014; Aslam et al., 2018).

This crisis extends beyond clinical settings with the environment significantly contributing to the emergence and spread of multidrug-resistant (MDR) bacteria and antibiotic resistance genes (ARGs) (Ahmad et al., 2021). Therefore, addressing AMR in a One Health perspective is essential to promote a comprehensive strategy that links human, animal, and environmental settings (Finley et al, 2013). In particular, the environmental resistome may represent a prolific source for the acquisition of resistances by clinically relevant bacterial strains found in natural environments (Nappier et al, 2020; Larsson & Flach, 2022).

Freshwater systems are significant route through which resistant bacteria can spread, since wastewater treatment plants effluents, healthcare facilities, industrial processes, and agriculture activities introduce pollutants (e.g. antibiotic residues, heavy metals, and pesticides) directly into rivers (Andersson and Hughes, 2012). These pollutants exert selective pressure on microbial communities and drive the co-selection of resistant determinants (Martinez et al., 2009; Zainab et al, 2020).

A factor amplifying the AMR is the formation of environmental biofilms. These assemblages of microorganisms, characterized by the proximity of cells, extracellular DNA (eDNA) and heterogeneous extracellular polymeric substances matrix (EPS), promote the ideal conditions for genetic exchange and spread of resistance genes (Flemming et al, 2021). These structures grow on various surfaces in the environment, including natural substrates like rocks and artificial as those formed on plastic debris floating in aquatic systems. (Oberbeckmann et al., 2014; Besemer, 2015; Michels et al., 2018). Plastic materials, due to their strong durability and the ability to adsorb pollutants, provide an ideal substrate for bacteria to attach and develop into biofilm. Specifically, biofilms growing on plastic, known as *plastisphere*, create a microenvironment where genetic exchange of ARGs via horizontal gene transfer (HGT) is promoted (Zettler et al., 2013; Amaral-Zettler et al., 2020).

HGT is another factor contributing to the propagation of AMR in the environment, amplified by mobile genetic elements (MGEs) as transposons, integron gene cassette array, and plasmids (Partridge et al., 2018). Class 1 integron, through site-specific recombination, is central in the acquisition and expression of ARGs (Hall and Collis, 1995). Its gene cassette can be mobilized through integration or excision at the recombination site, while the structure of the integron is not able to move itself but is often associated with other MGEs, enhancing the horizontal resistance gene transfer across and between different bacterial species (Boucher et al., 2007). This integron class is the most prevalent in MDR clinical isolates and the *intI1* gene, encoding for the enzyme integrase, is considered as a promising indicator of the anthropogenic pollution levels (Gillings et al., 2015).

The interplay of all the described factors creates a dynamic system where rivers are central conduits for both pollutants and resistant bacteria. Plastic materials introduced in freshwater environments, constitute a substrate for the whole cycle of biofilm development, which in turns facilitates the HGT of antibiotic-resistance genes by MGEs (Fig. 1A).

Additionally, due to their physiochemical characteristics, plastic particles float and move through the rivers, interacting with the surrounding microbial communities and enhancing the spread of AMR across various environments.

Following the WHO's classification of critical-priority bacteria, which includes third generation cephalosporin (3GC)-resistant *Enterobacteriaceae* (Tacconelli et al., 2018), we focused our research on the characterization of critical resistant bacteria present in a central Italian river. To this purpose, caged plastic fragments were introduced into different sites along the Chienti river (Marche, Italy), then at different points in time they were collected, and the associated bacteria have been analyzed in parallel with those recovered from the surrounding river water (Fig.1B). Our research aimed to: (i) investigate the presence and distribution of cefotaxime (CTX)-resistant *Enterobacteriaceae*, (ii) characterize the antibiotic resistance profile of CTX-resistant isolates at phenotypic and genotypic levels, (iii) explore the molecular elements potentially involved in the spread of resistance determinants and (iv) use metagenomic approaches to provide a comprehensive analysis of the plastisphere, investigating the resistome of the bacterial communities attached to these efficient vehicles for ARGs transfer.

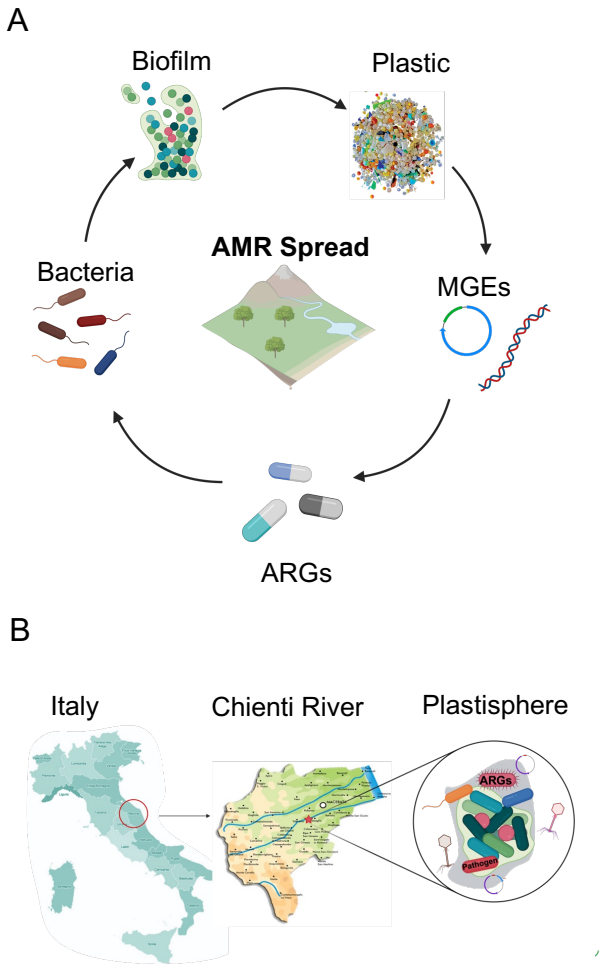

Fig1.The Plastisphere and AMR spread. (A) Scheme showing factors influencing the antimicrobial resistance (AMR) spread in freshwater ecosystems, including biofilm on plastic debris (plastisphere) and horizontal transfer of Antimicrobial Resistance Genes (ARGs) by Mobile Genetic Elements (MGEs). **(B)** Geographical location of the experiment. Two types of plastic materials were deliberately placed into the Chienti river in the Marche region of Italy in a time-course experiment. The resulting plastisphere and its associated ARGs were analyzed by a multidisciplinary approach.

2.0 Results

2.1 Experimental set-up

River pollution and in particular plastic contributes to the dissemination of AMR in the environment (Nnadozie et al., 2019; Lebreton et al., 2017). To study this problem focusing on rivers, where significant literature gaps are present, we established a protocol to analyze the AMR profile of coliforms present in river water, including those colonizing plastic fragments. Using microscopic, microbiological, and molecular techniques we provide a comprehensive overview of the plastisphere's role in the spread of the antibiotic resistance threats in aquatic ecosystems (Fig.2).

The experimental procedure was divided into several steps. Initially, to assess the presence of the total viable bacteria present on the plastic surfaces, they were stained with SYTO9 dye and visualized under Confocal Laser Scanning Microscopy (CLSM). Then, the bacteria were detached from the plastic fragments using a mild sonication. The sonicated solution was then cultured on Coliform Chromogenic Agar (CCA) supplemented with cefotaxime (10 μg/ml). River water samples were in parallel processed using the same protocol. After 24 hours of incubation at 37°C, *E. coli* and coliform isolates were identified. Fifty CTX-resistant enterobacteria were isolated, biochemically characterized using API 20E strips and the phenotypic antimicrobial profile characterized through antibiotic susceptibility test according to EUCAST guidelines. Resistance phenotypes were confirmed using ESBLs, and KPC/MBL and OXA-48 confirmation kits. Additionally, we investigated the molecular profiles of the antimicrobial resistance genes. Genomic DNA was extracted and used for

PCR amplification using specific primers targeting antibiotic resistance genes (ARGs) for the classes of sulfonamides, quinolones, beta-lactams and carbapenems. The presence of Class 1 Integron was detected, and the gene cassettes were sequenced to identify their associated ARGs. As last step, we performed shotgun metagenomic analysis on the total DNA extracted from biofilm associated to PE and PP, characterizing the microbial community, resistome, and mobile genetic elements residing on it. This allowed to enhance and broad our knowledges on the complete microbial community associated to the plastisphere, including uncultivable bacteria, and to complement information provided by microbiological and molecular assays besides the CTX-resistant *Enterobacteriaceae*.

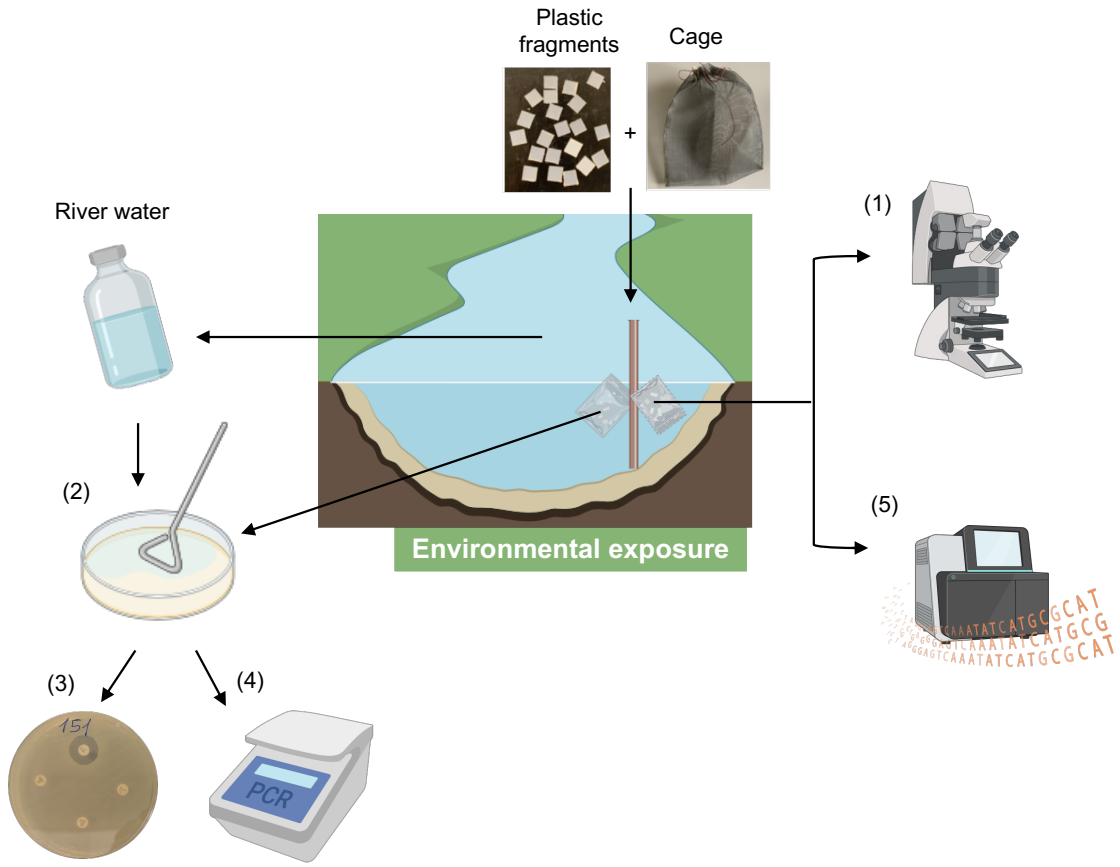

Fig.2 Overview of the study. PE and PP fragments were inserted into filter cages and submerged in the river in three distinct sites (Pontelatrave, Corridonia, Montecosaro). River water and plastic fragments were collected and analyzed weekly. Workflow of the study included: (1) Confocal Laser Scanning Microscopy analysis to detect biofilm associated to plastic fragments; (2) Bacterial isolation on CCA (Chromogenic Coliform Agar) medium with 10 mg/L Cephotaxime and biochemical identification; (3) Antimicrobial susceptibility

testing (AST); (4) Molecular analysis to identify ARGs and Class 1 Integron gene cassettes; (5) Metagenomic analysis to study the total microbial community describing the plastisphere, the associated ARGs and MGEs.

2.2 Visualization of biofilm on plastic fragments by CLSM

The presence of viable bacteria on plastic surfaces for each sampling week was assessed using confocal laser scanning microscopy (CLSM). This analysis confirmed microbial colonization of both polyethylene (PE) and polypropylene (PP) fragments (Fig. S1). Biofilm adhering to plastic substrates were detected in each image analysis with differences observed between the plastic fragments in the spatial distribution of the cells and dimension of the clusters. Specifically, PP showed localized clusters of cells, while PE demonstrated a more homogeneous distribution of the cells on its surface (Fig. Supp.1).

2.3 Antibiotic susceptibility of CTX- Resistant *Enterobacteriaceae*

The environment facilitates the transmission of resistant bacteria to humans and animals (Larsson and Flach, 2022). To analyze their presence in rivers, we characterized the antibiotic resistance profile of CTX- resistant *Enterobacteriaceae* isolated from both river water and plastic fragments.

Out of 267 CTX-resistant isolates collected, 50 strains were selected for phenotypic analysis: 25 from river water and 25 from plastic surfaces. These isolates were biochemically identified and tested for antimicrobial susceptibility (AST) to assess their resistance profiles.

The biochemical identification showed the presence of: *Citrobacter freundii* (2), *Escherichia coli* (13), *Enterobacter aerogenes* (1), *Klebsiella pneumoniae* (8), *Citrobacter brakii* (1) associated to plastic, while *Citrobacter freundii* (1), *Escherichia coli (16), Enterobacter cloacae* (1), and *Klebsiella pneumoniae* (7) associated to river water.

The AST results indicated the presence of a high number of isolates from both sample sources resistant to multiple antibiotics (Fig 3A-B). Notably 7 strains (1 from plastics and 6 from river water) were resistant to meropenem a last resort antibiotic for the treatment of multi-drug-resistant infections (Papp-Wallace et al., 2011).

The frequency of resistance, other than cefotaxime, showed similar rates for ceftazidime (CAZ), gentamycin (CN), and trimethoprim-sulfamethoxazole (SXT) between the two sample types. However, higher resistance to cefoxitin (FOX), meropenem (MEM), levofloxacin (LEV), and tigecycline (TGC) were present in river water enterobacteria (Fig.3C). A significant percentage (78%) of isolates exhibit multidrug resistant traits, while a minority (22%) display dual-drug resistance (DDR) characteristics. A higher percentage of MDR bacteria, up to 84% (21/25), was detected among the *Enterobacteriaceae* from water, with only the 16% (4/25) being DDR isolates. On the other hand, plastic harbored 72% (18/25) of MDR bacteria and 28% (7/25) DDR. (Fig.3D).

To further understand the resistance mechanisms to third-generation cephalosporins (3GCs) such as cefotaxime and ceftazidime, we investigated the production of extended-spectrum beta-lactamases (ESBLs), a key resistance mechanism in clinical isolates. (Fig. 3E). The results demonstrated a high prevalence of ESBL producers among the *Enterobacteriaceae* isolated from both plastic-associated and river water samples. Specifically, 80% of the strains from plastic and 56% from river water were identified as ESBL producers. In addition to ESBLs, AmpC beta-lactamase producers were identified, which constituted 8% of the plastic isolates and 24% from river water samples. This data suggests that the plastisphere may select for ESBL-producing bacteria, while river water have higher diversity of resistance mechanisms, including higher percentage of AmpC (Fig. 3F).

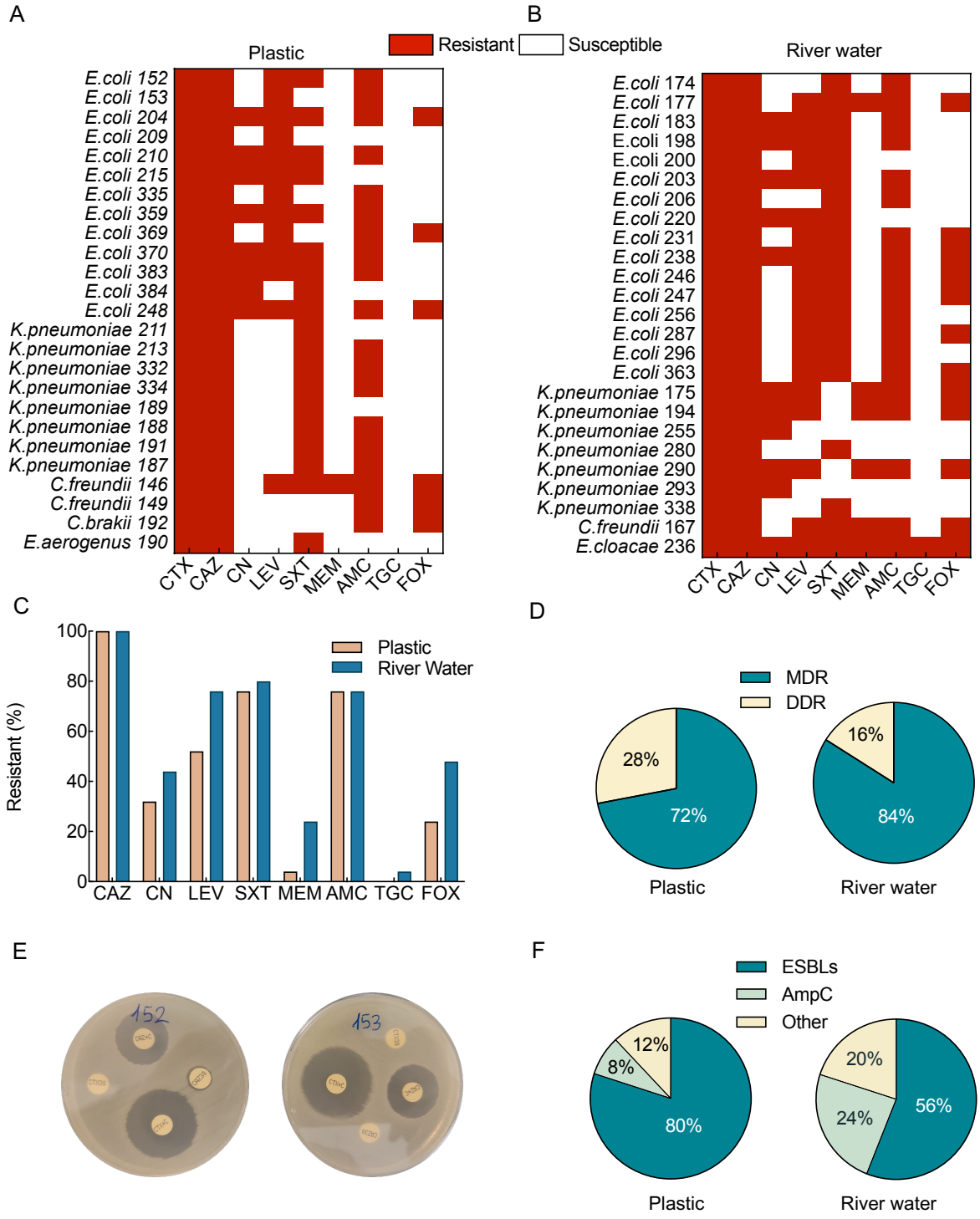

Fig.3 Characterization of antibiotic resistant Enterobacteria. Antimicrobial susceptibility test (AST) of bacteria isolated from plastic **(A)** and raw water **(B)** according to EUCAST guidelines. For each sample, 25 CTX-resistant enterobacteria were tested against eight antibiotics covering six drug classes. **(C)** Resistance rates other that Cefotaxime. **(D)** Drug resistance classification types: Multi-Drug Resistant (MDR), Dual-

Drug Resistant (DDR). **(E)** Two examples of isolates producing Extended-Beta Lactamases (ESBLs) using the phenotypic test. **(F)** Eighty percent of the resistant strains from plastic and 56% from raw water are ESBL producers.

2.4 Characterization of carbapenems resistant *Enterobacteriaceae* isolates

To characterize the seven meropenem-resistant strains, we conducted a phenotypic test to detect the carbapenemases production.

Five of these strains (*C.freundii* 146, *K.pneumoniae* 175, *K.pneumoniae* 194, *E.cloaceae* 236, *K.pneumoniae* 290) were KPC-producers, while two strains (*C.freundii* 167, *E.coli* 177) produced metallo-β-lactamases (MBLs). The presence of carbapenems resistance genes was by molecular analysis. The bla_{KPC} gene was found in the strains *C.freundii* 146, *K.pneumoniae* 175, *K.pneumoniae* 194, *E.cloaceae* 236, *K.pneumoniae* 290; and the bla_{NDM} (New Delhi metallo-β-lactamase), in the *C.freundii* 167, *E.coli* 177 strains. Additionally, the bla_{VIM} gene (encoding a different metallo-β-lactamase) was detected in *C.freundii* 146 isolated from plastic associated biofilm.

2.5 Multi-Locus Sequence Typing of KPC-producers *K. pneumoniae strains*.

On the three Meropenem-resistant and KPC-producers *K. pneumoniae* isolates mentioned above, Multi-Locus Sequence typing (MLST) analysis was conducted to determine the genetic association between these environmental isolates and the currently circulating lineages. These strains exhibited a consistent multidrug resistance pattern, including resistance to CTX, CAZ, LEV, CN, MEM, AMC, and FOX, and tested positive for *blaTEM*, *blaSHV*, and KPC genes. Based on the results of DNA sequences of seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB)* we determined their allelic profile, which is used to identify the sequence type (ST) of the isolates according to the Pasteur Institute scheme.

The analysis revealed that all three isolates shared the same allelic profile: *gapA*: 54, *infB*: 3, *mdh*: 1, *pgi*: 1, *phoE*: 1, *rpoB*: 9, *tonB*: 79, which corresponds to the sequence type ST1519.

2.6 Molecular Detection of ARGs

We performed molecular analysis targeting ARGs including *qnrB, qnrS, qnrA (*quinolones*),* bla_{SHV}, bla_{TEM}, bla_{CTX-M}, *ampC04, ampC05* (beta-lactamases), *sul1, sul2,* and *sul3* (sulfonamides).

All targeted ARGs were identified in the samples except for *ampC04, ampC05* and *qnrA*. Comparing the AST results of the isolates with the ARGs detected, we observed that: 1) *sul* genes were consistently found in 100% (39/39) of trimethoprim/sulfamethoxazole (SXT) resistant strains; 2) the *qnr* genes (*qnrA*, *qnrB* and *qnrS*) were found in 40,6% (13/32) of levofloxacin (LEV) resistant strains; and at least one of the *bla* genes was found in 78% (32/41) of ESBL-positive strains.The frequency of detected ARGs were: sul1 = sul2 (30/50) > bla_{TEM} (21/50) > bla_{SHV} (18/50) > qnrB (14/50) > qnrS (13/50) > bla_{CTXM} (2/50) > sul3 (1/50) > qnrA = ampC04 = ampC05 (0/50). The *sul* genes were detected from both the sources, with at least one of the three genes present in the 80% of the total isolates, suggesting widespread presence of the resistance genes to sulfonamides in this environment (Fig.4A-B).

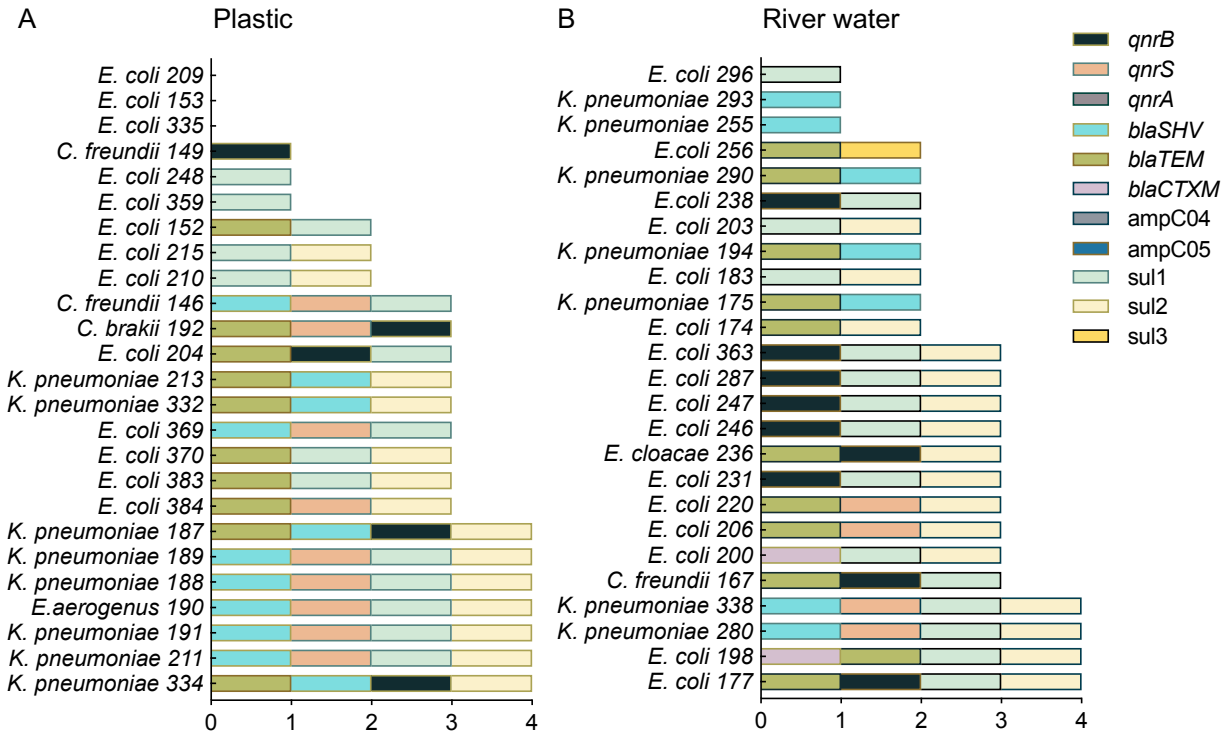

Figure 4. ARGs prevalence. Molecular detection by PCR of 11 ARGs in the bacteria isolated from plastic **(A)** or river water **(B)**.

Class 1 Integron and the associated ARGs cassettes play a central role in the resistance genes acquisition within Gram-negative bacteria (Gillings, 2014).

Seven different cassette arrays were detected, containing ARGs associated to aminoglycosides (*aadA2, aadA5 and ant(2'')-Ia*), trimethoprim (*dfrA17, dfrA12, and dfrA1*), chloramphenicol (catB3), and rifampicin (*ARR-3*). Additionally, in *E. coli* 203, 200, 183, and 296, a gene cassette composed of *aadA5* and *dfrA17* was detected. Notably, the Class 1 integrase gene was absent in these 4 isolates, including *E. coli* 296, which carried *Integrase 2*. Overall, we observed a consistent presence of aminoglycoside resistances among isolates. The *E. coli* 369 from plastic contained *blaOXA-1* gene, encoding an oxacillinase, consistent with its ESBLs production. Similarly, *C. freundii* 146, carried the *blaVIM-1* gene encoding a metallo-beta-lactamase, in concordance with the AST showing resistance to meropenem and with molecular analysis. Furthermore, we identified a gene cassette containing Class 2 integrase (Int2) in *E. coli* 248 and *E. coli* 296. The Int2 gene is non-functional, representing a fragment that covers approximately 50% of the full sequence. Among the most frequently detected gene cassette arrays, we observed a high prevalence of ARGs associated to aminoglycosides and trimethoprim resistances (represented in the fig.5A-B in green and yellow). Notably, 12 plastic isolates exhibited this cassette array, compared to the 4 detected within river water isolates.

The *Int1* gene was detected in, 92% (23/25) of the plastic isolates, and with the 65% (15/23) of them harboring an associated gene cassette (Fig.5A). In contrast, only 44% (11/25) of the river water isolates carried the *int1* gene, with the 55% (6/11) of these isolates containing an associated gene cassette carrying ARGs (Fig.5B). Overall, the gene cassette arrays of Class 1 Integrons detected in *Enterobacteriaceae* from plastic samples showed a higher heterogeneity in inserted resistance genes than those from water samples, aligning with previous studies of bacteria associated to microplastic in aquatic environment (Zhang et al., 2020).

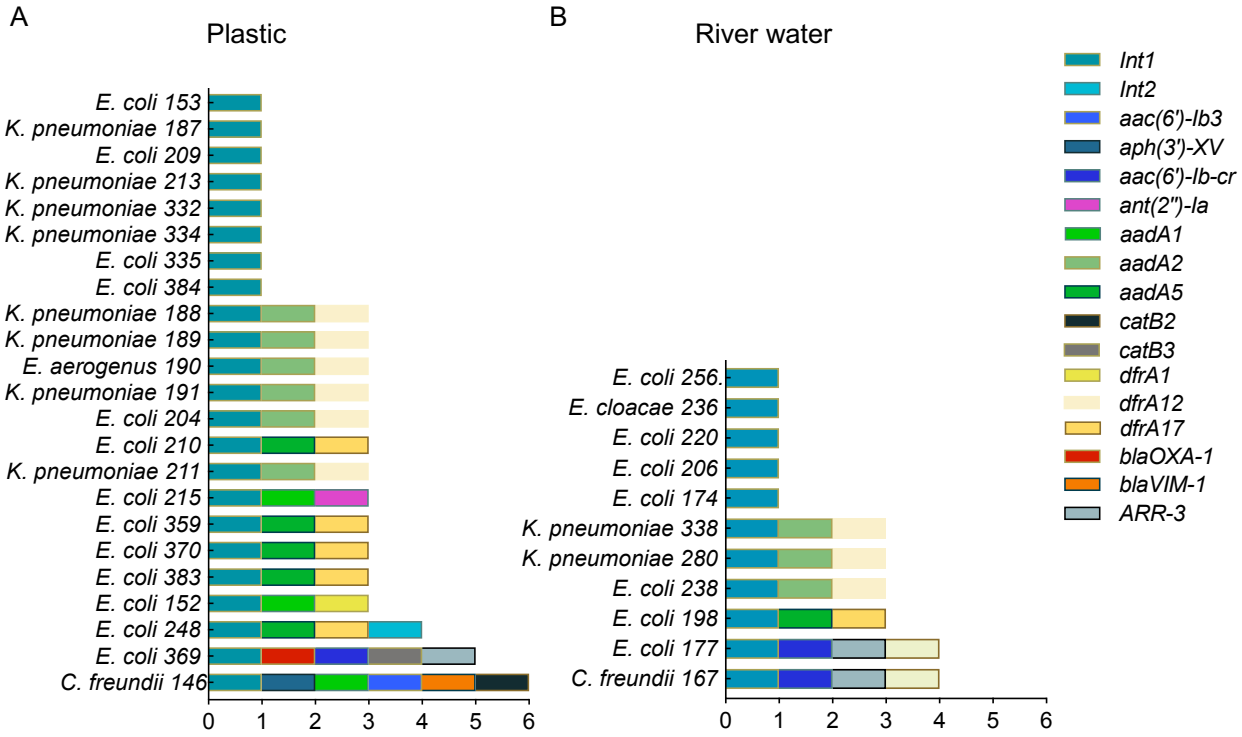

Fig.5 Class 1 Integron prevalence. Class 1 integron gene cassettes array in different bacterial species from plastic **(A)** and river water **(B)**. The presence of *Int1* was assessed by PCR, while ARGs associated to the gene cassettes were identified by Nanopore sequencing.

2.7 Plastisphere Metagenomic Analysis

Shotgun metagenomic analysis of the plastisphere provided a detailed overview of the taxonomic and AMR profiles of microbial communities associated with plastic samples P1, P2, and P3 collected at each sampling site, Pontelatrave, Corridonia and Montecosaro, respectively.

A core genome of 1,442,182 genes was shared among the three plastic samples analyzed (Fig.6A). The taxonomic composition of the samples was dominated by bacteria, which accounted for approximately 75-80% of the microbial community in all samples. Despite the predominance of bacterial species, archaea, viruses, and eukaryotes were also present, but in smaller proportion (Fig.6B).

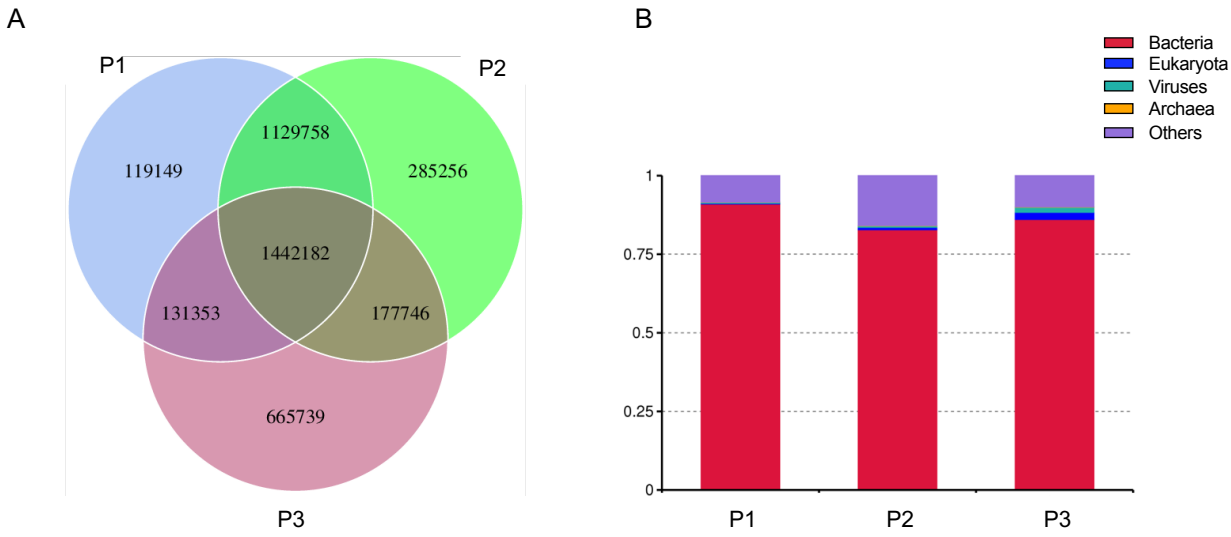

Fig.6 Core genome and composition of microbial communities on plastics. (A) Venn genome analysis based on the abundance of genes in every sample. The analysis identifies a Core-pan genome of approx. 1442182 genes. **(B)** Relative abundance for each sample at taxonomic level.

The taxonomic analysis revealed significant variations in the microbial composition at both the family and genus levels. Less dominant taxa and those unclassified in databases, accounted from 64-67% of the total family-level microbial composition, and from 77 to 82% at the genus-level composition (Fig. Supp. 2A-B). Among the dominant families, *Comamonadaceae*, *Sphaerotilaceae*, and *Flavobacteriaceae*, were consistently detected across all plastic types, but with variations in their relative abundance. For instance, P3 exhibited a higher abundance of *Flavobacteriaceae* and *Sphaerotilaceae*, and uniquely hosted *Filoviridae* (Fig. 7A). At the genus level, shared genera like *Rubrivivax*, *Aquabacterium*, and *Flavobacterium* formed a core microbiota across all plastic samples, likely playing crucial roles in biofilm dynamics. On the other site in P1 and P2 *Rubrivivax* is dominant while P3 was enriched in *Flavobacterium*. Additionally, *Ideonella,* known to be involved in plastic degradation, was present in all samples but more abundant in P3 (Fig. 7B).

The resistome analysis highlighted a broad diversity of antibiotic resistance genes (ARGs). Specifically, ARGs conferring resistance to clinically important antibiotics, such as vancomycin (*vanY*, *vanW*), tetracyclines (*tetC*), and beta-lactams (*TEM-206, CMY-132*), were detected in all plastic samples, with P3 exhibiting the highest diversity (Fig. 7C). Additionally, other notable ARGs were identified, such as *adeF*, which confers resistance to fluoroquinolone and tetracycline antibiotics; *AAC(6')-32* and *AAC(6')-IIa*, associated with aminoglycoside resistance; and *FosI* and *FosA5*, which provide resistance to phosphonic antibiotics. Beyond antibiotic resistance genes, the RGI (Resistance Gene Identifier) database used in this analysis identified the presence of genes involved in resistance to disinfectants, known to be involved in the co-selection mechanisms that promotes the persistence and spread of ARGs (Basiry et al., 2022). Specifically, genes *qacG* and *qacJ* coding to resistance quaternary ammonium compounds were identified in this study.

Importantly, integrons identified in the plastisphere were linked to specific bacterial hosts, based on accession numbers deposited in the Integrall database. All integrons identified belong to the Class 1, expect for the *CP001220.2* classified as the Class 2 Integron. Class 1 integron integrase genes were detected in *Hydrogenophaga sp.*(CP017311.1) *Melaminivora sp.* (CP017311.1), *Comamonas testosteroni* (CP001220.2), *Rhodobacter sp.* (CP017781.1), *Thauera humireducens* (CP014646.1), and among bacteria of clinical importance like *Citrobacter freundii* (AP025035.1), *Klebsiella aerogenes* (CP047669.1)

and *Pseudomonas sp. TUM18999* (AP023189.1), commonly harboring multidrug-resistant traits (Fig. 7D).

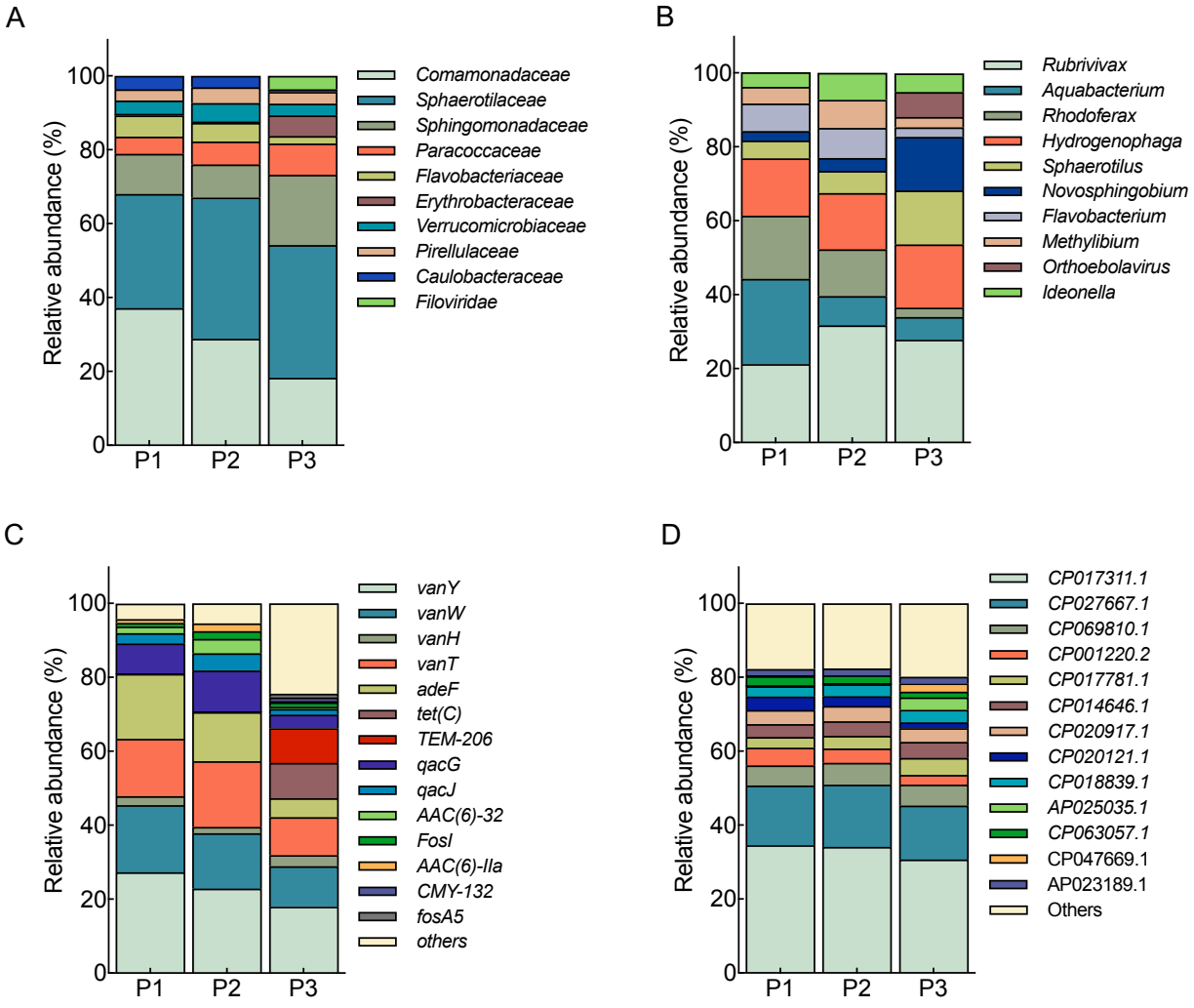

Fig.7. Taxonomic and AMR profiling of the bacteria associated to the plastisphere. Metagenomic analysis based on shotgun sequencing data from P1, P2, and P3 plastisphere samples. Relative abundance of predominant bacteria at family level **(A)**, and genus level **(B)** Distribution of antibiotic resistance genes (ARGs) **(C)** and integrons **(D)** identified from the contigs (>500 bp) in each sample.

3.0 Discussion

Plastic pollution is rising due to the steadily increase of global production. This phenomenon has a considerable impact in freshwater systems, which act as conduits for transferring pollutants from land to oceans (Law et al., 2017; Dauvergne et al., 2018). Concurrently, antimicrobial resistance (AMR) is recognized has a growing threat that extends far beyond clinical settings and require a One Health approach. A relevant issue in this regard is the worldwide spread, in the last decade, of *Enterobacteriaceae* resistant to third-generation cephalosporins. Very few therapeutical treatments are currently available when this resistance is combined with the resistance to other first line agents - such as fluoroquinolones or aminoglycosides – (Rohde et al, 2018).

Our study highlights the critical intersection of these two global threats, through a multidisciplinary approach involving the development of an experimental setup for the evaluation of biofilm formation on plastics simulating plastisphere formation in a riverine environment, the detection of multidrug-resistant (MDR) bacteria and their antibiotic resistance genes (ARGs).

In this study a high number of 3GC-resistant *E. coli* and coliform were isolated from plastics and river water. The isolated bacterial strains belong to species commonly found in riverine environment (Di Bonaventura MDR 2019; Delgado-Blas JF, 2021). Most of the isolates display a Multi Drug Resistant profile, representing a public health risk.

Notably, a higher prevalence of extended-spectrum beta-lactamase (ESBL)-producing bacteria was observed on plastic fragments (80%) compared to river water (56%), suggesting that the plastic materials may play a role in the persistence in the environment, providing a suitable substrate for the adhesion and proliferation of these clinically relevant microorganisms. These results suggest that both plastic and water environments support the proliferation of 3GC-resistant *Enterobacteriaceae*. Plastic surfaces may provide a favorable environment for the acquisition of ESBL genes, while in the river water samples a broader diversity of resistance mechanisms, including AmpC production. Both ESBL- and AmpC-producing strains are associated with treatment failures in clinical settings, highlighting the public health significance of these environmental resistant bacteria.
In this study among the 3GC resistant isolates we detected 7 carbapenems resistant strains

(CRE), in both river and plastics, confirming the public health risks posed by freshwaters in the spread critical priority pathogens that potentially may enter or re-enter humans by commensals pathways. Among the CRE, identification of 3 KPC-producing *Klebsiella pneumoniae* ST1519, a strain previously reported only in Italian hospitals, highlights the tight connection between clinical and environmental settings. Such findings raise questions about the mechanisms enabling these high-priority pathogens to persist in natural ecosystems and potentially move between environments, animals, and humans. Notably, there is a significant gap in the literature regarding the investigation of these critical priority bacteria in river ecosystems, with no other studies reporting the presence of *CRE* in Italian rivers.

The evidence that the plastisphere represents a hotspot for the acquisition and spread of antimicrobial resistance genes (ARGs) (Stevenson EM, 2024) is supported by our findings of an increased prevalence of Class 1 integrons, which showed greater heterogeneity and length of associated gene cassette, in plastic isolates compared to those from river water. These gene cassettes encode a diverse array of resistance determinants that can be horizontally transferred across different species, enhancing the adaptive potential of bacterial populations (Stalder et al., 2021). The presence of integrons in both environmental and clinical bacteria may also indicate their role as mediators of genetic exchange and their ability to mobilize ARGs between non-pathogenic and pathogenic bacteria, enhancing the entire adaptability of microbial communities (Bhat et al., 2023).

Among ARGs identified by metagenomic analysis, the most abundant resistance determinants were *TEM-206*, *CMY-132* (beta-lactams), *tetC* (tetracyclines), and *vanY*, *vanW* (vancomycin). The concomitant detection of disinfectant resistance genes *qacJ* e *qacG*, further highlights the plastisphere's potential to adapt to and spread resistance beyond antibiotics, particularly in environments exposed to anthropogenic pressures (such as agriculture runoff or farming practices). Disinfectants are known to exert selective pressure, not only promoting the persistence of resistant bacteria but also increasing the mutation rate and enhancing their ability to acquire antibiotic resistance (Randall et al., 2004).

In conclusion, our results demonstrate that the plastisphere may act as a genetic reservoir for antibiotic resistances, amplifying the risk of ARGs being transferred to clinically significant pathogens. Further research is needed to elucidate mechanisms involved in ARGs mobilization within rivers impacted by pollution. Such evidence will be fundamental to

contain the spread of AMR from environmental reservoirs to clinical settings, as well as better preventing the transfer of clinically derived resistances from hospitals into natural environments.

4.0 Material and Methods

4.1 Study area and sampling sites

The study area is in central Italy (Marche Region). Three different geographical locations along the course of the Chienti river were selected as sampling sites, namely Pontelatrave (PLT), Corridonia (CORR) and Montecosaro (MONT) (Fig. 8). They are characterized by different features and their selection was performed considering data emerged from the last report on the monitoring of Chienti river by ARPAM (Agenzia Regionale per la Protezione Ambientale delle Marche).

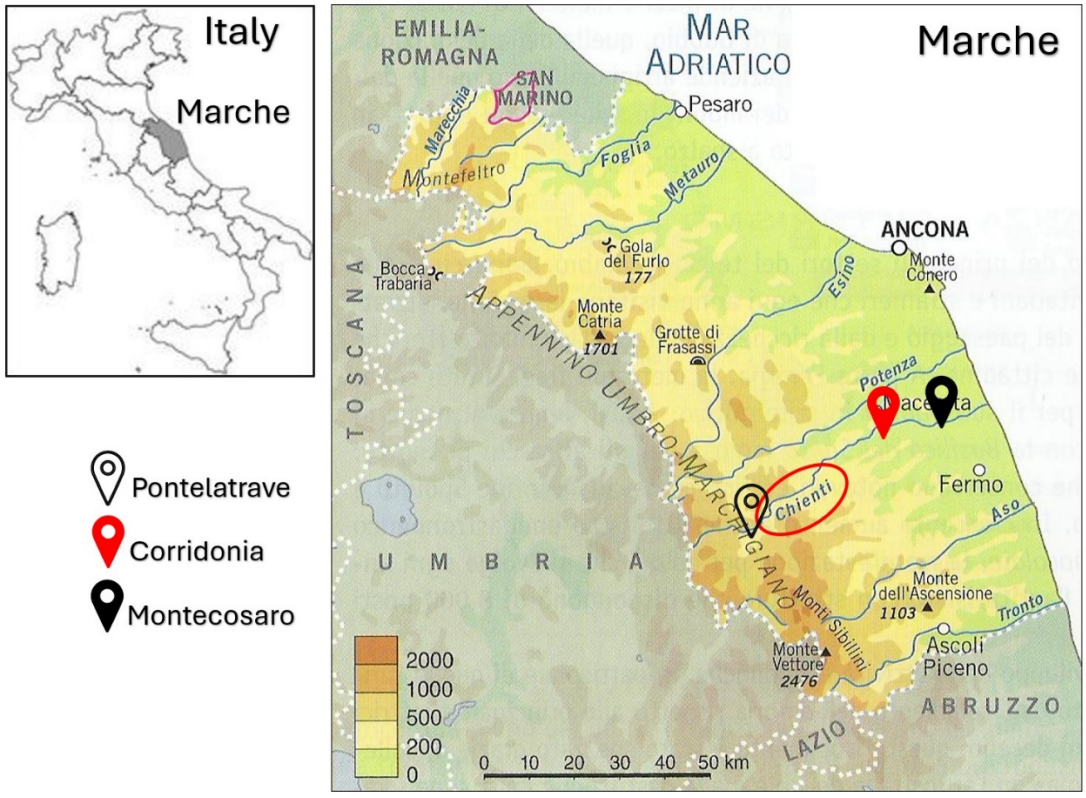

Figure 8. Geographical location of sampling sites along the course of Chienti river (Italy, Marche).

Selected sampling sites show the following main features:

The site of Pontelatrave (x: 2363793; y: 4771352; ARPAM monitoring station: R110195CH)

is the closest to the source of the river (14 km). In this section the watercourse has moderate current velocity, with an avarage flow rate of 1923 l/s in 2021. The use of the surrounding land is mainly agricultural, so the environmental pressure is represented by agricultural use and transport. In this site ARPAM reported the presence of pesticides and microbial pollution by *E. coli*.

The site of Corridonia (x: 2399783; y: 4792022; ARPAM monitoring station: R1101913CH) is 40 km downstream from Pontelatrave site along the river. The watercourse is affected by daily variation of the flow due to the release of the hydroelectric power stations located upstream. The surrounding area shows a higher population density compared to PLT and the land use is for agriculture and industry. Different environmental pressures were reported: chemical pollution (pesticides, phthalates, metals), urban runoff, agriculture and transport.

Montecosaro (x: 2409404; y: 4791483; ARPAM monitoring station: R1101914CH) is located 7 km upstream the river mouth and in this section the current velocity is moderate, with limited turbulence. Similarly to Corridonia, the surrounding area shows a higher population density compared to PLT and its land use is for agriculture and industry. Environmental pressures were reported: chemical pollution (pesticides, metals), nitrogen pollution, urban runoff, agriculture use and transport (Report BACINO CHIENTI 2018-2020, ARPAM).

4.2 Field experiment sampling

Two hundred plastic fragments (both PE and PP) were artificially inserted in the three previously described sites of Chienti river, inside nylon bags exposed to the surface layer of the water. The bags were firmly anchored to the side banks through a steel wire. Plastic fragments (25 PP and 25 PE) and water samples (1L) were collected from each site for three weeks weekly. The samples were immediately refrigerated at +4°C, transported to the laboratory and analyzed within 24h.

The plastic fragments were processed according with the following scheme:

- 3 fragments for each plastic type were stained using Syto9 and observed under a confocal fluorescence microscope to visualize the biofilm formed on the surface.
-12 fragments were subjected to a mild sonication to detach the cells from the surface of the plastic without compromising their viability.

4.3 Experimental set-up

The experimental procedure was divided into several steps. Initially, to assess the presence of the total viable bacteria, plastic fragments were stained with SYTO9 dye and visualized under Confocal Laser Scanning Microscopy (CLSM). Then, to detach the bacteria from the plastic fragments, mild sonication (Misonix Sonicator 3000 Ultrasonic Cell disruptor) was performed on 12 fragments for sample under the following conditions: 4 pulses of 45 seconds each at 9W, separated by 1-minute intervals. The sonicated solution was then either cultured or filtered through nitrocellulose filters (with a pore diameter of 0.45 microns) and subsequently plated on the Coliform Chromogenic Agar (CCA) selective medium, supplemented with cefotaxime (10 μg/ml). River water samples were in parallel processed using the same protocol. After 24 hours of incubation at 37°C, *E. coli* and coliform isolates were identified from both plastic and raw water. 50 CTX-resistant enterobacteria were isolated, biochemically characterized using API 20E strips (Biomerieux) and the phenotypic antimicrobial profile characterized through antibiotic susceptibility test according to EUCAST guidelines. Resistance phenotype was confirmed using ESBLs, KPC/MBL and OXA-48 confirmation kits. Additionally, we investigated the molecular profile of the antimicrobial resistances. Genomic DNA was extracted and used for PCR amplification using specific primers targeting antibiotic resistance genes (ARGs) involved in the resistance to the classes of sulfonamides, quinolones, beta-lactams and carbapenems. The presence of Class 1 Integron was detected, and the gene cassettes were sequenced to identify their associated ARGs. As last step, we performed shotgun metagenomic analysis on the total DNA extracted from biofilm associated to PE and PP, characterizing the microbial community, resistome, and mobile genetic elements residing on it (Fig.3B). This allowed to enhance and broad our knowledges on the complete microbial community associated to the plastisphere, including uncultivable bacteria, and to complement information provided by microbiological and molecular assays besides the CTX-resistant *Enterobacteriaceae*.

4.4 CLSM Analysis

Fragments of polypropylene (PP) and polyethylene (PE) were stained with SYTO9 dye (LIVE/DEAD® BacLight Bacterial Viability Kit-Thermofisher). Each plastic fragment was immersed in saline containing SYTO9 dye (1 μl/ml) and incubated at room temperature in the dark for 15 minutes. Subsequently, fragments were mounted between a slide and a

coverslip and observed using a 100× objective under a C2+ Laser Scanning Confocal Microscope (Nikon, Tokyo, Japan).

4.5 Isolation of Cefotaxime-Resistant Coliforms

Twelve fragments of PP and twelve fragments of PE from each sampling were sonicated using a microtip in 15 ml of saline (9 g/L NaCl) under the following conditions: 4 pulses of 45 seconds each at 9W, separated by a 1-minute interval. The post-sonicated solution in parallel with river water was cultured to isolate cefotaxime-resistant coliforms and *E. coli* by the membrane filtration method. The nitrocellulose membranes (0.45 µm Millipore) were transferred into Coliform Chromogenic Agar medium (CCA) supplemented with cefotaxime (8 µg/ml). Plates were incubated at 37°C for 24 hours, and then colonies were counted and isolated. Cefotaxime-resistant strains were stored in 20% glycerol stocks at -80°C for the subsequent analysis.

4.6 Biochemical Identification

Isolates resistant to cefotaxime were biochemically identified using API 20 E^{TM} strips in combination with APIWEB software (Biomerieux).

4.7 Antibiotic Susceptibility Testing

The antibiotic susceptibility profiles of cefotaxime-resistant *Enterobacteriaceae* strains were determined using the disk diffusion method (www.eucast.org). The antibiotics tested were cefotaxime (CTX, 5 µg), ceftazidime (CAZ, 10 µg), gentamicin (CN, 10 µg), levofloxacin (LEV, 5 µg), cefoxitin (FOX, 30 µg), trimethoprim/sulfamethoxazole (SXT, 1.25/23.75), meropenem (MEM, 10 µg), amoxicillin/clavulanic acid (AMC, 20/10 µg), and tigecycline (TGC, 15 µg). After incubation at 37°C for 18±2 hours, the diameter of inhibition zones was measured and interpreted according to EUCAST guidelines.

4.8 Beta-Lactamases and carbapenemases Phenotypic Tests

ESBL Confirm Kits (ROSCO Diagnostica Denmark) were used to detect Extended-Spectrum Beta-Lactamases (ESBLs) The kit includes antibiotic tablets (Cefotaxime 30 µg, Ceftazidime 30 µg and Cefepime 30 µg) and their combination with Clavulanate, an inhibitor of ESBLs. After the incubation at 35±1°C for 18±2 hours the diameter of the inhibition zones

was measured. Similarly, the Meropenem-resistant strains were tested using the KPC/MBL and OXA-48 Confirm Kit – (ROSCO Diagnostica, Denmark) for the in vitro identification of the mechanisms of resistance to carbapenems.

4.9 DNA Extraction

The genomic DNA of the isolated cefotaxime-resistant *Enterobacteriaceae* strains was extracted using the GenElute™ DNA Kit (Sigma-Aldrich). Bacterial cultures were grown overnight in LB media at 37°C, and DNA extraction was performed according to the manufacturer's instructions.

4.10 DNA amplification of antibiotic resistance genes, *IntI* gene, and class 1 integron gene cassette by PCR

PCR amplification of ARGs and Integrase 1 gene (Table 1) (Zhang Y et al., 2020) was performed using Takara Taq™ DNA Polymerase (Sigma-Aldrich). The variable region of the Class 1 integron was amplified using primers targeting the conserved regions flanking this segment: the 3′ conserved segment (3′-CS), which includes genes associated with disinfectant resistance (*qacEΔ1*) and sulfonamide resistance (*sul1*), and the 5′ open reading frame (*orf5*). The reaction mixture (25 µl) contained: Takara Taq™ DNA Polymerase 0.63 units, 1X PCR Buffer,200 µM dNTP, 2 µl of template DNA, 0.5 µM forward and reverse primers each. The amplification conditions were the following: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing according to table 1for 30 seconds, and extension at 72°C for 30 seconds. A final extension step was carried out at 72°C for 1 minute. For Class 1 gene cassette amplification (Table 1) the Jump Start Accu Taq™ DNA Polymerase was used. The reaction mixture (25 µl) contained Jump Start Accu Taq™ DNA Polymerase at a concentration of 1.25 units, 1X PCR Buffer, 200 µM dNTPs, 2 µl of template DNA, 0.5 µM forward and reverse primers each. The thermal cycler conditions were as follows: 5 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C for annealing, and 5 minutes at 72°C for extension. A final extension step at 72°C for 10 minutes was included. PCR products were then analyzed by electrophoresis on agarose gel.

4.11 Sequencing

The Class 1 gene cassette PCR products were purified using the GenElute™ PCR Clean-up Kit (Sigma-Aldrich) and quantified by Nanodrop spectrophotometer. Purified DNA was sequenced at Plasmidsaureus (USA), by Nanopore Sequencing.

4.12 MLST

The Multi-Locus Sequence typing analysis was conducted on three Meropenem- resistant, KPC-producers *K. pneumoniae* isolates according to the Pasteur Institute. (https://bigsdb.pasteur.fr/cgiibin/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef&page=profiles). This analysis is based on the sequencing of seven housekeeping genes: *gapA, infB, mdh, pgi, phoE, rpoB,* and *tonB.* For each gene, amplification product was sequenced, and the allelic profiles were determined by comparing the sequence data to the database provided by the Pasteur Institute. Sequence types (STs) were assigned according to the allelic profiles of the isolates. The primers used for the amplification of each gene are listed in Table 2 (Diancourt et al., 2005).

4.13 Metagenomic Analysis

A duplicate of plastic samples, collected from Pontelatrave (P1), Corridonia (P2) and Montecosaro (P3) was analyzed.

Bacterial communities were detached from 90 plastic fragments per sample (mixing PE and PP) using a mild sonication protocol (see section 4.5). Total DNA was extracted using EZNA water DNA kit (Omega Bio-Tek) and assed for quality through gel electrophoresis and Nanodrop spectrophotometry. Libraries were prepared and then sequenced by Novogene Europe (Germany), using Illumina sequencing technology. Bioinformatic analyses were performed on 30G of raw sequence data. After quality control, the clean data from each sample was used for metagenome assembly (>500bp). MEGAHIT software was used for assembly of clean data. Based on the number of reads aligned and the length of gene, the abundance of each gene in each sample is calculated. The annotation begins by aligning unigene sequences using DIAMOND software (https://github.com/bbuchfink/diamond/) (Buchfink B, 2015) with Micro_NR database, which includes sequences from bacteria, fungi, archaea, and viruses extracted from NCBI's NR database

(https://www.ncbi.nlm.nih.gov/). The alignment is performed using the blastp algorithm with a parameter setting of 1e-5 (Karlsson FH, 2013).

For antibiotic resistance genes detection, the CARD database was used. Integrons, were identified by aligning unigenes against Integrall database.

Table1. Oligonucleotides targeting ARGs and *Int1* genes

Gene	Orientation	Sequence*	Amplicon size (bp)	Annealing t(°C)
sul1	FW	CGCACCGGAAAACATCGCTGCAC	162	67
	RV	TGAAGTTCCGCCGCAAGGCTCG		
sul2	FW	TCCGGTGGAGGCCGGTATCTGG	190	65
	RV	TTCGTTCACGCCTTACCCAGC		
sul3	FW	TCCGTTCAGCGAATTGGTGCAG	127	63
	RV	TTCGTTCACGCCTTACACCAGC		
qnrA	FW	AGGATTTCTCACGCCAGGATT	124	59
	RV	CCGCTTTCAATGAAACTGCA		
qnrB	FW	CAGATTTYCGCGGCGCAAG	134	55
	RV	TTCCCACAGCTCRCAYTTTTC		
qnrS	FW	GTATAGAGTTCCGTGCGTGTGA	189	60
	RV	GGTTCGTTCCTATCCAGGATT		
ampC	FW	TCCGGTGACGCGACAGA	64	60
	RV	CAGCACGCCGGTGAAAGT		
blaCTX-M	FW	GGAGGCGTAGACGGCTTTT	53	60
	RV	TTCAGTGCGATCCAGACGAA		
blaSHV	FW	TCCATGATGGCACCTTTAAA	90	58
	RV	TTCGTCACCGGCATCCA		
blaTEM	FW	AGCATCTTACGGATGGCATGA	101	60
	RV	TCCTCCGATCGTTGTCAGAAGT		
Int1	FW	GGCTTCGTGATGCCTGCT	146	60
	RV	CATTCCTGGCCGTGGTTCT		
Int1V	FW	TCATGGCTTGTTATGACTGT	variable	55
	RV	GTAGGGCTTATTATGCACGC		

*Primer sequences used as reported by Zhang Y et al., 2020.

Table2. MLST characterization oligonucleotides.

Gene	Orientation	Sequence*	Amplicon size (bp)	Annealing T°
rpoB	FW	GTTTTCCCAGTCACGACGTTGTAGG CGAAATGGCWGAGAACCA	1119	50
	RV	TTGTGAGCGGATAACAATTTCGAGT CTTCGAAGTTGTAACC		
gapA	FW	GTTTTCCCAGTCACGACGTTGTATG AAATATGACTCCACTCACGG	706	50
	RV	TTGTGAGCGGATAACAATTTCCTTC AGAAGCGGCTTTGATGGCTT		
mdh	FW	GTTTTCCCAGTCACGACGTTGTACC CAACTCGCTTCAGGTTCAG	800	50
	RV	TTGTGAGCGGATAACAATTTCCCGT TTTTCCCCAGCAGCAG		
pgi	FW	GTTTTCCCAGTCACGACGTTGTAGA GAAAAACCTGCCTGTACTGCTGGC	610	50
	RV	TTGTGAGCGGATAACAATTTCCGCG CCACGCTTTATAGCGGTTAAT		
phoE	FW	GTTTTCCCAGTCACGACGTTGTAAC CTACCGCAACACCGACTTCTTCGG	646	50
	RV	TTGTGAGCGGATAACAATTTCTGAT CAGAACTGGTAGGTGAT		
infB	FW	GTTTTCCCAGTCACGACGTTGTACT CGCTGCTGGACTATATTCG	506	50
	RV	TTGTGAGCGGATAACAATTTC CGCTTTCAGCTCAAGAACTTC		
tonB	FW	GTTTTCCCAGTCACGACGTTGTACT TTATACCTCGGTACATCAGGTT	583	50
	RV	TTGTGAGCGGATAACAATTTCATTC GCCGGCTGRGCRGAGAG		

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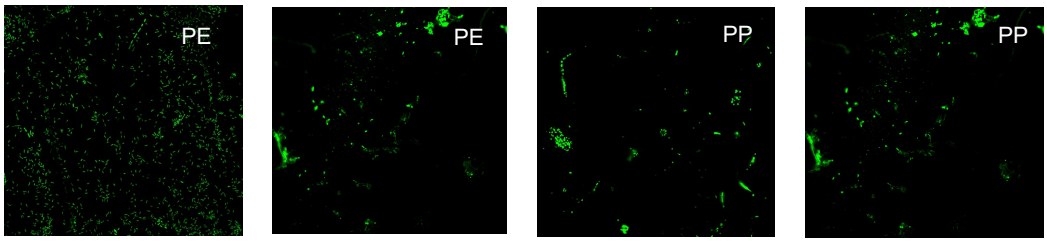
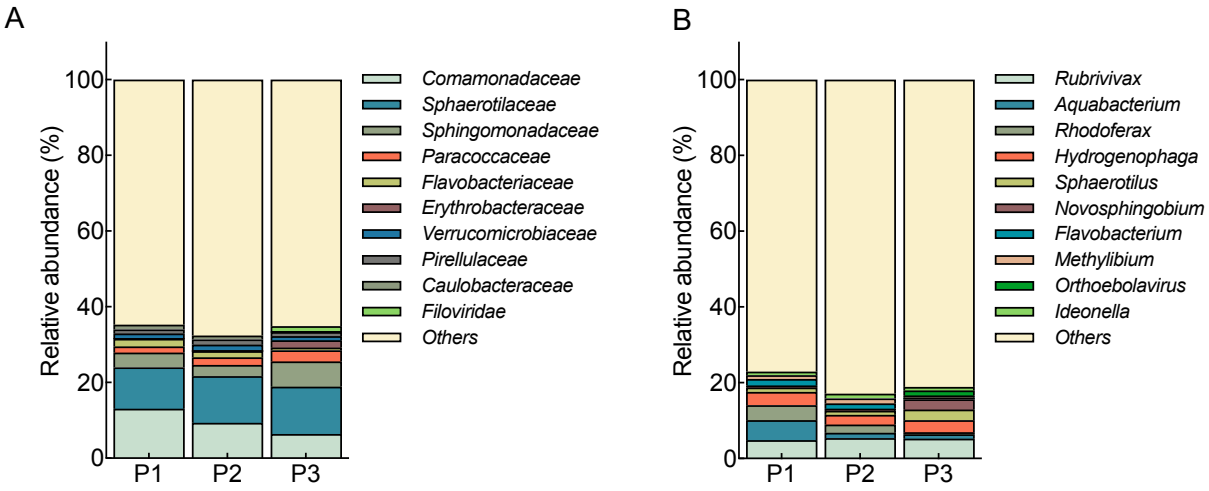
Supplementary Materials



S1. Plastisphere visualization. Bacteria attached to PE and PP fragments (two sample each) after being exposed to river water, stained by Syto9 and observed by Confocal Laser Scanning Microscopy (CLSM). The square images show biofilm projection through the x-y plane. The scale bars are 10 µm.



S2. Plastisphere taxonomic composition. Relative abundance of **(A)** bacterial families, and **(B)** bacterial genera identified from the contigs in each plastic sample (P1, P2, P3).

4 Surveillance of Antimicrobial Resistance Genes and Class 1 integrase system.

AMR surveillance and monitoring is essential for tackling the spread of drug-resistant microorganisms in humans, animals, and the environment. In fact, understanding the emergence, evolution, and transmission of AMR is essential to fully assess the extent of the problem and to develop sustainable fallback strategies. In this context, the manuscript of this section focuses on the development of a versatile CRISPR/Cas12a toolbox aimed to detect two ARGs, namely *blaCTX-M-15* and *floR* genes.

The methodology described here involves target DNA amplification by PCR, combined with Cas12a-mediated detection of ARGs, optimized for use in different environments, from high-tech laboratories to field applications. In field settings, the use of lateral flow assays (LFA), allows real-time, on-site detection of ARGs providing a direct "snapshot" of the panel of genes operating in a specific environmental setting.

In addition, applying the detection system to the anthropogenic Class1 integron, commonly found in environments influenced by human activities, enables the researcher to assess the presence of genetic elements involved in the insertion and excision of ARGs. The integrase gene, specifically Class 1 integrase (*intI1*), is investigated here as a potential marker of ARGs, as it often harbors multiple resistances associated with the gene cassette arrays. Detection of integrase 1 in environmental samples may indicate the presence of an anthropogenic pressure that impact on the ARG spread among microbial ecosystems.

The integration of a rapid, cost-effective and versatile detection system could significantly enhance our ability to monitor the spread of resistance genes and to use it as a tool for application-specific surveillance directly in the field, an approach that could significantly expand our view of the multifaceted phenomena of AMR.

4.1 Versatile and Portable Cas12a-mediated Detection of Antibiotic Resistance Markers

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Keywords: Antibiotic Resistance Gene, Cas12a, *blaCTX*, *floR*, Integrase.

Abstract (217/250)

Antimicrobial resistance (AMR) is a global public health problem particularly accentuated in low- and middle-income countries, largely due to a lack of access to sanitation and hygiene, lack of awareness and knowledge, and the inadequacy of molecular laboratories for timely and accurate surveillance programs. This study introduces a versatile molecular detection toolbox (C12a) for antibiotic resistance gene markers using CRISPR/Cas12a coupled to PCR. Our toolbox can detect less than $3x10^{-7}$ ng of DNA (100 attoMolar) or 10^2 CFU/mL. High concordance was observed when comparing the C12a toolbox with sequenced genomes and antibiotic susceptibility tests for the *blaCTX-M-15* and *floR* antibiotic resistance genes (ARGs), which confer resistance to cefotaxime and other β-lactams, and amphenicols, respectively. $C12a^{INT}$, designed to detect the Integrase 1 gene, confirmed a high prevalence of the integrase/integron system in *E. coli* containing multiple ARGs. The C12a toolbox was tested across a wide range of laboratory infrastructure including a portable setup. When combined with lateral flow assays (LFA), C12a exhibited competitive performance, making it a promising solution for on-site ARG detection. Altogether, this work presents a collection of molecular tools (primers, crRNAs, probes) and validated assays for rapid, versatile, and portable detection of antibiotic resistance markers, highlighting the C12a toolbox potential for applications in surveillance and ARG identification in clinical and environmental settings.

Introduction

The discovery of antibiotics allowed control of pathogenic bacterial proliferation, enabling surgeries, treating infections, and boosting farm productivity, ultimately benefiting humans. Their use in clinical treatments has significantly increased life expectancy (1). However, the combination of excessive and inappropriate antibiotic use, complex ecological factors (2, 3), and horizontal gene transfer have favored the spread and selection of resistant strains (4, 5). Extended Spectrum Beta Lactamases (ESBLs) and amphenicol resistance are of particular concern for human and animal health. In Gram-negative bacteria, the *blaCTX-M-15* and *floR* antibiotic resistance genes (ARGs) confer resistance to β-lactam and amphenicol antibiotics, used to treat infections in clinical and veterinary settings, respectively (6, 7). Both ARGs have been monitored in various environments such as hospitals, air, water, farms, soil, and wastewater (8)(Figure 1A). Monitoring *blaCTX-M-15* and *floR*, the most frequently detected variants within their respective classes, is essential for assessing the scale of their spread across populations and ecological systems (9–12).

At the molecular level, mobile genetic elements (MGEs) play a crucial role in the spread of antimicrobial resistance genes (ARG), due to their ability to physically move within different hosts, intra- or interspecies (13). In *Enterobacteriaceae*, like *E.coli*, Class 1 integrons are important contributors to ARG dissemination (14), and the most prevalent in clinical isolates (15–17) (Figure 1 B). Class 1 Integron consists of three coding modules: i) the *intI1* gene located at the 5' end, which codes for an integrase; ii) a recombinase site (*attI*) followed by an array of various gene cassettes; and; iii) a genome-integrated region at the 3' end, which varies between environmental and clinical Class 1 Integrons (18). The structure of the cassette array can be shaped by the selective pressure imposed by anthropogenic activities and could be used as an indicator of the presence of antibiotic-resistance genes. In addition to antibiotics, they are associated with resistance to disinfectants, heavy metals, and other pollutants (18). The detection of anthropogenic Class 1 Integron in environmental samples through the application of precise and affordable methods, is a necessary step to assess the impact of human activities and ecological genetic pollution (17).

Different methods for detecting antimicrobial resistance (AMR) are currently in use, with the most common being antimicrobial susceptibility testing (AST) and molecular techniques such as qPCR and DNA sequencing (19, 20). However, there is a need for a tool that

combines the speed of ARG detection provided by molecular methods with AST costs. Target pre-amplification followed by nucleic acid detection using CRISPR technology has shown promising results due to the high specificity, sensitivity, low costs, and speed of the assay (21). The molecular detection mechanism involves crRNA, a targeting sequence homologous to the gene of interest. Hybridization with the complementary target region activates Cas12a, which then engages in trans-cleaving activity, non-specifically cutting ssDNA (22). When using a fluorescence donor/quencher FRET pair placed into a ssDNA, this trans-cleaving activity leads to an increase in fluorescence (23) (Figure 1C).

Here, we use CRISPR technology to develop the C12a toolbox, a set of molecular tools and assays to detect genetic elements conferring antibiotic resistance or involved in ARG propagation. C12a^{bCTX} and C12a^{FLO} detect *blaCTX-M-15* and *floR* genes, respectively, while C12a^{INT} detects the Class 1 integrase gene as a rapid indicator of the potential presence of other ARGs. We also provide crRNAs designed for detecting ARGs commonly associated with MGEs. The C12a toolbox was optimized for three different laboratory setups (Figure 1C) including high-, and low-equipped in addition to a portable setting. Altogether, the C12a toolbox developed here entails a set of novel molecular assays to achieve rapid and efficient ARG detection.

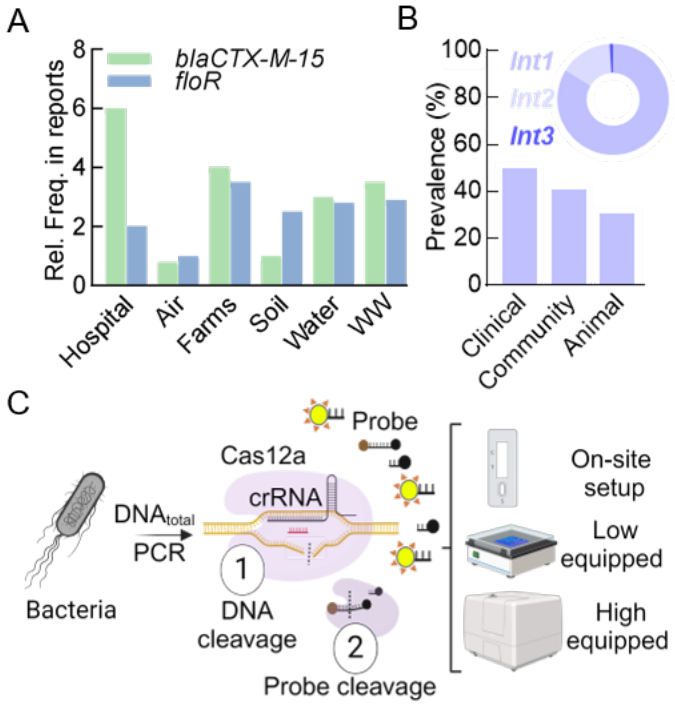

Figure 1. *blaCTX* and *floR* epidemiological context and the C12a rationale. (A) Frequency distribution of *blaCTX-M-15* and *floR* across various environments, based on publication frequency from 1990 to 2020 (8). WW: wastewater. **(B)** Prevalence of the Class 1 Integron across clinical, community, and animal settings(15, 24). The pie chart shows integron prevalence in clinical isolates (25). **(C)** Scheme showing the C12a toolbox and the molecular mechanism of Cas12a-mediated detection, highlighting signal development, versatility, and portability.

Materials and Methods

The Extended Methods in the Supplemental Material file provides details on biological components, microbiology assays, and bioinformatic analysis.

Assay Optimization

DNA amplification and analysis: PCR conditions for amplifying the *blaCTX-M-15* and *floR* targets included an initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95° for 30 sec, annealing at 59°C for 30 sec, extension at 72°C for 30 sec, and a final extension at 72°C for 5 minutes. For the *intI1* gene target, an annealing temperature of 56°C was used. The master mix included 2 ng/µl Taq DNA polymerase, 0.2 µM primers in the RPB1X buffer (Table S1), and DNA concentrations ranging from 1 to 10 ng/µl, in a final volume of 20 µl. PCR products were visualized using agarose gel electrophoresis (Cat # CSL-AG500, Cleaver Scientific). Gel concentrations were optimized at 1.7% for all genes. Electrophoresis was performed in 1X TBE buffer (Table S1) at 80 V for 1 hour. A 5 µl aliquot of PCR product was mixed with 1 µl of 6X Trick-Track – SYBR Gold (Cat # R1161, ThermoFisher), and loaded onto the agarose gel. A 100 bp molecular weight marker (Cat # SM0242, Thermo Scientific) was included. Agarose gels were imaged with a gel documentation system using the Sybergold option (Gel Doc-BioRad). Band intensity was quantified by calculating the area under the curve (AUC) with Gel Analyzer v23 (www.gelanalyzer.com).

crRNA/Cas12a-mediated detection: the crRNA was refolded through a sequential incubation, first at 65°C for 10 minutes, followed by 10 minutes at 25°C. The crRNA/Cas12a complex (0.1 µM Cas12a, 0.15 µM crRNA, and 2 µM FAM probe [6-FAM/TTATT/3IABkFQ]) was incubated in CRB1 buffer (Table S1) in the dark for 10 minutes. Afterward, 5 µl of amplification reaction was mixed with 10 µl of the crRNA/Cas12a complex in CRB2 buffer (Table S1) in a black 96-well plate (Cat #23710,

ThermoFisher), in a volume of 100 µl. Fluorescence was measured using a plate reader (Synergy H1, BioTek Instruments) with excitation at 491 nm and emission at 525 nm.

Template and Mg2+ optimization: A six-point curve of total DNA was established using a serial dilution ranging from 1 ng/µl to 10-5 ng/µl to optimize the template concentration. A magnesium concentration curve was performed, ranging from 7.5 mM to 25 mM in CRB1 buffer (Table S1), using 2 ng/µl of genomic DNA and 30 amplification cycles.

LoB and LoD determination: Ten susceptible *E.coli* isolates (negative controls) were evaluated to determine the LoB (Limit of Blank) as the cut-off ratio, using the formula LoB = Avg_NF_{NTC} + 2 SD (where "Avg" is average NF_{ntc} values, and "SD" is standard deviation)(26). The LoD (Limit of Detection) was determined for both amplification products and bacterial cell counts for the *blaCTX-M-15* and *floR* targets. Amplicons were purified using the Oligo Clean Kit (Cat # D4060, Zymo Research). Serial ten-fold dilutions of the purified product, from 1 aM to 10 nM, were prepared, resulting in 10 dilution points. The LoD was then calculated using the formula LoD = LoB + 2 (SD_low concentration sample)(26). For the bacterial cell LoD determination, a curve was generated using an *E. coli* isolate positive for the antimicrobial resistance gene. Starting from a cell density of 0.5 McFarland, 7 ten-fold dilutions were prepared in duplicate. One set of dilutions was plated on LB agar plates, while the other set was analyzed using the CRISPR/Cas12a method.

Lateral Flow Assay (LFA): Target genes were amplified by PCR from a small set (N = 14) of stored purified DNA from stool samples and detected using the described Cas12a assays. After denaturation and refolding of 0.15 µM crRNA, the crRNA/Cas12a complex (0.2 µM Cas12 and 5 µM of probe [6-FAM/TTATTATT/BIOTIN]) was mixed in CRB1 buffer and incubated in the dark for 10 minutes. Then, 10 µl of the crRNA/Cas12a complex was mixed with 6 µl of PCR products and CRB2 buffer, in a volume of 95 µl. This mixture was incubated for 30 minutes in the dark. Next, polyethylene glycol was added to a final concentration of 5% (Cat # 25322-68-3, Merck), and a LFA strip (Cat # 3822-9000, Milenia Biotec GmbH) was submerged in each mixture. The signal on the strips was detected after 5-10 minutes.

Results

C12a Development

To define the optimal sets of primers, we first compared 61 *blaCTX-M-15* sequences and 46 *floR* sequences from *E. coli* isolates available in GenBank (27) (Supplementary Dataset 1 and Dataset 2, respectively). Based on the consensus sequences for each gene, we selected two primer sets for *blaCTX-M-15* and one for *floR* (Table S2) (Figure 2A), which amplify efficiently a DNA region containing both the crRNA complementary sequence and a PAM region (TTTN) (28)(Figure S1). Agarose gel electrophoresis revealed defined bands at 216 bp and 270 bp for *blaCTX-M-15,* and *floR* amplicons, respectively. Negative controls showed no DNA amplification, as determined by gel electrophoresis (Figure S2). Amplicons were sequenced to confirm the presence of the expected target DNA for each gene (Table S3). To optimize the crRNA/Cas12a reaction, two candidate crRNAs for each target gene were designed (Table S2). To determine sensitivity parameters, a test carried out using increasing Mg^{2+} concentrations revealed an optimal value of 20 mM (Figure S3), which was used in all assays. Both *blaCTX-M-15* and *floR* amplicons were recognized by the crRNA/Cas12a complex, resulting in an increase in fluorescence over time. However, primer set 2, combined with $crRNA^{CTX-2}$, showed greater fluorescence intensity compared to $crRNA^{CTX-1}$. For *floR*, $crRNA^{FLO-1}$ exhibited a higher fluorescence change than $crRNA^{FLO-2}$ (Figure S1). Notably, regardless of template concentration, $crRNA^{CTX}$ consistently generated a fluorescence signal faster than the *floR* crRNAs (Figure 2B and Figure S1). Thus, the selected crRNAs for *blaCTX-M-15* and *floR* were $crRNA^{CTX-2}$ and $crRNA^{FLO-1}$, respectively.

For better comparison with previous reports, this study used a fluorescence signal normalized to the no-template control (NTC), indicated as NF_{NTC} (a.u.). To calculate NF_{NTC}, each raw fluorescence data point was divided by its corresponding data point from the NTC (Figure 2C). For both target genes, the measured NF_{NTC} for positive controls was 4- to 14-fold higher than for negative controls. Specifically, for the *blaCTX-M-15* gene, positive samples exhibited an NF_{NTC} of at least 14-fold, while negative samples showed values similar to the NTC, with NF_{NTC} values near 1. Similarly, for the *floR* gene, positive samples showed a 4-fold increase in NF_{NTC} compared to negative controls (Figure 2C and Figure S1). For *blaCTX-M-15* evaluation, the $C12a^{bCTX}$ detection system was used, which incorporates the primer set 2 and $crRNA^{CTX-2}$ (Table S2). On the other hand, the presence of *floR* was

evaluated using the C12a^{FLO} detection system using the primer set 1 in combination with crRNA^{FLO-1} (Table S2).

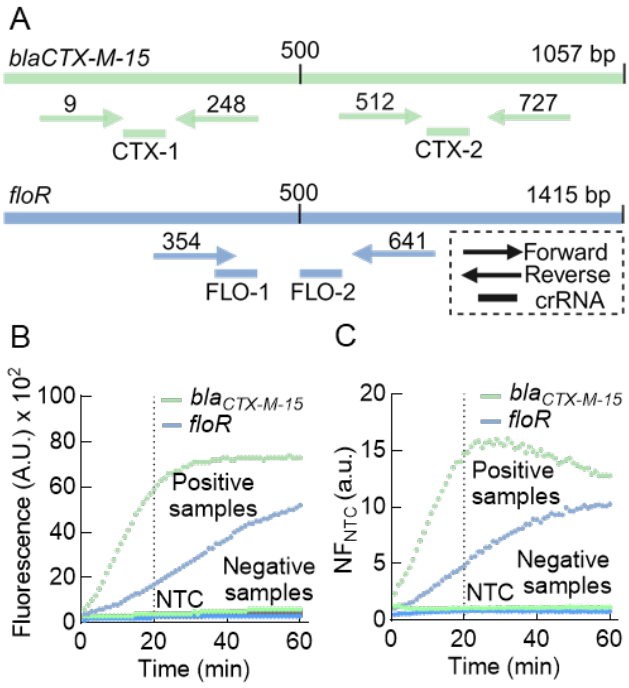

Figure 2. Experimental setup for CRISPR/Cas12a-based assay development. **(A)** Primers and crRNA location on *blaCTX-M-15* and *floR* gene targets, ensuring coverage of PAM regions and complementary crRNA areas. **(B)** Example of raw fluorescence readouts from CRISPR/Cas12a assays, showing a time-dependent increase of the signal. Positive and negative controls, alongside the Non-Template Control (NTC), are represented to illustrate the assay's ability to differentiate samples with or without ARGs. **(C)** Example of normalized fluorescence ratio NF_{NTC} (a.u.) over time. In **(B)** and **(C),** the dotted line indicates the 20-minute detection point, selected as the cut-off reading time for analysis.

C12a Analytical Sensitivity

Two key analytical parameters describe a laboratory assay for analyte detection: the limit of blank (LoB; also referred to as the cut-off) and the limit of detection (LoD). The LoB was determined using total DNA from ten susceptible *E. coli* isolates. The LoB was calculated as the average NF_{NTC} plus the standard deviation multiplied by a confidence factor (LoB=Avg + c x SD, where c=2) (26). The average NF_{NTC} values for negative samples using C12a^{bCTX} or C12a^{FLO} were 1.04 ± 0.02 and 0.94 ± 0.02 after 20 minutes of incubation (Figure S4). Thus, the resulting LoB values were 1.07 NF_{NTC} (a.u.) for C12a^{bCTX} and 0.97 NF_{NTC}

(a.u.) for C12a^{FLO}. Based on these values, we can state that 95.45% of the values below the determined LoB were likely to be classified as nonspecific background signals (i.e., signals in the absence of the analyte) (Figure S4).

To estimate the LoD for both detection systems, purified and quantified amplicons were tested at increasing concentrations (from 1 attoM to 10 nanoM). Averaged NF_{NTC} (a.u.) values, measured at 10-minute intervals were used to define the lowest detectable target concentration and the shortest reaction time. The LoD was calculated for each time interval, allowing the estimation of LoD as a function of incubation time (Figure 3A). The time dependence indicates that after 21 minutes for C12a^{bCTX} and 17 minutes for C12a^{FLO}, the LoD reached half of its maximum value, suggesting that a 20-minute reaction time is a good compromise between assay speed and analytical sensitivity. The calculated LoD for C12a^{bCTX} and C12a^{FLO} at 20 minutes was 70 aM and 50 aM, respectively (Figure 3B). Taking NF_{NTC} values at times shorter than 20 minutes would result in a tenfold loss in sensitivity (Figure 3A).

We then assessed the analytical sensitivity of the CRISPR/Cas12a system in terms of the number of cells carrying the resistance genes (Figure 3C). Fresh bacterial cultures of *E.coli* ATCC25922 ranging from 3 to 3x10^6 CFU/mL were used (Figure S5). Non-linear fitting of the NF_{NTC} dependence on cell number (CFU) allowed the calculation of the LoD. C12a^{bCTX} reliably detected the *blaCTX-M-15* gene at 77 CFU/mL, while C12a^{FLO} required 173 CFU/mL to detect the *floR* gene (Figure 3C). Both ARG detection systems showed low LoB values and enabled the detection of extremely low concentrations of the target DNA (LoDs) with a 20-minute incubation at room temperature. Additionally, the lowest target concentration that could be empirically detected for the two target genes, was 10 to 100 times lower compared to gel electrophoresis analysis of PCR amplification (Figure 3D,E, and Figure S6). Altogether, the detection and sensitivity performance of C12a^{bCTX} and C12a^{FLO} provide a solid foundation for testing the detection systems on a larger number of samples (see below).

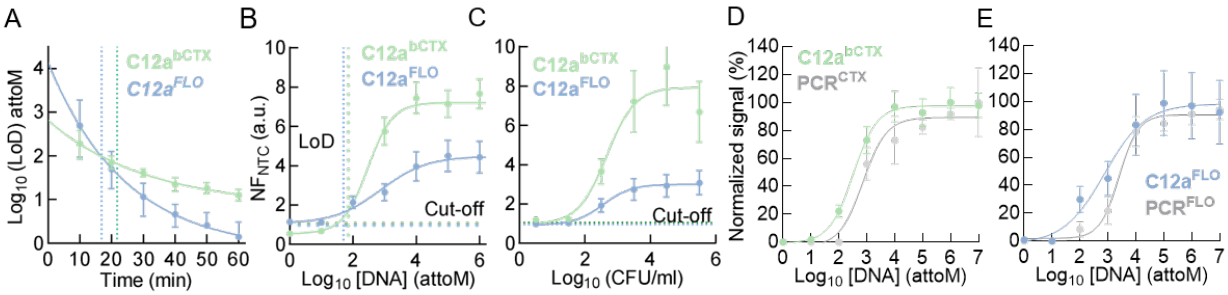

Figure 3. C12a^{bCTX} and C12a^{FLO} Limit of Detection (LoD). (A) Time-resolved determination of LoD values for both ARGs Cas12a reactions, plotted on a logarithmic scale. The graph illustrates the LoD decrease for C12a^{bCTX} and C12a^{FLO} over incubation time, with dotted lines marking the time-point when the LoD reaches half its maximum value. **(B)** Normalized fluorescence ratio as a function of target concentration for ARG detection assays. The X-axis dotted lines represent the LoD value calculated at 20 minutes, while the Y-axis dotted lines represent NF_{NTC} LoB (cutoff). **(C)** Normalized fluorescence ratio over bacterial cell concentration for both C12a assays. The dotted lines indicate average NF_{NTC} cut-off values of 1.04 and 0.94 for C12a^{bCTX} and C12a^{FLO}, respectively. **(D)** Normalized assay signals across target concentrations for C12a^{bCTX} detection compared to PCR-based detection evaluated by gel electrophoresis. The fluorescent ratio for the Cas12a reaction and electrophoresis gel band intensity, recorded as Area Under the Curve (AUC) values, are normalized as percentages to the highest value. **(E)** Same as (D), but for C12a^{FLO}. Error bars represent standard deviations from 3 replicates.

C12a^{bCTX} and C12a^{FLO} performance in E. coli isolates

To assess the presence of antibiotic resistance genes (ARGs) and the phenotypic resistance in *E. coli* isolates, two distinct assays were conducted: (i) molecular detection using the C12a toolbox and (ii) antimicrobial susceptibility testing (AST). A total of 32 *E. coli* isolates were selected, each containing either the *blaCTX-M-15* gene, the *floR* gene, or neither, from a previously published sample repository (NIH R01AI108695-01A1) (29). Of these, 15 samples were tested for beta-lactam resistance and 17 for amphenicol resistance (Table S4), using the combined disk (CD) test and broth microdilution for minimum inhibitory

concentration (MIC) determination, respectively. Finally, the C12a assay was applied to all samples of the collection.

All isolates positive to the *blaCTX-M-15* gene (7 samples) were ESBL producers, confirming the resistance to beta-lactam antibiotics. Similarly, resistant isolates harboring the *floR* gene (11 samples) exhibited MIC values ranging from 256 to 128 µg/mL, while susceptible isolates (6 samples) had MIC values below 32 µg/mL (30)(Table S4). When assessing the performance of C12a^{bCTX} and C12a^{FLO} systems, NF_{NTC} values for ARG-positive samples ranged from 11 to 16 for C12a^{bCTX} and 3 to 4.5 for C12a^{FLO} after 20 minutes of incubation, indicating the detection of the target genes. In contrast, ARG-negative samples showed no NF_{NTC} values higher than 1.0 with either C12a assay, indicating the absence of the target genes (Figure 4A-B). A strong concordance was observed between the presence of resistance genes using the C12a toolbox, genetic data, and the phenotypic resistance determined by AST assays (Figure 4C-D).

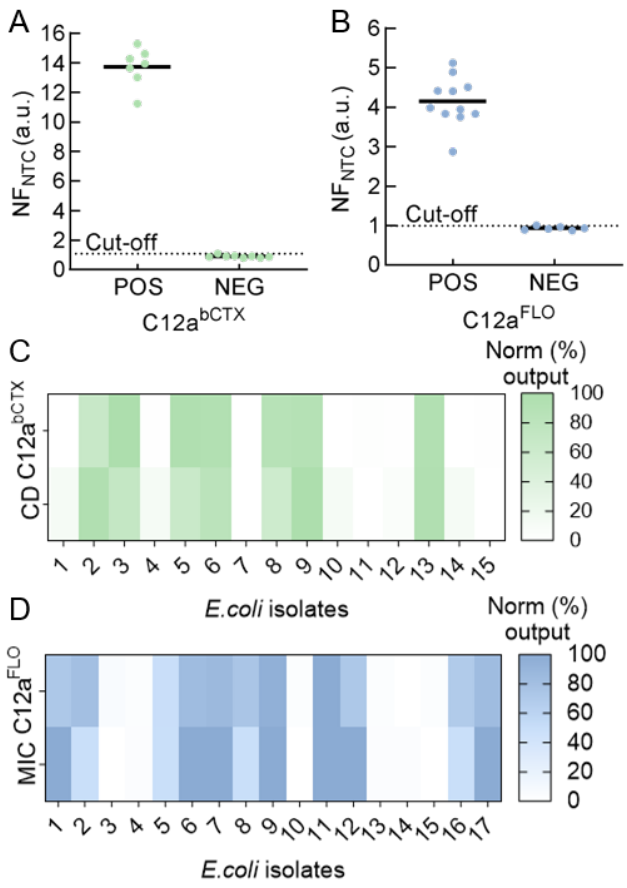

Figure 4. C12a^{bCTX} and C12a^{FLO} comparison with AST methods. (A) Distribution of *blaCTX-M*-15 in 17 isolates tested using the C12a^{bCTX} assay. The dotted line represents the

LoB at 1.1. **(B)** Similar to (A), but for the C12a^{FLO} assay. The dotted line indicates the LoB at 1.0. **(C)** Heat map comparison of the C12a^{bCTX} assay with the disk diffusion test for AMR (CD). Results were normalized to percentages, using the maximum value obtained for each dataset as 100%. **(D)** Similar to (C) but for the C12a^{FLO} assay with microdilution test (MIC), showing that *floR* positive samples were phenotypically resistant to florfenicol.

C12a^{INT} detects the intI1 gene

The spread of ARGs can be mediated by Class 1 Integron, which is known to host genes conferring resistance to aminoglycosides, sulfonamides, beta-lactams, and trimethoprim. The modularity and versatility of the Ca12a toolbox could allow a rapid adaptation to detect other genetic elements relevant to the AMR problem. Bioinformatic analysis of ARG-positive *E. coli* isolates (Fig. 4 A) revealed that 77.77% (14/18) contained the *intI1* gene, encoding Integrase I. Nearly all integrase-positive isolates also carried a cassette array (78.57%), indicated by the presence of the attC recombination sites. Three isolates had *intI1* integrase but lacked a cassette array, while one lacked the integrase but carried a cassette array. For samples carrying the Class 1 integron, deeper bioinformatic analysis was performed on 18 genomes, to identify the ARG genes present in the cassette array. Genes conferring resistance to aminoglycosides, trimethoprim, phenicols, lincosamide and sulfonamides were the most prevalent, with frequencies of 91.67%, 75%, 33%, 25%, and 16.67%, respectively. Additionally, genes conferring tolerance to disinfectants such as *qacE* were observed in 5 isolates (41.67%) (Figure 5A). The *in silico* analysis revealed that transposons localized near Class 1 Integron, were probably involved in the mechanism of acquisition and transfer of the observed cassette array. Neither *blaCTX-M-15* nor *floR* genes were detected as part of the cassette array in any of the analyzed isolates (Table S5).

Thus, we designed a collection of primers and crRNAs for detecting the representative ARGs for each antibiotic family (Table S6) and for the *intI1* gene. From this collection, we further tested the C12a using the C12a^{INT} assay and the *E. coli* isolates library. Agarose gel electrophoresis revealed defined bands at 146 bp only for *intI-1* positive samples using a reported set of primers (31) (Figure S7). Among the three candidate crRNAs, crRNA^{int3} showed better NF_{NTC} values at 20 minutes reaction time under standardized C12a reaction conditions (Figure 5B). Using the C12a^{INT}, we detected the presence of the *intI1* gene in 16 out of 18 ARG-positive samples (Figure 5C). Detection of *intI1* could indicate the presence

of other resistances frequently associated with Class 1 Integron (17, 32, 33). Furthermore, although *intI1* could be a good predictor of resistance to some antibiotic classes, direct detection of the ARG may be preferable for certain setups Here we provide a set of primers and crRNAs designed by bioinformatic analysis with the aim to detect the most prevalent ARGs found in the Class 1 integron cassette array (Table S6), a target region suitable for CRISPR/Cas technology. Therefore, this could be a solid starting point for studies aiming to direct detection of relevant ARGs.

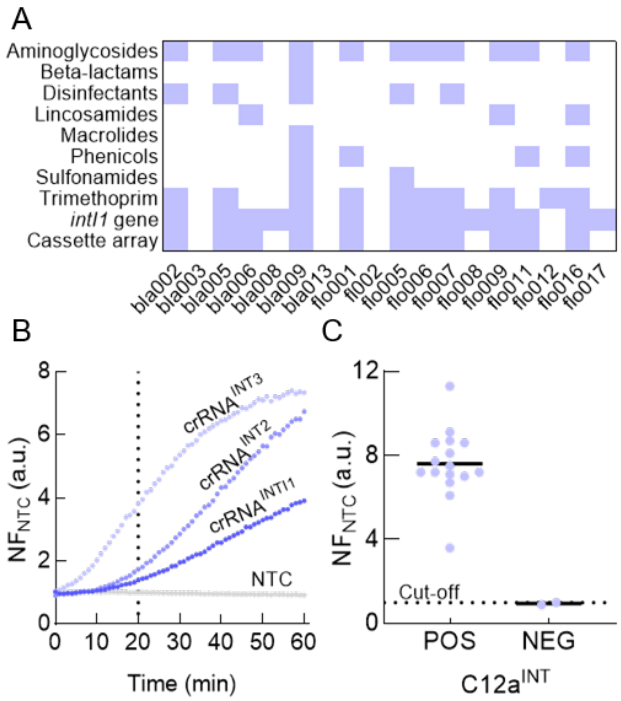

Figure 5. C12a^{INT} detects the *intI1* gene, a potential marker of multidrug-resistant *E.coli*. (A) Heat map summarizing the antibiotic resistance genes related to different antibiotic families identified within the Class 1 integron cassette array of the ARG-positive *E. coli* isolates used in this study. ARGs were identified by bioinformatic analysis of sequencing data (BioProject number PRJNA821865) using CARD. **(B)** Time-course of normalized fluorescence development for the C12a^{INT} assay with three crRNA designed to detect the *intI1* gene. The X-axis dotted line represents the incubation time used for further analysis. **(C)** CRISPR-Cas detection of *intI1* gene in 18 ARG-positive isolates tested using the C12a^{INT} assay. The dotted line represents the LoB at 1.0.

Efficient C12a-mediated ARGs detection in different Lab setups

Differences in equipment availability and portability may impact the analytical performance of the C12a detection toolkit. To address this, we adapted our detection assays to three laboratory setups: i) Low-C12a–for low-equipped labs with minimal tools, such as a thermocycler and transilluminator, for qualitative readouts; ii) OnSite-C12a–for portable setups with equipment-free readouts using the Lateral Flow Assay (LFA) platform and transportable workstation like BentoLab®, and iii) High-C12a– for a high-equipped setup with a multimode microplate reader for precise quantitative analysis, as used here for the C12a validation (Figure 6).

Fourteen purified total DNA available from the stool sample repository (BioProject number PRJNA821865) were assessed with the C12a^{bCTX} (7 samples) and C12a^{FLO} (7 samples) using all three laboratory set-ups. Using Low-C12a^{bCTX}, direct visualization of fluorescence allowed detection for the five positive samples. Negative samples did not show any fluorescence signal. When using Low-C12a^{FLO}, six samples tested positive by direct observation (Figure 6A). The high-equipped setup showed that C12a^{bCTX} detected *blaCTX-M-15* in five samples, and two samples tested negative, respectively. All positive samples showed NF_{NTC} (a.u.) values exceeding 6 within 20 minutes. Similarly, for the C12a^{FLO}, NF_{NTC} (a.u.) values surpassing 3 were recorded at 20 minutes (Figure 6B). When using a portable setup with OnSite-C12a^{bCTX} and OnSite-C12a^{FLO}, the presence of the ARG is indicated by the appearance of two bands: the first is the C line (control) and an additional band that is the T line (test), which can display an intensity equal to or greater than that of the C line. Both bands were observed exclusively in the positive samples for both ARG evaluated (Figure 6C). Under our experimental conditions, no difference in detection performance was observed in all three setups evaluated. Although the sample size was limited, these results provide an initial indication that our toolbox demonstrates sufficient analytical sensitivity for direct ARG detection in total DNA samples across different laboratory setups.

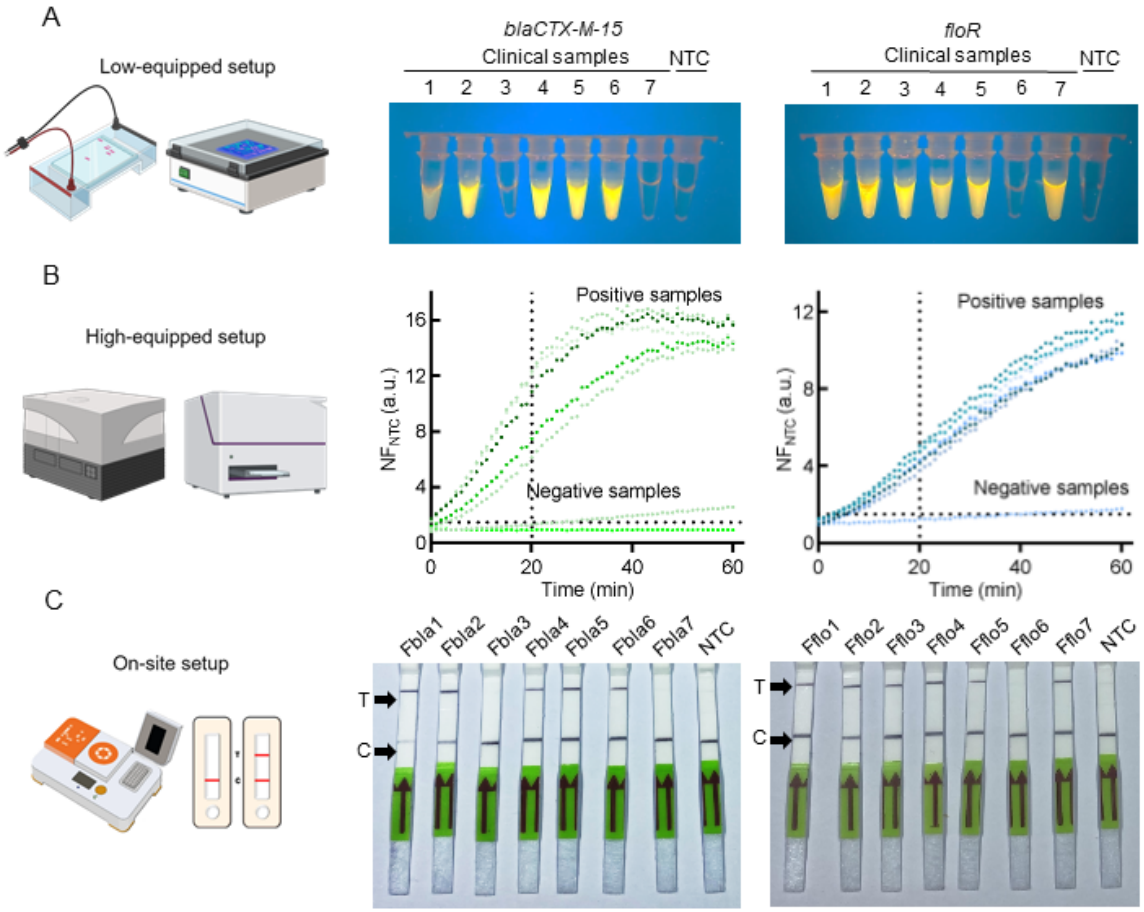

Figure 6. C12a application across different laboratory setups. (A) *blaCTX-M-15* and *floR* detection in a low-equipped laboratory setup. Fluorescence was observed by direct visualization only for ARG-positive samples, photographed using a smartphone camera with default settings using a blue light transilluminator. **(B)** Similar to (A), but for a high-equipped laboratory. NF_{NFC} (a.u.) values were measured over 60 minutes with a 20-minute detection cutoff. The Y-axis dotted line represents an average NF_{NTC} cut-off value of 1.0. **(C)** Similar to (A), but for an On-site setup. Detection by LFA strips at 15 minutes incubation, where the T band is positive and C the quality control. NTC refers to non-template control.

Discussion

Antimicrobial resistance is a growing threat beyond clinical settings, confirmed by the detection of AMR pathogenic bacteria and mobile genetic elements in the environment, including anthropogenic integrons in *Enterobacteriaceae* (33). The detection of *blaCTX-M-15* and *floR* gene is of increasing importance due to their widespread distribution in antimicrobial resistance (Figure 1A). *blaCTX-M-15* is one of the most prevalent extended-spectrum β-lactamase (ESBL) genes globally, conferring resistance to a wide range of β-lactam antibiotics, including third-generation cephalosporins (34). Its widespread dissemination among Enterobacteriaceae, particularly *Escherichia coli*, poses a serious threat to public health by limiting treatment options for bacterial infections. Similarly, the *floR* gene confers resistance to amphenicols like florfenicol, an antibiotic used in veterinary medicine (35). The presence of these genes facilitates the survival and spread of multidrug-resistant bacteria in various environments, making their detection crucial for effective surveillance and control measures.

Therefore, our research presents an optimized molecular detection toolbox for two of the most widespread genes conferring resistance to beta-lactams (*blaCTX-M-15)* and amphenicols (*floR*) and *Int1* integrase, using the CRISPR-Cas technology. This toolbox is composed of a collection of primers, crRNAs, and well-defined protocols to be applied in laboratories with different equipment availability. Detection of *blaCTX-M-15* and *floR* with C12a^{bCTX} and C12a^{FLO}, display distinctive features, such as incubation times as short as 20 minutes and competitive low limits of detection, that make them suitable for various applications. The analytical performance of Cas-based assays competes well with established molecular techniques; yet, they are simpler, faster, and more versatile. Our C12a detection systems can recognize the target genes with similar analytical sensitivity as commercial qPCR kits for ARGs detection (36). Furthermore, they have proven to be robust and effective with a limit of detection in the range of 75 to 180 bacterial cells per reaction, as the limit of detection, indicating high sensitivity.

Anthropogenic integrons in *Enterobacteriaceae* are frequently associated with multidrug resistance and are influenced by human activities (17, 37). Global studies have frequently reported the widespread Class 1 integron/integrase systems carrying resistances to aminoglycosides, sulfonamides, beta-lactams, and trimethoprim. Research conducted in wastewater treatment plants (WWTPs) has also demonstrated that the Class 1 integron/integrase system can be a potential indicator of ARGs abundance, making it a

promising indicator for environmental surveillance (38). Furthermore, various studies indicate that ARGs can be acquired from animal sources through horizontal gene transfer, illustrating complex AMR dissemination pathways (39). For instance, genetic analyses have shown similarities in plasmids carrying the *floR* gene in *E. coli* strains from both human and livestock samples, suggesting a possible zoonotic vector for ARG transmission (7, 40). This hypothesis is further supported by studies linking contaminated poultry meat consumption to potential horizontal gene transfer of *floR* to human commensals (41). In this scenario, our C12a toolbox has the potential to be adopted and used as a rapid surveillance system in the aforementioned situations. Our findings, in agreement with previous studies, show that C12a^{INT} finds that *E.coli* isolates carrying the *intI1* gene are also positive for *blaCTX-M-15*. However, bioinformatic analysis shows that *blaCTX-M-15* is not located within the mobile cassette array. Similarly, although a high correspondence between the *floR* and *intI1* presence, *floR* was not found within the integron cassette array. A molecular rationale behind these observations remains to be discovered. Mechanisms of DNA shuffling involving mobile genetic elements, like transposons or insertion sequences may facilitate the acquisition, loss, and spread of ARGs within the integron cassette array.

Although our study involved a limited number of *E. coli* isolates, the results perfectly conform with those obtained from established molecular and microbiological tests, indicating a high degree of reliability. The high specificity of C12a systems aligns with other molecular detection methods, thanks to the dual recognition steps mechanism, which involves both primer selection and crRNA binding. Of special interest, is the implementation of Cas12a systems into a large range of laboratory equipment availability. The performance of our C12a detection systems was consistent across all setups (Figure 6), showing low sensitivity losses (<10-fold) in low-equipped or portable setups as compared to a high-end laboratory. Remarkably, the portable system, albeit using lateral flow assay (LFA), that represents a leap toward field-ready applications, allowing direct and on-site ARG detection. The development of a simplified detection system for ARGs is crucial for investigating AMR across a spectrum of contexts, from clinical settings to environmental and veterinary surveillance. The technical features of our toolbox are valuable because they enable accurate, consistent, and cost-effective monitoring/surveillance efforts. These aspects are particularly beneficial for resource-limited settings lacking an adequate, cutting-edge, laboratory infrastructure.

Ethics

The *E. coli* isolates used in this study were derived from a strain collection of a longitudinal study approved by the ethics committee of Universidad Peruana de Cayetano Heredia (UPCH, SIDISI: 65178).

Authors Contribution

M.V-R., R.A., M.P., and P.M. conceived the project. M.V-R., R.A., S.A., and P.M. designed experiments. M.V-R., R.A., and S.A. performed experiments. M.V-R., R.A., K.P., and P.M. analyzed the data, and elaborated figures, and tables. R.S. and D.P. helped with data interpretation. M.V-R., R.A., S.A., K.P., and P.M. wrote the manuscript with the input of D.P., R.S., and M.P..

Disclosure of interest

The authors report there are no competing interests to declare.

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Supplementary Materials

Extended Methods

Biological reagents preparation

Total DNA purification: Total DNA was extracted from an *E. coli* culture grown to OD_{600} = 0.7 using the Quick DNA Miniprep kit (Cat # D4069, Zymo Research) following the manufacturer's protocol. DNA was quantified using a NanoDrop spectrophotometer and stored at -20°C.

Oligo design: To identify resistance genes, all available *E. coli* resistance gene sequences in GenBank as of January 2022 were collected. Individual alignments for *blaCTX-M-15* and *floR* were performed using AliView and MUSCLE, focusing on conserved regions [1,2] (Supplementary Dataset 1 and Dataset 2, respectively). The resulting sequences were 1057 bp for *blaCTX-M-15* and 1415 bp for *floR*. Primer sets were designed for the *blaCTX-M-15* [3] and *floR* [4] genes. For crRNA guide design and evaluation, CRISPRscan, Chop-Chop, and RNAfold bioinformatics tools were used [5 – 7]. Primers and crRNA sequences were sourced from Macrogen Inc. (Seoul, South Korea). crRNA was synthesized by *in vitro* transcription using the TranscriptAid T7 High Yield Transcription Kit (#K0411, ThermoFisher Scientific) and purified with the RNA Clean & Concentrator Kit (Cat #11-353B, Zymo Research).

crRNA production: crRNAs were produced by *in vitro* transcription using T7 RNA polymerase. Double-stranded constructs were designed to include the T7 promoter, an adaptor, and complementary crRNA sequences (Table 1). *In vitro* transcription of the dsDNA templates yielded a range of 3000 to 9000 pmoles of RNA transcript per 50 µL reaction, with concentrations ranging from 61 to 160 µM, sufficient for 4 to 12 thousand assays.

Enzymes production: Taq DNA polymerase and Cas12a proteins were sourced from locally expressed and purified stocks stored at -80°C, as detailed in [8]. Our standardized protocol yields approximately 23 mg of Taq DNA polymerase and 78 mg of Cas12a per 1 L of induced culture. Stock concentrations of 24.5 µM for Taq DNA polymerase and 18.8 µM for Cas12a were used in this study.

Antimicrobial susceptibility test

Disk diffusion: The antibiotic susceptibility profile of 32 *E. coli* isolates was determined by the disk diffusion method, using *E. coli ATCC* 25922 (Cat # 0335, Lab Elite) as the control

strain [9]. *E.coli* isolates from glycerol stocks were streaked on LB agar plates and incubated overnight at 37°C. Isolated colonies were resuspended in 0.9% saline solution to a density of 0.5 McFarland and swabbed onto Mueller-Hinton agar plates (Cat # M173-500G, Himedia). Antibiotic disks, including cefotaxime (CTX) 30 µg (Cat # 9017, Liofilchem), and ceftazidime (CAZ) 30 µg (Cat # 9019, Liofilchem) were placed on the surface of the medium. After 18 hours of incubation at 37°, inhibition zones were measured and interpreted following the CLSI guidelines. A confirmatory test for ESBL production was performed using CTX 30 µg and CAZ 30 µg disks combined with clavulanic acid: CTX/AC 40 µg (Cat # 9182, Liofilchem), and CAZ/AC 40 µg (Cat # 9145, Liofilchem). Plates were incubated for 18 hours at 37°C, and ESBL producers were detected by measuring the difference in the inhibition halo of the antibiotic in the presence of clavulanic acid (>5 mm) [9].

Microdilution assay: Florfenicol (Cat #F1427-500MG, Merck) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 mg/ml. Serial dilutions, ranging from 256 µg/ml to 0.125 µg/ml were prepared in a 96-well plate (Cat # 3596, ThermoFisher) using Mueller-Hinton Broth II (Cat # 610218, Liofilchem) [10]. Each well was inoculated with 1.5 X 10^6 CFU/ml of cells. After 18 h of incubation at 37°C, the MIC was determined. The breakpoint for discriminating susceptible from resistant isolates was 32 µg/mL [11].

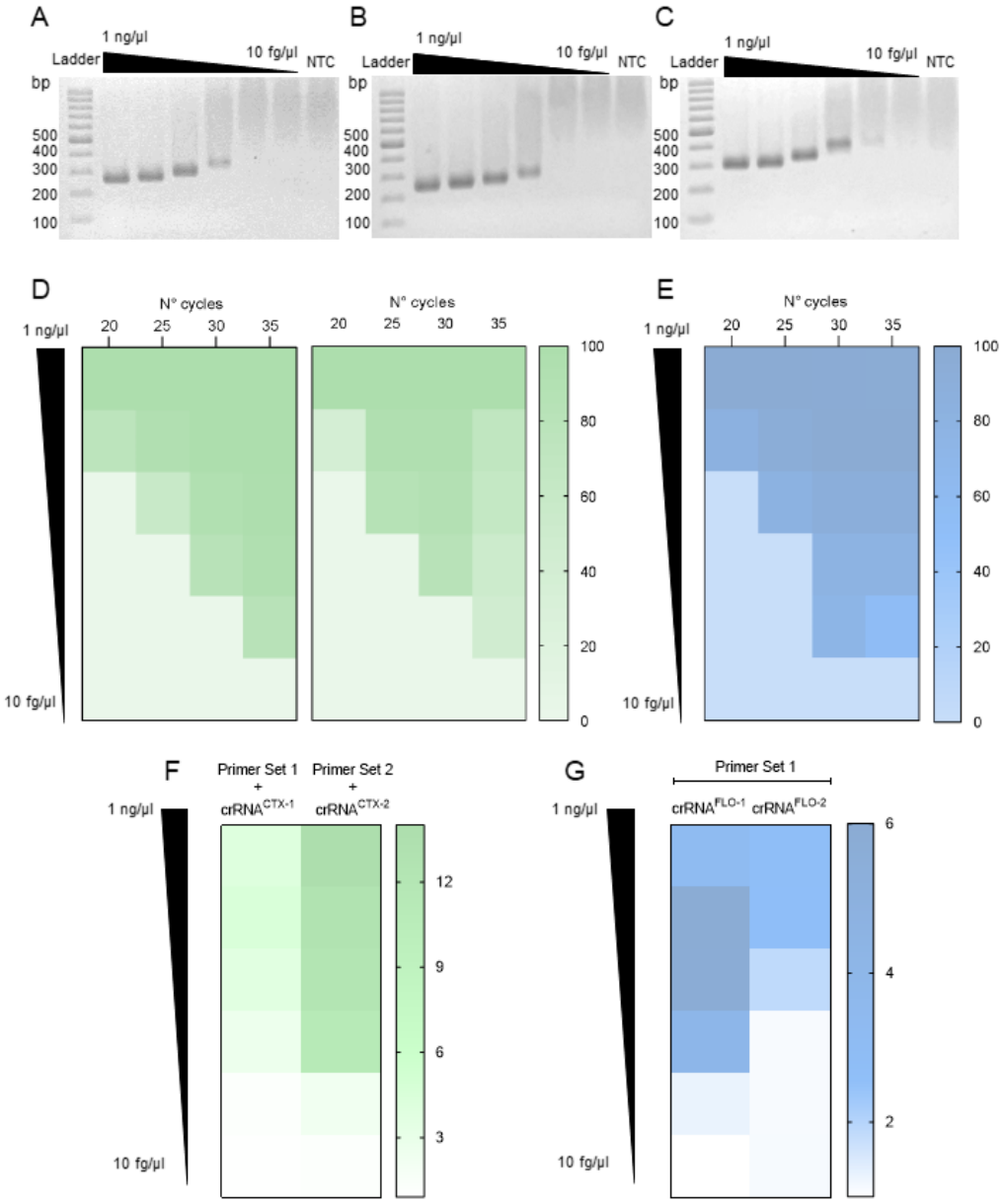
Bioinformatic analysis

DNA sequencing data were downloaded from the BioProject web portal of the NCBI (https://www.ncbi.nlm.nih.gov/bioproject/) using accession code PRJNA821865. ARG presence was evaluated using the ResFinder platform (http://genepi.food.dtu.dk/resfinder) v4.6.0 [12] with default parameters. Briefly, a minimum length of 60% and a threshold of 90% were set up for ARG and disinfectant-resistance gene identification. *E.coli* was defined as the species input. For the identification of the integrase-integron class 1, the VRprofile2 platform (https://tool2-mml.sjtu.edu.cn/VRprofile/) [13] and the IntegronFinder tool (v2.0.5) [14] on the Galaxy EU server (https://usegalaxy.eu/) were used simultaneously with default parameters.

Data Analysis and Software

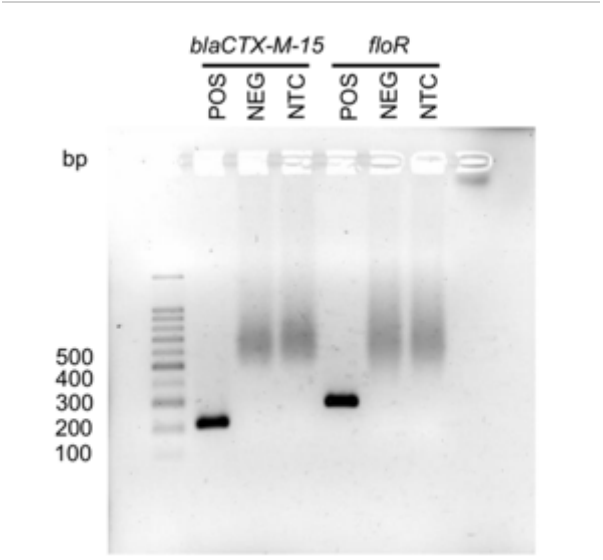
Fluorescence data from the Synergy H1 plate reader were collected using Gen 5 software and exported to Excel. NF_{ntc} values were calculated during data processing and analysis as appropriate. All fluorescence data analysis and chart preparation were performed using GraphPad Prism version 10.0.0 for Windows, www.graphpad.com.

Supplementary figures

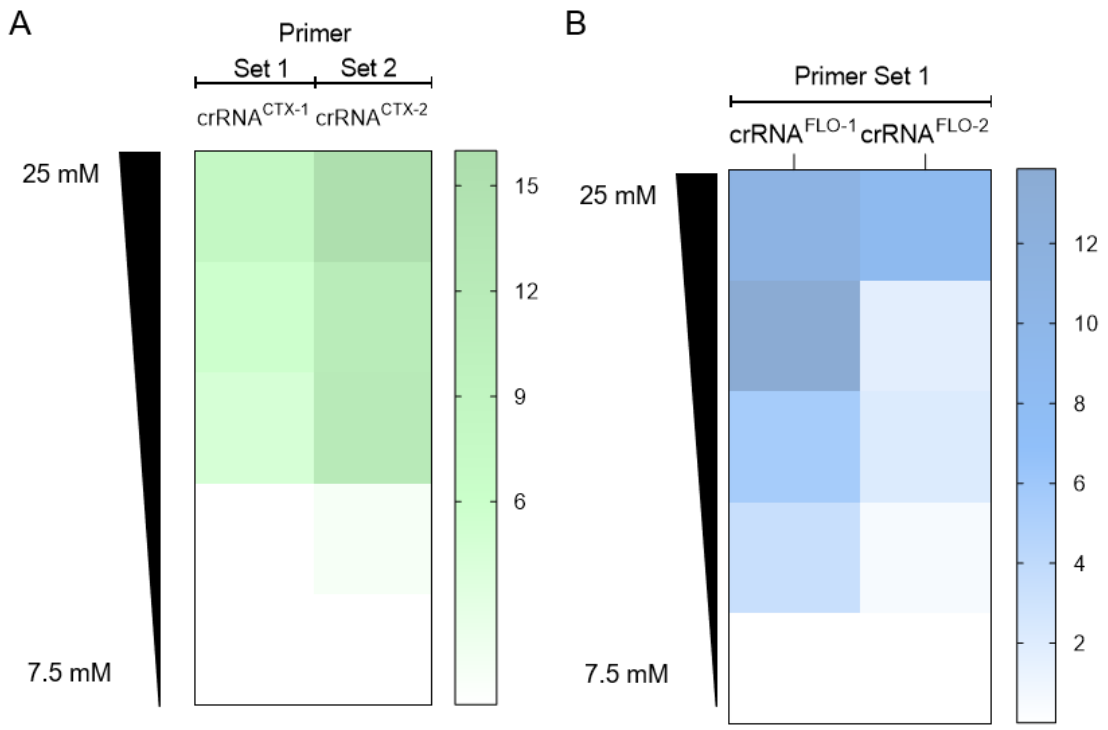


Supplementary Figure 1. Overview of the optimization process for the CRISPR/Cas12a detection systems. (A), (B), and **(C)** show agarose gel images illustrating the results of the 30-cycle PCR for the *blaCTX-M-15* primer set 1, primer set 2, and the *floR* primer set, respectively. The observed decrease in migration with reduced fragment concentration is attributed to an interaction with the SYBR Gold dye, as documented in the literature [15]. **(D)** Heatmap displaying the AUC (Area Under the Curve) values of the amplification bands observed in an agarose gel for the *blaCTX-M-15* primer sets. A template titration was conducted in a standard PCR with an increasing number of amplification cycles.

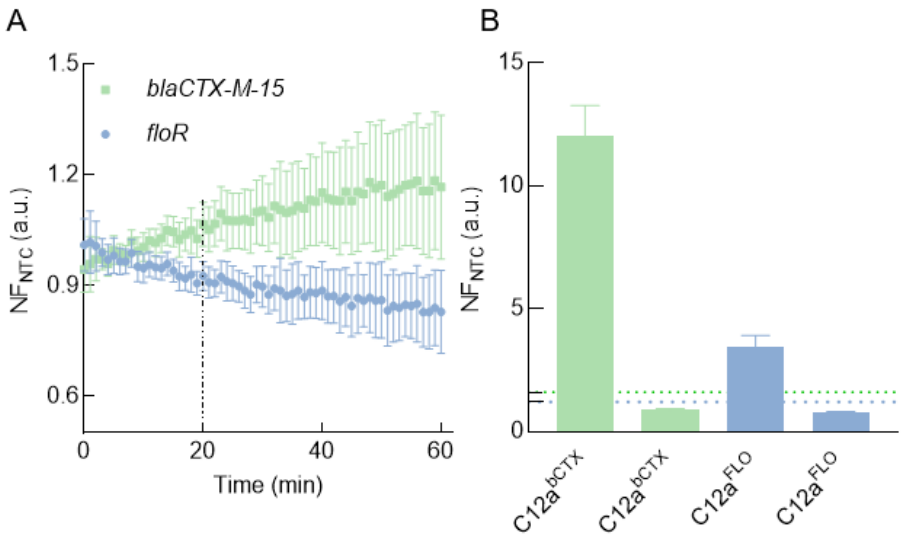
(E) Similar to (D), but for the *floR* primer set. **(F)** Heatmap displaying fluorescent ratio values for the crRNA candidates designed for the *blaCTX-M-15* gene, obtained from CRISPR-Cas-based detection of 30-cycle PCR amplification products across a template concentration range from 10 fg/µl to 1 ng/µl. **(G)** Similar to (F), but for *floR.*



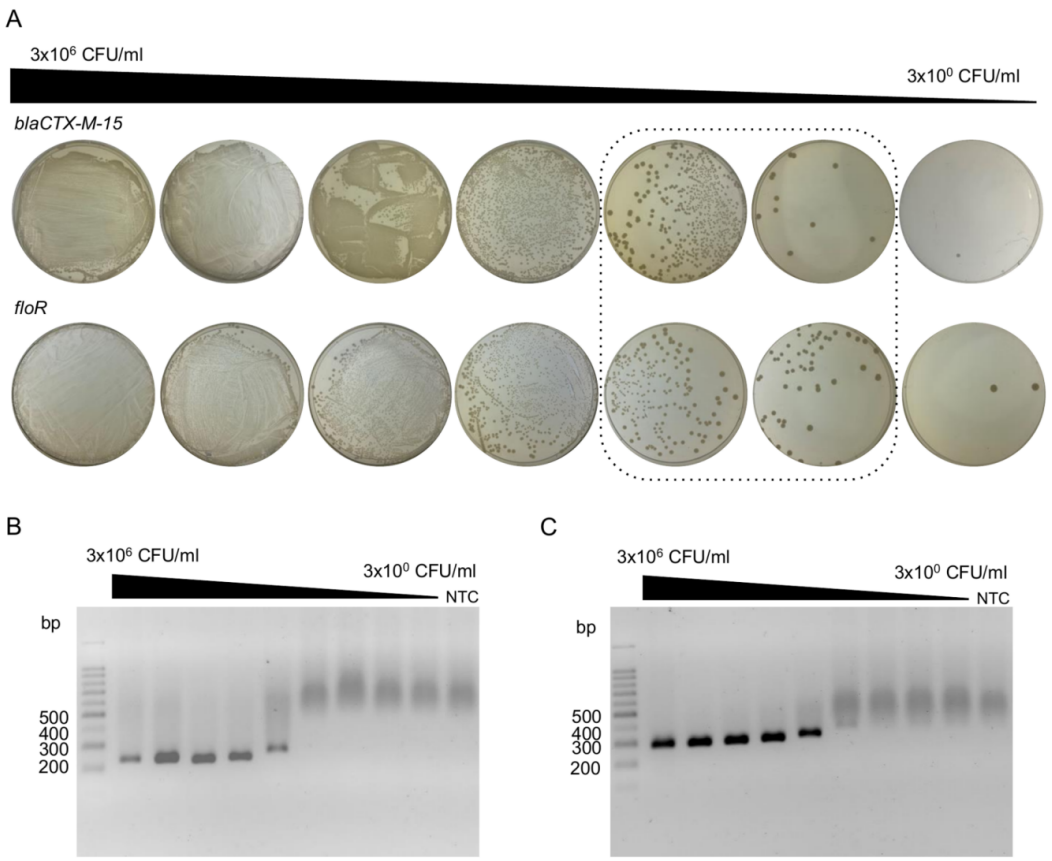
Supplementary Figure 2. Agarose gel displaying PCR amplification products for the ARG evaluated. Amplified products obtained from a standard 30-cycle PCR were analyzed using 1.7% agarose gel electrophoresis, with a 100 bp ladder for size reference. Amplicons were observed exclusively in positive samples. POS indicates a reaction containing the target gene; NEG indicates a reaction with template DNA missing the target gene; NTC indicates a reaction without template DNA. Specifically, the primer set 2 targeting the *blaCTX-M-15* gene produced a 216 bp amplicon, while the gene-specific primer for *floR* produced a 270 bp amplicon.



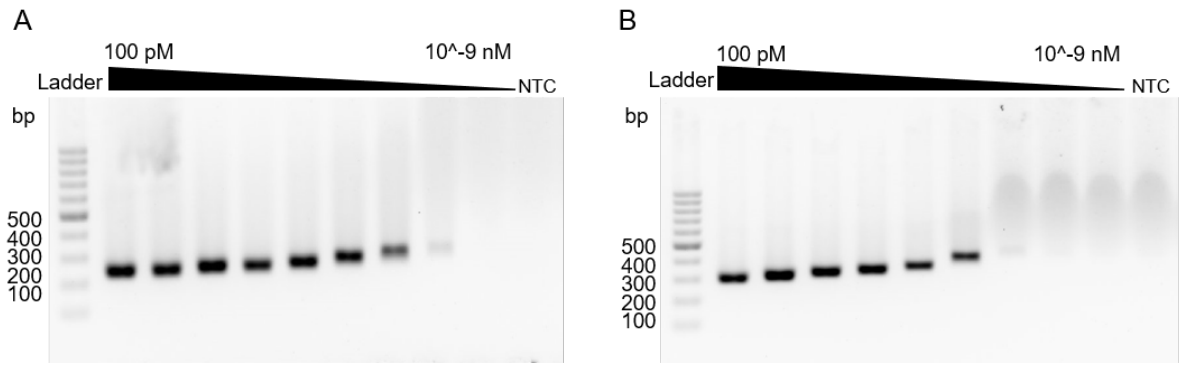
Supplementary Figure 3. Evaluation of Mg2+ concentration on the CRISPR-Cas system performance. Heatmaps displaying the NF_{NTC} values of the $MgCl^{2+}$ titration for the crRNA candidates designed for *blaCTX-M-15* and *floR*. The concentrations ranged from 7.5 to 25 mM. PCR amplicons were obtained using a 30-cycle PCR protocol with primer set 2 for *blaCTX-M-15* and primer set 1 for *floR*. The NF_{NTC} values were measured at 15 min.



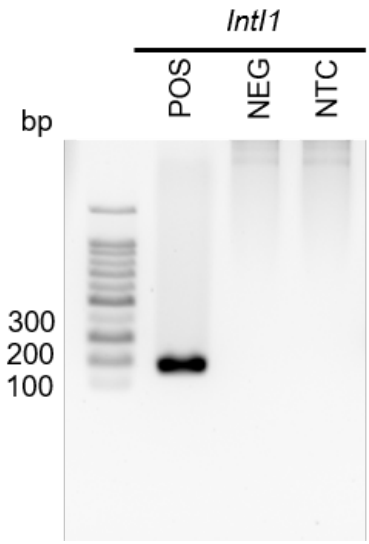
Supplementary Figure 4. Determination of Limit of Blank (LoB) and Day-to-Day Reproducibility of C12a^{bCTX} and C12a^{FLO} (A) Normalized fluorescence ratio (NF_{NTC}) values over time (minutes) for ten negative samples were analyzed to determine the Limit of Blank (LoB). The average NF_{NTC} values for negative samples after 20 minutes (dotted line) were 1.04 ± 0.02 for *blaCTX-M-15* and 0.94 ± 0.02 for *floR*. Error bars represent the standard deviation of at least ten consecutive measurements. **(B)** NF_{NTC} values for positive and negative samples at 20 minutes detection point across 34 experimental days. The bar represents the mean NF_{NTC} values with the error bars representing the standard deviation. The dotted lines correspond to the LoB values calculated for each gene.



Supplementary Figure 5. Determination of the Limit of detection based on CFU quantification. To determine the LoD in CFU/ml, 7 ten-fold dilutions of a cell culture of *E.coli* ATCC25922 at 0.5 Mc Farland were prepared. **(A)** *E.coli* cells were plated on LB agar after 18 hours of incubation at 37°C. The dilution curve was prepared using 1 mL of LB medium, and 50 µL of each dilution was spread on the LB plates. The CFU/ml titer was determined using the CFU number observed in the dilutions framed in the dotted box. **(B)** Amplified products obtained from each dilution using a standard 30-cycle PCR were analyzed using 1.7% agarose gel electrophoresis, with a 100 bp ladder for size comparison. For *blaCTX-M-15* an amplicon of 216 bp was expected. **(C)** Similar to panel (B), but for *floR,* with an expected amplicon size of 270 bp. The reduced migration at lower fragment concentrations is caused by an interaction with the SYBR Gold dye, as documented in the literature [15].



Supplementary Figure 6. Agarose-gel electrophoresis for comparison of analytical sensitivity between CRISPR-Cas-based fluorescent and naked-eye agarose electrophoresis detection. Target amplification across different template concentrations over a 30-cycle PCR. Amplified products were resolved using 1.7% agarose gel electrophoresis, alongside a 100 bp ladder for size comparison. **(A)** An amplicon of 216 bp was expected for *blaCTX-M-15*. **(B)** Same as (A), but for *floR* with an expected amplicon size of 270 bp. The observed decrease in migration with reduced fragment concentration is attributed to an interaction with the SYBR Gold dye, as documented in the literature [15].



Supplementary Figure 7. Agarose gel displaying PCR amplification products for the *intI1* gene. Amplified products obtained from a standard 30-cycle PCR were analyzed using 1.7% agarose gel electrophoresis, with a 100 bp ladder for size reference. Amplicons were observed exclusively in positive samples. Specifically, the primer set targeting the *intI1* gene produced a 146 bp amplicon.

Supplementary files

Supplementary Dataset 1. DNA sequences for *blaCTX-M-15* alignment.

Supplementary Dataset 2. DNA sequences for *floR* alignment.

Supplementary File 1. Excel File with supplementary tables:

- **Sheet 1:** Supplementary Table 1, List of buffers used in this study.
- **Sheet 2:** Supplementary Table 2, List of oligonucleotides used in this study.
- **Sheet 3:** Supplementary Table 3, List of DNA sequences for *blaCTX-M-15* and *floR* genes amplified with the selected primer sets. DNA sequences were obtained by SANGER sequencing.
- **Sheet 4:** Supplementary Table 4, Database with NGS, CRISPR/Cas, and Antimicrobial Susceptibility Testing data for all evaluated samples.
- **Sheet 5:** Supplementary Table 5, List of ARGs identified in the *blaCTX-M-15* and *floR*-positive *E. coli* isolates, including those found in the genome and in the Class 1 integron.
- **Sheet 6:** Supplementary Table 6, List of candidate crRNA and primers for additional ARGs detection

Table S1

Buffer	Purpose	Components
RPB 4X	Taq Pol Buffer	200 mM Tris-HCl (pH 8.4 25°C), 300 mM KCl, 12 mM MgCl2, 40% trehalose, 40 mM DTT, 0.4 mM EDTA, 1.6 mM dNTP mix
TBE 5X	Electrophoresis	445 mM Tris Base, 445 mM Boric Acid and 10 mM de EDTA
CRB1	CRISPR Reaction	10 mM TrisHcl – 50 mM NaCl - 100 ug/ul BSA
CRB2		CRB1X – 15 Mm MgCl2
LB	AST test	10 g triptone, 10 g Nacl, 5 g Yeast
LB agar		1 L LB and 15 g agar

Table S2

List of primers						
Gene	**Set**	**Orientation**	**Sequence**	**Amplicon size (bp)**	**ealing temper**	**Source**
blaCTX-M-15	1	Forward	GCAAAAACTTGCCGAATTAGAG	241	56 °C	This study
		Reverse	CTTTTCCGCAATCGGATTATAG			
	2	Forward	GCGCTACAGTACAGCGATAA	216	50 °C	Ramadan et al., 2019
		Reverse	TTTACCCAGCGTCAGATTCC			
floR	1	Forward	GCGCAACGGCTTTCGTCATT	270	58 °C	Qian et al., 2021
		Reverse	GCATCGCCAGTATAGCCAAA			
intI1	1	Forward	GGCTTCGTGATGCCTGCTT	146	59°C	Zhang et al., 2020
		Reverse	CATTCCTGGCCGTGGTTCT			

List of crRNAs					
Gene	**Set**	**PAM**	**Sequence**	**Length**	**Source**
blaCTX-M-15	crRNACTX-1	TTTA	TAATTTCTACTAAGTGTAGATACAGATTCGGTTCGCTTTCACTT	23	This study
	crRNACTX-2	TTTC	TAATTTCTACTAAGTGTAGATGTCTCCCAGCTGTCGGGCGAACG	23	
floR	crRNAFLO-1	TTTA	TAATTTCTACTAAGTGTAGATTGCCAACCGTCCTGAGGGTGTCG	23	
	crRNAFLO-2	TTTG	TAATTTCTACTAAGTGTAGATTCGCTTTCCGTCTACTTCAAGCA	23	
intI1	crRNAINT1	TTTG	TAATTTCTACTAAGTGTAGATAAGGCGCGCTGAAAGGTCTGGTCA	24	
	crRNAINT2	TTTG	TAATTTCTACTAAGTGTAGATCGCAGCACACGCATTCGACCGATC	24	
	crRNAINT3	TTTT	TAATTTCTACTAAGTGTAGATGCGCAGCACACGCATTCGACCGAT	24	

crRNA repeat sequence is highlighted in red.

Table S3

Amplicon sequence obtained by SANGER sequency

Gene	Length	DNA sequence
blaCTX-M-15	184	CAGCGATAACGTGGCGATGAATAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCAC CGCGTTCGCCCGACAGCTGGGAGACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGTT AAACACCGCCATTCCGGGCGATCCGCGTGATACCACTTCACCTCGGGCAATGGCGCAAAC TCTG
floR	213	GTCATTGCGTCTCTGGGAGCAGCTTGGTCTTCAACTGCACCGGCCTTTGTCGCTTTCCGTCT ACTTCAAGCAGTGGGCGCGTCGGCCATGCTGGTGGCGACGTTCGCGACGGTTCGCGACGTT TATGCCAACCGTCCTGAGGGTGTCGTCATCTACGGCCTTTTCAGTTCGATGCTGGCGTTCGT GCCTGCGCTCGGCCCTATCGCCGGAACA

Database codebook_Table S4

Codebook	Description
1	Positive
0	Negative
CTX	Cefotaxime
CTX/CA	Cefotaxime and clavulanic acid
CAZ	Ceftazidimae
CAZ/CA	Ceftazidime and clavulanic acid
R	Resistant
S	Susceptible

Table S4

Code	Type of sample	NGS	Status	Disk difussion								MIC		CRISPR		LFA	
				CTX (mm)	CTX/CA (mm)	Difference (mm)	Status	CAZ (mm)	CAZ/CA (mm)	Difference (mm)	Status	MIC (µg/ml)	MIC status	Ratio	Status	Bands	Status
bla001	*E.coli* isolate	0	S	30	32	3	S	21	22.0	1	S	na	na	0.8	S	na	na
bla002	*E.coli* isolate	1	R	6	23	17	R	12	21	9	R	na	na	9.1	R	na	na
bla003	*E.coli* isolate	1	R	6	19	13	R	14	18.6	5	R	na	na	13.4	R	na	na
bla004	*E.coli* isolate	0	S	21	24	3	S	24	25.5	1	S	na	na	0.9	S	na	na
bla005	*E.coli* isolate	1	R	9	21	12	R	13	21.2	8	R	na	na	12.8	R	na	na
bla006	*E.coli* isolate	1	R	6	22	15	R	14	21.2	8	R	na	na	12.5	R	na	na
bla007	*E.coli* isolate	0	S	30	31	1	S	31	31.5	1	S	na	na	0.8	S	na	na
bla008	*E.coli* isolate	1	R	7	18	11	R	13	19.3	6	R	na	na	11.7	R	na	na
bla009	*E.coli* isolate	1	R	6	24	18	R	14	22	7	R	na	na	12	R	na	na
bla010	*E.coli* isolate	0	S	19	22	3	S	26	26.2	1	S	na	na	0.8	S	na	na
bla011	*E.coli* isolate	0	S	19	20	1	S	22	23.3	2	S	na	na	1	S	na	na
bla012	*E.coli* isolate	0	S	27	29	2	S	21	23	2	S	na	na	0.9	S	na	na
bla013	*E.coli* isolate	1	R	6	23	17	R	14	21.8	8	R	na	na	12.5	R	na	na
bla014	*E.coli* isolate	0	S	24	26	3	S	21	21.6	1	S	na	na	0.8	S	na	na
bla015	*E.coli* isolate	0	S	26	26	1	S	22	22.8	1	S	na	na	0.9	S	na	na
flo001	*E.coli* isolate	1	R	na	na	na	na	na	na	na	na	>256	R	3.2	R	na	na
flo002	*E.coli* isolate	1	R	na	na	na	na	na	na	na	na	128	R	3.5	R	na	na
flo003	*E.coli* isolate	0	S	na	na	na	na	na	na	na	na	8	S	0.9	S	na	na
flo004	*E.coli* isolate	0	S	na	na	na	na	na	na	na	na	16	S	0.8	S	na	na
flo005	*E.coli* isolate	1	R	na	na	na	na	na	na	na	na	128	R	2.4	R	na	na
flo006	*E.coli* isolate	1	R	na	na	na	na	na	na	na	na	>256	R	3.6	R	na	na
flo007	*E.coli* isolate	1	R	na	na	na	na	na	na	na	na	>256	R	3.7	R	na	na
flo008	*E.coli* isolate	0	S	na	na	na	na	na	na	na	na	128	S	3.3	S	na	na
flo009	*E.coli* isolate	0	S	na	na	na	na	na	na	na	na	>256	S	4	S	na	na
flo010	*E.coli* isolate	0	S	na	na	na	na	na	na	na	na	8	S	0.8	S	na	na
flo011	*E.coli* isolate	1	R	na	na	na	na	na	na	na	na	>256	R	4.2	R	na	na
flo012	*E.coli* isolate	1	R	na	na	na	na	na	na	na	na	>256	R	3.2	R	na	na
flo013	*E.coli* isolate	0	S	na	na	na	na	na	na	na	na	16	S	0.8	S	na	na
flo014	*E.coli* isolate	0	S	na	na	na	na	na	na	na	na	16	S	0.7	S	na	na
flo015	*E.coli* isolate	0	S	na	na	na	na	na	na	na	na	8	S	0.8	S	na	na
flo016	*E.coli* isolate	1	R	na	na	na	na	na	na	na	na	128	R	3.1	R	na	na
flo017	*E.coli* isolate	1	R	na	na	na	na	na	na	na	na	>256	R	3.6	R	na	na
Fbla1	Total DNA	na	na	na	na	na	na	na	na	na	na	na	na	6.2	R	1	R
Fbla2	Total DNA	na	na	na	na	na	na	na	na	na	na	na	na	10.9	R	1	R
Fbla3	Total DNA	na	na	na	na	na	na	na	na	na	na	na	na	1.4	S	0	S
Fbla4	Total DNA	na	na	na	na	na	na	na	na	na	na	na	na	12.4	R	1	R
Fbla5	Total DNA	na	na	na	na	na	na	na	na	na	na	na	na	12.1	R	1	R
Fbla6	Total DNA	na	na	na	na	na	na	na	na	na	na	na	na	7.5	R	1	R
Fbla7	Total DNA	na	na	na	na	na	na	na	na	na	na	na	na	0.9	S	0	S
Fflo1	Total DNA	na	na	na	na	na	na	na	na	na	na	na	na	4.2	R	1	R
Fflo2	Total DNA	na	na	na	na	na	na	na	na	na	na	na	na	5.1	R	1	R
Fflo3	Total DNA	na	na	na	na	na	na	na	na	na	na	na	na	4.8	R	1	R
Fflo4	Total DNA	na	na	na	na	na	na	na	na	na	na	na	na	4.3	R	1	R
Fflo5	Total DNA	na	na	na	na	na	na	na	na	na	na	na	na	3.8	R	1	R
Fflo6	Total DNA	na	na	na	na	na	na	na	na	na	na	na	na	1.3	S	0	S
Fflo7	Total DNA	na	na	na	na	na	na	na	na	na	na	na	na	4.3	R	1	R

Table S5_Summary

Summary ARG found			
aac	aminoglycoside	*antibiotic inactivation*	*3-N-acetyltransferase*
aadA	aminoglycoside	*antibiotic inactivation*	*nucleotidyltransferase*
aph	aminoglycoside	*antibiotic inactivation*	*3'-O-phosphotransferase*
arr	rifamycin/macrolide	*antibiotic inactivation*	*ribosyltransferase*
bla	penam	*antibiotic inactivation*	*beta-lactamase*
cat	phenicols	*antibiotic inactivation*	*acetyltransferase*
cml/mdf	phenicols	*antibiotic efflux*	*efflux pump*
dfrA	trimethoprim	*antibiotic target replacement*	*dihydrofolate reductase*
erm	macrolide	*antibiotic target alteration*	*methyltransferase*
floR	phenicols	*antibiotic efflux*	*efflux pump*
fosA	fosfomycin	*antibiotic inactivation*	*thiol transferase*
lnu	lincosamide	*antibiotic inactivation*	*nucleotidyltransferase*
mcr	polymyxin	*antibiotic target alteration*	*phosphoethanolamine transferase*
mph	macrolide	*antibiotic inactivation*	*phosphotransferase*
qac	disinfectant	*antibiotic efflux*	*efflux pump*
qnr	quinolone	*antibiotic target protection*	*structure mimic*
sul	sulfonamide	*antibiotic target replacement*	*dihydropteroate synthase*
tet	tetracycline	*antibiotic efflux*	*efflux pump*

Table S5

Code	Out of Class 1 integron																		Within Class 1 Integron										MGE near Class 1 Integron	
	aac	*aad*	*aph*	*bla*	*cat*	*cml*	*dfrA*	*erm*	*floR*	*forn*	*fosA*	*lnu*	*mcr*	*mph*	*qac*	*qnr*	*sul*	*tet*	*ABC*	*aac*	*aad*	*ARR*	*bla*	*cat*	*cml*	*dfr*	*lnu*	*qac*	*sul*	Insertion element	Transposons
bla002				*blaCTXM15*		*cmlA*				*formA*						*qnrS1*	*sul2*				*aadA1*						*dfrA1*		*qacE*			
bla003	*aac(3)-IID*			*blaCTXM15*		*cmlA*		*erm(B)*		*formA*				*mph(A)*				*tet(B)*		*aac(6')-Ib-cr*	*aadA5*	*ARR-3*	*blaOXA-1*	*catB3*		*dfrA17*		*qacE*	*sul1*	IS6100	
bla005				*blaCTXM15*		*cmlA*				*formA*						*qnrS1*															
bla006				*blaCTXM15*		*cmlA*				*formA*						*qnrS1*															
bla008				*blaCTXM15*		*cmlA*				*formA*						*qnrS1, qnrB19*	*sul2*				*aadA1*					*dfrA1*		*qacE*			
bla009			*aph(3)-Ia, aph(3)-Ib, aph(6)-Id*	*blaCTXM15, blaTEM176*		*cmlA*				*formA*							*sul2, sul3*	*tet(A)*			*aadA1, aadA2*							*lnu(F)*			*ISEc38*	*TnpR_TnAs1, TnpA_TnAs1*
bla013	*aac(6)-Ib.cr*			*blaCTXM15, blaOXA1*	*catB3*	*cmlA*	*dfrA14*	*erm(B)*		*formA*				*mph(A)*																	
flo001	*aac(3)-Iid*			*blaTEM176, blaCTXM65*		*cmlA*	*dfrA2*		*floR*	*formA*	*fosA*						*sul2, sul3*	*tet(A), tet(M)*			*aadA1, aadA2*				*cmlA1*	*dfrA12*				IS1006, ISVsa3, ISAba33	TnpR_TnShes11, TnpA_TnEcO26
fl002	*aac(3)-Iva*		*aph(4)-Ia*	*blaTEM1B, blaCTXM65*		*cmlA*			*floR*	*formA*						*qnrD1*															
flo005	*aac(3)-Iid*	*aadA2*		*blaTEM176, blaCTXM65*		*cmlA*	*dfrA2*		*floR*	*formA*	*fosA3*	*lnu(F)*					*sul2, sul3*	*tet(A), tet(M)*			*aadA1*				*cmlA1*	*dfrA12*	*lnu(F)*			IS1006, ISVsa3	TnpR_TnShes11, TnpA_TnEcO26
flo006	*aac(3)-Via*		*aph(3)Ib, aph(6)Id, aph(A)IIa*	*blaCMY2, blaCTXM55, blaTEM1B*		*cmlA*			*floR*	*formA*	*fosA3*				*qacE*	qnrB19	*sul1*	*tet(A)*	*sitABC*												
flo007	*aac(3)-IId*		*aph(6)-Id, aph(3)-Ib,*	*blaCTXM65, blaTEM1B*		*cmlA*			*floR*	*formA*	*fosA3*			*mph(A)*				*tet(A)*	*sitABC*		*aadA5*					*dfrA17*		*qacE*	*sul1*	IS6100	TnpR_TnShess11
flo008			*aph(6)-Id, aph(3)-Ib, aph(3)-Iia*	*blaTEM1B, blaCTXM55*		*cmlA*			*floR*	*formA*	*fosA2*	*lnu(G)*					*sul2*	*tet(A), tet(M)*			*aadA2*					*dfrA12*					
flo009	*aac(3)-IIA*			*blaCTXM27*		*cmlA*			*floR*	*formA*	*fosA3*						*sul3*	*tet(A)*	*sitABC*		*aadA1, aadA2*				*cmlA1*						
flo011	*aac(3)-Iva*		*aph(4)-Ia*	*blaTEM1B, blaCTXM65*		*cmlA*			*floR*	*formA*	*fosA3*						*sul2*	*tet(A)*													
flo012	*aac(3)-Iva*		*aph(4)-Ia*	*blaTEM1B, blaCTXM65*		*cmlA*			*floR*	*formA*	*fosA3*						*sul2*	*tet(A)*			*aadA5*					*dfrA17*		*qacE*			
flo016	*aac(3)-Via*		*aph(6)-Id, aph(3)-Ib, aph(3)-Iia*	*blaCMY2, blaCTXM55, blaTEM1B*		*cmlA*			*floR*	*formA*	*fosA3*				*qacE*		*sul1, sul2*	*tet(A)*	*sitABC*												
flo017	*aac(3)-Iid*		*aph(3)-Ia*	*blaCTXM65*		*cmlA*			*floR*	*formA*	*fosA3*		*mcr-1.1*			*qnrS1*	*sul2, sul3*	*tet(A)*			*aadA1*					*dfrA14*	*lnu(F)*				TnpR_TnAs

Table S6

Genes name	crRNA 1	crRNA 2	Primer Forward	Primer Reverse	Reference
aac(6')-Ib-cr	TTTGAGAGGCAAGGTACCGTAACCACC	TTTCTTCTTCCCACCGTCCGTCCCCGCT	TTGCGATGCTCTATGAGTGGCTA	CTCGAATGCCTGGCGTGTTT	A. Robicsek 2006
aadA1	TTTCATCAAGCCTTACGGTCACCGTAA	TTTGTCAGCAAGATAGCCAGATCAATGT	TATCAGAGGTAGTTGGCGTCAT	GTTCCATAGCGTTAAGGTTTCATT	L. P. Randall 2004
aadA2	TTTGTAAGCAGGATAGCTAGATCAATG	TTTCGCTCATCGCCGGCCCAGTCGGGCT	TGTTGGTTACTGTGGCCGTA	GATCTCGCCTTTCACAAAGC	L. P. Randall 2004
aadA5	TTTCCCTGCACAAGTTTTCAAGCAGCTG	TTTGGTACAGCGCTTCAACTGGTCTCAT	AGCTTTTAAGTCGCGTCTTTGT	ACGCAAGATTCTCTCAATCGTT	This study
ARR-3	TTTAAGTCCTCCAACGAATCCAACATTC	TTTCCCGGTAATCCAACACAGTCCTATA	ACATAGTTGAGCCAACAGGACC	GTTTGATGGCTTTGTTATGCAA	This study
blaOXA1	TTTGTGTCCGCACTTACAGGAAACTTG	TTTGATTATGGAAATCAAGACTTCTCTG	TTTTCTGTTGTTTGGGTTTT	TTTCTTGGCTTTTATGCTTG	Sugumar 2014
cmlA1	TTTATTGGCATCACTCGGCATGGACATG	TTTGGCATAAACGGAACTGCTGGCAAGT	TAGTTGGCGGTACTCCCTTG	GAATTGTGCTCGCTGTCGTA	This study
dfrA1	TTTACATCTGACAATGAGAACGTAGTG	TTTGTATATCTCCCCACCACCTGAAACA	TGGTAGCTATATCGAAGAATGGAGT	TATGTTAGAGGCGAAGTCTTGGGTA	M. Grape 2007
dfrA12	TTTCGCAGACTCACTGAGGGAAAAGTC	TTTGATGTACCTCAGATAGAAACATGCC	ACTCGGAATCAGTACGCA	GTGTACGGAATTACAGCT	Guerra 2001
dfrA14	TTTCTTCCCGAGTATTCCAAATACCTT	TTTCTCCGCCACCAGACACTATAACGTG	TTAACCCAGGATGAGAACCT	CGATTGCATAGCTTTGTTAA	Miranda 2016
drfA17	TTTGTCTACTGTTCACGTTGAAGTCGA	TTTGACCCCCGCCAGAGACATATACATG	ACATTTGACTCTATGGGTGTTCTTC	AAAACTGTTCAAAAACCAAATTGAA	M. Grape 2007
sul1	TTTCGCGAGGGTTTCCGAGAAGGTGATT	TTTACAGGAAGGCCAACGGTGGCGCCCA	GCTATTGGTCTCGGTGTCGC	GCATGATCTAACCCTCGGTCT	Zhao 2016
catB3	TTTCCGGACCGTGATGACGTTGATAAGT		AAGGCAAGCTGCTTTCTGAG	GATAAAGGAAGCCCCACTCC	Szczepanowski 2009

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5 Discussion

This thesis provides a comprehensive overview of the combined threat of antimicrobial resistance and plastic pollution in riverine environments, focusing on a river of central-east Italy as a case study.

The initial evidence for this thesis came from the collection and characterization of MPs present in the Chienti River. Even though this river does not cross major cities and is not considered heavily polluted, the study revealed the widespread presence of microplastics, with PE being the most abundant polymer, accounting for over 60% of detected plastic debris. The results of this study, which are presented as short communication, provided the foundation for exploring the intersection of plastic pollution and AMR within riverine ecosystems. This complex phenomenon, which is difficult to monitor and quantify due to the challenges posed by sampling and irregular distribution of plastic particles, is influenced by environmental and hydrological factors such as flow velocity, storm events, and land-based pollution sources (Faure F, 2015; Lebreton LC, 2017). MPs are ubiquitous in freshwater systems, and the resistome carried by the associated bacteria constitutes a long-term risk in terms of AMR spread, which is difficult to detect when associated to these artificial substrates. To fully understand this complex and interconnected ecosystem, a multifaceted approach was presented that included microscopic, microbiological and molecular analyses, firstly focusing on bacteria of clinical importance. Subsequently, a metagenomic approach was used to investigate and analyze the whole bacterial composition of the plastisphere, potentially enhancing the spread of resistance determinants.

The field experiment performed in the year 2023 demonstrates the alarming presence of MDR bacteria in both river water and plastic substrates samples, underscoring the widespread nature of antibiotic-resistant bacteria in river environments. Specifically, the results of this case study revealed that bacteria grown as structured communities (biofilms) on plastic materials harbor a high percentage of MDR bacteria (72%) and ARGs, while the *Enterobacteriaceae* isolated from river water exhibited an even higher percentage of MDR bacteria (84%). These alarming data highlight the role of rivers as conduits, and the constantly floating MPs as efficient vehicles for AMR transfer across ecosystems and into the marine environment. The wide presence of ARB and ARGs in aquatic environments is closely related with anthropogenic pollution, with these bacteria accumulating from various

sources, including agricultural runoff, wastewater effluents, and urban discharge, further intensifying the dissemination of resistance determinants (Barros and Seena, 2021).

The isolation and identification of 3GC-resistant *Enterobacteriaceae* in the Chienti River is an issue of great concern. These bacteria are categorized by the WHO as a critical priority due to limited treatment options available, and to the high rates of morbidity and mortality associated with infection (WHO report, 2024). In addition to cephalosporin resistance, an association with resistance to other antibiotic classes was observed, specifically high frequencies of resistance to levofloxacin, gentamicin (CN), and trimethoprim-sulfamethoxazole (SXT), along with a lower, but notable, frequency of carbapenem resistance. Association of multidrug resistance patterns in Cefotaxime-resistant *Enterobacteriaceae* increases the difficulty in treating infections displaying these critical profiles and highlights the potential public health risks they pose also in environmental contexts.

This study has identified carbapenemases-producing *Klebsiella pneumoniae* sequence type ST1519 in river water, a strain not previously reported in natural environments in Italy. The emergence of such resistant strains in rivers highlights the potential for the environmental resistome to act as a reservoir for clinically significant resistance genes. As these strains enter the environment, potentially through sewage and wastewater discharge (Ahmed W, 2019), they can colonize and spread via plastic debris, which act as mobile, highly firm and steady substrates within aquatic systems. Once released into the environment, these bacteria can dissociate from plastic surfaces and persist in the water column, further amplifying the spread of AMR to other ecosystems. The capability of the pathogenic bacteria to enter or re-enter humans through environmental pathways, raises concern about the hazards connected with this potential chain reaction. The exact mechanisms through which these critical resistances move across clinical and environment settings, however, remain still largely unclear.

MGEs, a recognized key factor involved in ARGs transmission, have been investigated in this study. Consistent with previous studies on the plastisphere, our results point out the plastic substrate as an ideal microbial habitat that facilitates horizontal gene transfer and microbial adaptation (Zettler ER, 2013; Amaral-Zettler LA, 2020). The close physical proximity of bacterial cells within the plastisphere, may favor the mobilization of *intI1*, presumably in association with other MGEs (Gillings MR, 2015). In our study, has been detected a steadily higher frequency of Class 1 Integrase and their associated gene cassettes in *Enterobacteria* isolated from plastic samples, compared to those isolated from raw water.

Moreover, the wider variety of gene cassettes found within the variable region of Class 1 Integrons in bacterial strains recovered from plastics, supports the increasing evidence on its role in recombining structure to promote microbial survival, fitness and adaptation. This can potentially affect the composition of the surrounding aquatic resistome by promoting not only the gene exchange within bacteria of the attached biofilm but also between the surrounding free-living bacteria (Arias-Andres M, 2018).

Metagenomic analysis allowed us to identify the whole community associated to the plastisphere characterizing both critical and non-critical bacteria, the latter playing a significant role in contributing the spread of resistance through MGEs.

All the data emerged from a study conducted in the Chienti River, which is a small river system compared to major Italian rivers, and less exposed to anthropogenic impact than wider reservoirs running through large metropolitan areas. Nevertheless, the presence of critical resistant bacteria detected within this small river highlights the extent of the threat, and the need of extending AMR surveillance to all the freshwater systems, regardless of their size or presumed pollution levels. For these reasons, there is a pressing need to develop monitoring systems that stick out beyond healthcare settings to encompass environmental contexts. In this thesis, a CRISPR/Cas12-based assay was developed to detect ARGs and the integrase gene, as a useful marker to evaluate the presence of genes coding for resistance determinants. This assay is the “core” of a promising and rapid tool for real-time ARG identification. In prototype testing, it proved to be versatile and adaptable for various laboratory setups and, with further optimization, holds the potential for on-site monitoring, thereby enhancing our ability to conduct efficient and timely AMR surveillance immediately after collecting water samples, at any river sampling site.

6 Conclusion

Few studies have explored antimicrobial resistance (AMR) within river ecosystems, and even fewer have focused on Italian environments. This study aims to address this knowledge gap by investigating the role of the plastisphere and river water in the spread of MDR bacteria. The high percentage of MDR bacteria associated to isolates of both river water and plastic biofilms, highlights the river system as environmental reservoir contributing to the spread and maybe the enhancement of AMR-related public health issues. As plastic pollution is projected to increase globally, the need to understand its impact within freshwater systems is urgent. This study provides a foundation for future research aimed at integrating basic research with environmental monitoring of both AMR and plastic pollution, through a One Health approach. In a future perspective, the next steps for research should entail an inventory of potential sources of contamination, devise a strategy to efficiently monitor the contamination sources of plastic and ARB, expand knowledge driving the mechanisms of horizontal gene transfer and integrate rapid and accessible tools for real-time surveillance. By expanding the detectable horizon beyond clinical areas, including the environmental resistome, we will be able to identify AMR hotspots and to track emerging resistance patterns, with the goal to mitigate the public health risks posed by AMR dissemination in natural ecosystems.

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8 Research activities

1) Conferences (Poster)

- ASM Microbe 2024 - Atlanta, USA.
- SIM 2024 - Pavia, Italy.
- IUMS 2024- Firenze, Italy

2) Co-supervisor (Master Thesis)

- Francesca Racciatti thesis titled “Isolation and characterization of cefotaxime-resistant *Enterobacteriaceae* from water and plastics in Chienti river”.

3) Participation in the SEA-Europe Bio-Tune Summer Course 2023 (Online) and in Bio-Tune final meeting 2024, Buenos Aires.

5) Early-Stage Researcher within

- Marie Skłodowska-Curie Actions (MSCA-RISE) Bio-Tune Project, at the Universidad Peruana de Ciencias Aplicadas (UPC), Lima, Peru.
- PhD program at the Technical University of Crete, Chania, Greece.
- PhD program at Biolab srl, Ascoli Piceno, Italy.

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