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XXXVII CYCLE

In the context of the PhD scholarship of the National Operational Programme for Research and Innovation 2014-2020 (CCI 2014IT16M2OP005), funded by REACT-EU, Action IV.5 " PhD programmes on green related topics"

Maria Clara La Rosa

PhD Thesis

Green strategies for Public Health: the application of waste-water epidemiology to monitor and prevent antimicrobial resistance and pandemics

Supervisor: *Prof. Antonella Agodi*

PhD Coordinator: *Prof. Carlo Vancheri*

Academic year *2023-2024*

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1. INTRODUCTION

1.1. Wastewater-Based Epidemiology (WBE)

Modern societal needs are reshaping perceptions of wastewater, driving paradigm shifts in wastewater research. This shift encourages viewing wastewater not as an unwanted byproduct but as a valuable resource [1-3]. These changes underscore the importance of innovative wastewater management approaches that address both current challenges and future demands. Municipal wastewater traditionally contains a variety of organic compounds, including pharmaceuticals, personal care products (PPCPs), and illicit drugs [4,5]. The presence of these substances and their metabolites in wastewater reflects human habits and lifestyle, providing valuable insights into chemical exposure and the prevalence of certain diseases [6,7]. The advanced analysis of wastewater for assessing population exposure and health status is known as wastewater-based epidemiology (WBE). This approach allows for the monitoring of public health trends and environmental impacts by analyzing the chemical and biological components found in wastewater. WBE serves as a comprehensive tool for evaluating exposure to infectious agents, as well as biological and chemical pollutants. By examining wastewater, which contains urine, feces, and other bodily substances, WBE delivers a real-time overview of community health and environmental conditions. At present, WBE is primarily focused on raw, untreated wastewater from centralized municipal sewer systems [8-10].Research can be conducted at multiple scales, including regions, cities, districts, and airports, or at specific locations like nursing homes, prisons, hospitals, university residences, and schools, as well as on boats and planes [11]. WBE has its roots in studies dating back to the poliomyelitis epidemic in Charleston in 1939, although the concept was first documented in the 1970s and has seen significant advancements over the past 15 years. Early in the 20th century, researchers discovered that the poliovirus could be recovered from various bodily fluids, including stool. However, it wasn't until 1939 that wastewater was first used for poliovirus surveillance. Despite over 99% of poliovirus infections being asymptomatic, John Paul and his colleagues [12-14] successfully detected the virus in wastewater. This discovery enabled indirect epidemic monitoring through sewage analysis, even during periods of low incidence. For more than six decades, this approach remained largely unexplored until the World Health Organization (WHO) formally adopted WBE for poliovirus monitoring in 2003 [15]. The WHO's WBE program was designed to identify regions of ongoing polio transmission and ultimately confirm the eradication of the disease.

Building on years of successful poliovirus containment through WBE, academia has significantly broadened the application of WBE techniques. Initially focused on tracking infectious diseases, WBE has evolved to encompass the monitoring of illicit drug use, pharmaceutical prescriptions, and various health biomarkers. This expansion now includes: (i) licit and illicit drugs, (ii) human viral infections such as polio and hepatitis A [16,17], and (iii) more recently, SARS-CoV-2 [18-22].

WBE also holds great promise for the rapid assessment of the impact of chemical contaminants and dietary nutrients on public health. Over the past decade, WBE studies have expanded their scope to monitor a wide range of biomarkers, including pharmaceuticals (such as antibiotics and benzodiazepines), pesticides, plasticizers, alcohol, tobacco, and indicators of oxidative stress (such as isoprostanes). This makes WBE particularly well-suited for studying the exposome, which encompasses all the exposures an individual encounters over their lifetime and how these exposures relate to health. WBE can be easily implemented to provide regular exposomic characterization at community, regional, national, and even global scales. One key advantage of WBE is that it allows for the simultaneous monitoring of exposure and health markers (such as endogenous metabolites) within the same wastewater matrix. This comprehensive approach enables a more integrated understanding of public health and environmental interactions [23,24]. Biomarkers can be categorized into two main types: biomarkers of exposure and biomarkers of effect. i. Biomarkers of Exposure: These are chemicals excreted by humans as a result of detoxification processes after exposure to substances like pesticides or illicit drugs. By understanding the excretion rates and measuring their concentrations in wastewater, researchers can estimate the population's exposure to these xenobiotics. ii.Biomarkers of Effect: These are molecules secreted by the body during the development of diseases. For instance, isoprostanes can indicate oxidative stress levels within a population, while other biomarkers may signal the prevalence of conditions like diabetes or cancer. Accurate quantification of these biomarkers is challenging due to their low concentrations in complex wastewater matrices. Extensive sample preparation is necessary to ensure precise measurements. Additionally, understanding the stability of these biomarkers in wastewater is crucial to avoid underestimation and obtain meaningful data. Therefore, while the concept of WBE is straightforward and offers significant advantages for public health monitoring, it presents several challenges. As mentioned, one key issue is the highly diluted nature of biomarkers in wastewater, especially when compared to more concentrated sources like urine. Additionally, the complex composition of the wastewater matrix adds another layer of difficulty. Wastewater contains a vast array of chemical and biological substances, providing

detailed insights into the contributing population. However, this complexity also complicates the extraction and analysis of specific targets, making it a challenging task to obtain accurate and reliable data [25]. Another challenge is the variability introduced by dynamic populations, such as those influenced by tourism or commuting patterns [26,27]. Variations in population size introduce uncertainties that can complicate infectious disease surveillance within wastewater. For instance, the presence of tourists or commuters in a catchment area can obscure the true origin of a detected pathogen, making it difficult to determine whether it originated from a local resident or a transient visitor. However, even if the pathogen's source is external, its detection in wastewater still offers critical insights. It suggests that members of the local population may have been exposed to the infection, potentially signaling the early stages of disease emergence within the community. This early warning allows for timely preparation and response, mitigating the impact of potential outbreaks. Despite these challenges, WBE has proven to be a valuable tool in various fields, adapting its focus to address different public health concerns. Over the past decades, WBE has increasingly focused on detecting illicit drugs to estimate consumption levels within populations [28,29]. By analyzing the geographic and temporal distribution of drug residues and correlating them with demographic factors such as age, economic status, and spatial distribution, researchers can gain a deeper understanding of drug use patterns. For example, some studies have shown a spike in the use of cocaine and 3,4-methylenedioxymethamphetamine (MDMA) for recreational purposes during weekends, while the use of methadone and 3,4-methylenedioxypyrovalerone (MDPV) showed no significant variation based on the day of the week [26,30]. Overall, WBE provides valuable insights into the health status of an entire population through the analysis of wastewater rather than individual blood or urine samples. Additionally, WBE is a non-invasive method that does not require personal consent, as only the approval of the authorities responsible for wastewater collection and treatment is needed. The resolution of WBE can vary based on the sampling area. For example, sampling at the entrance of a wastewater treatment plant yields data representing the entire city. In contrast, collecting samples from various points in the sewer network can isolate specific districts or neighborhoods, facilitating geographical and socio-demographic comparisons. These results can be correlated with population surveys or used to generate estimates in the absence of such data, such as in the case of illicit drug consumption [6,26,31]. More recently, the systematic surveillance of polioviruses in wastewater enabled British researchers to identify the early onset of an epidemic in London caused by a variant of the virus originating from the oral polio vaccine [32]. Advancements in environmental pathogen detection techniques, including gene amplification and sequencing, have broadened the scope

of WBE to encompass bacteria, parasites, and viruses that are well-known for being excreted in stool. This method has been particularly effective in studying the spread of antibiotic-resistant bacteria, particularly those originating from hospitals and farms [33,34]. However, until recently, the main goal of this research was to determine the effectiveness of sanitation practices and to evaluate the health risks posed by these pathogens in wastewater treatment plants (WWTP) and their potential effects on surface water, recreational waters, and shellfish that humans consume [11]. Surveillance of pathogens through WBE under the One Health framework is crucial for the prevention and early detection of future pandemics. The One Health approach operates on the premise that human, environmental, and animal health are inherently interconnected. By considering all three aspects, it is possible to develop solutions that not only address specific health problems but also mitigate their sources [35]. A prominent example of this interdependence is the global Antimicrobial Resistance (AMR) crisis, which illustrates the interconnectedness of humans, animals, and the environment. WBE has the potential to be a powerful tool in combating AMR, as it provides a comprehensive view of its dynamics across the human population at a lower cost compared to individual sampling. Moreover, WBE is particularly valuable in high-risk settings where there is a greater chance of pathogen spillover into humans before an outbreak occurs. Continuous surveillance of pathogens, especially zoonotic ones, in wastewater from livestock farms, wet markets, and surrounding areas is therefore of utmost importance. This approach can help identify emerging threats early, allowing for timely interventions to protect public health.

1.2. The Role of Wastewater Surveillance (WWS) in Pathogen Detection

Wastewater serves as a dynamic collection point where a wide array of physical, chemical, and biological substances from society converge. The presence and concentration of pathogens in sewage are determined by several factors, including the site of replication in the host (such as the gastrointestinal tract, upper respiratory tract, nose, skin, or internal organs), the duration and quantity of pathogen release, the initial concentration at the source, the prevalence of infected individuals, and environmental influences like water dilution (due to per capita water use, precipitation, or industrial discharge) and seasonal variations [36]. One of the most significant factors is the daily shedding of pathogens through bodily fluids, feces, skin, and urine. Since pathogens excreted in urine and feces are typically released multiple times throughout a 24-hour period, this results in a continuous influx of pathogens into the sewage system. Wastewater surveillance (WWS) is particularly effective for detecting pathogens of fecal origin, which can

cause gastrointestinal infections as well as a range of other infections, including urinary tract infections, pneumonia, skin infections, surgical wound infections, soft tissue infections, and septicemia. These pathogens often have a sufficiently high bacterial load in wastewater to be detectable [36]. For instance, bacteria from the Enterobacteriaceae family, such as E. coli and K. pneumoniae, are more frequently found in sewage compared to other bacteria like methicillin-resistant Staphylococcus aureus (MRSA) or opportunistic pathogens like P. aeruginosa and A. baumannii, which primarily shed from human skin. Enteric and respiratory pathogens are frequently detected in sewage due to their prolonged release during infection, the large quantities excreted, and the prevalence of asymptomatic cases. A key advantage of wastewater monitoring is that most pathogens of interest can remain viable in the sewer environment for several days. Enteric and respiratory agents are particularly stable, though there is limited data on viral encephalitis agents, as their transmission through water and other liquid media is uncommon. Moreover, with molecular detection methods, the pathogens do not need to be viable to be identified, extending the period during which they can be detected in wastewater. In some cases, simply detecting the presence of certain high-risk pathogens in the sewage system can be sufficient to warrant further investigation, regardless of whether the organisms are still viable [36]. However, when the pathogen load per infected individual is low, a large number of infected individuals may be required to detect the pathogen in the sewage system. Additionally, if an outbreak involves only a small number of patients, or even a single patient, detecting the resistant pathogen through WWS can be challenging. Moreover, comparing WWS data on antibiotic-resistant bacteria (ARB) to clinical evidence, as has been done with SARS-CoV-2, is often difficult. It requires comprehensive expert interpretation, including an analysis of ongoing events within the community and the potential public health implications.

2. INTRODUCTION TO HUMAN CORONAVIRUSES: FROM SARS TO SARS-COV-2

Human Coronaviruses (HCoVs) are a subset of Coronaviruses (CoVs) that cause a range of respiratory illnesses, from the common cold to more severe conditions like bronchiolitis and pneumonia [37]. Coronaviruses are prevalent infectious agents in both animals and humans. In humans, they can lead to a variety of illnesses, from mild conditions such as the common cold—caused by strains like HCoV-NL63, HCoV-229E, Beta-CoV1, and HCoV-HKU1—to severe diseases including SARS and MERS [38]. These viruses possess an unsegmented, single-stranded, positive-sense RNA genome, approximately 30,000 nucleotides in length. Under electron microscopy, coronaviruses appear rounded with spicule-like projections on their surface, creating a structure reminiscent of a solar corona, from which their name is derived [39]. Known for their rapid evolution, these viruses exhibit high rates of nucleotide substitution and recombination [40], allowing them to adapt swiftly and potentially increase their virulence or transmission efficiency. Since the start of the 21st century, HCoVs have periodically emerged in different regions, causing significant outbreaks of severe pneumonia[41]. The first major CoV outbreak, caused by severe acute respiratory syndrome coronavirus (SARS-CoV), began in November 2002 in Foshan, China [42], and escalated into a global epidemic in 2003 with a fatality rate of 10% [43]. A decade later, in June 2012, the Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in Jeddah, Saudi Arabia [42], resulting in a global fatality rate of 35% [44]. The most recent major HCoV outbreak occurred in December 2019 in Wuhan, China, caused by a novel strain closely related to SARS-CoV, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This outbreak rapidly escalated into the COVID-19 pandemic, the most significant global health crisis of the 21st century[45]. The unpredictable emergence, rapid spread, and severe impact of these HCoVs, particularly SARS-CoV-2, underscore the continuous threat they pose to global health and economies. The experience gained from dealing with SARS-CoV and MERS-CoV has been crucial in informing the global response to SARS-CoV-2, yet the unprecedented scale of the COVID-19 pandemic has highlighted the need for innovative monitoring tools, such as WBE, to better track and manage future outbreaks.

2.1. Structural, Pathogenic, and Immunological Insights into Pandemic Coronaviruses

HCoVs are enveloped viruses with a non-segmented, single-stranded, positive-sense RNA genome, primarily infecting vertebrates [46]. These viruses frequently require classification of newly emerged strains associated with severe or fatal human diseases into existing genera or new species [47]. Currently, the Coronaviridae family includes 39 species distributed across 27 subgenera, 5 genera, and 2 subfamilies, within the suborder Cornidovirineae, order Nidovirales, and realm Riboviria [48]. HCoVs belong to the subfamily Coronavirinae and are divided into four genera: AlphaCoV, BetaCoV, GammaCoV, and DeltaCoV, as classified by the International Committee on Taxonomy of Viruses [41]. Although the history of coronaviruses dates back to the 1940s [49], the first human coronaviruses were identified in the 1960s as causative agents of mild respiratory diseases, specifically HCoV-229E and HCoV-OC43 [50,51]. The SARS-CoV outbreak in 2002 spurred significant research into novel coronaviruses, leading to the identification of HCoV-NL63 in 2004 and HCoV-HKU1 in 2005 [52]. Other notable HCoVs include MERS-CoV, SARS-CoV, and SARS-CoV-2. The first four HCoVs—HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1—are widespread and contribute to about one-third of common colds in humans [53]. In severe cases, they can cause bronchiolitis and life-threatening pneumonia, particularly in immunocompromised individuals and children [37,54]. Additionally, these HCoVs have been associated with enteric and neurological diseases [55-58]. SARS-CoV, MERS-CoV, and SARS-CoV-2 are well-known pandemic HCoVs. Taxonomically, HCoV-229E and HCoV-NL63 are classified as AlphaCoVs, while HCoV-HKU1, HCoV-OC43, SARS-CoV, and MERS-CoV fall under BetaCoVs. These groups primarily infect mammals, whereas GammaCoVs and DeltaCoVs typically infect birds but can occasionally infect mammals [59]. After infecting humans, HCoVs are primarily transmitted through close person-to-person contact. Proximity to an infected individual within a 3-foot distance has been identified as a significant factor in the transmission of coronaviruses [60].

2.2. SARS-COV-2

In late 2019, a novel coronavirus (nCoV) emerged in Wuhan, China, prompting the WHO to issue a global health alert as the virus rapidly spread to other countries. Consequently, the WHO declared a Public Health Emergency of International Concern (PHEIC) on January 30, 2020, and later classified the outbreak as a pandemic on March 11, 2020 [61]. Genetic studies revealed that this new virus belongs to the *Coronaviridae* family, which includes the viruses responsible

for severe acute respiratory syndrome (SARS-CoV) and Middle East respiratory syndrome (MERS-CoV), both of which cause severe respiratory infections in humans and have led to significant epidemics in various countries [45,62]. In addition to symptoms typical of an upper respiratory infection, such as fever, cough, and sore throat, SARS-CoV-2 can occasionally lead to severe and life-threatening conditions like pneumonia, sepsis, and major organ failure. Over 6 million people, primarily older adults, have died due to complications from the virus. The pandemic has also resulted in enormous economic losses worldwide due to efforts to contain its spread [63]. Initially referred to as the “new coronavirus 2019” and the "2019 novel coronavirus (2019-nCoV)," the International Committee on Taxonomy of Viruses later named this virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses,2020). The disease caused by this virus was officially named coronavirus disease 2019 (COVID-19) [61]. The viral genome of SARS-CoV-2 contains a 5’ cap structure and a 3’ poly-A tail, allowing it to function as messenger RNA (mRNA). This mRNA is translated to produce non-structural proteins (nsps) essential for the replication and maturation of viral proteins. Additionally, the genomic RNA generates subgenomic mRNAs, which produce other viral proteins. These subgenomic mRNAs share a common 3’ end, leading to the term "nested," which is the origin of the order name Nidovirales, to which coronaviruses belong [64]**.** The viral genome comprises at least 13 open reading frames (ORFs). Processing of some polypeptides results in multiple mature proteins. The virus has four structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N), along with at least 16 non-structural proteins, known as nsp1 to nsp16 [65]. In particular, viral RNA is encapsulated by the nucleocapsid (N) protein and surrounded by an envelope, which is a lipid bilayer containing the S, M, and E proteins. Structural proteins form the viral particle, with the S protein being the most antigenic and prominent, contributing significantly to the virion's crown-like appearance. In SARS-CoV-2, the S protein recognizes and binds to the host cell receptor ACE2 (angiotensin-converting enzyme 2). The N protein protects the viral genome and is involved in RNA synthesis, the M protein provides structure and stability, and the E protein functions as an ion channel during virion assembly and release from the host cell [39,66]. The structural protein coding regions include other ORFs encoding accessory proteins, such as ORF3a, ORF6, ORF7a, ORF7b, ORF8, ORF9b, and potentially ORF14 [65]. These accessory proteins are not essential for the coronavirus replication cycle but interact with host proteins to influence antiviral responses [67]. Among the 16 non-structural proteins are helicase, triphosphatase, methyltransferase, and nuclease. Notably, nsp5 is one of the virus's two main proteases, known as Mpro (main protease) or 3CLpro (3C-like protease). This chymotrypsin-

like protease is crucial for the maturation of 12 viral proteins, acting on 11 cleavage sites in the viral genome translation products [39].

Since its initial identification, SARS-CoV-2 has continued to evolve, with variants such as Alpha, Beta, Gamma, Delta, and Omicron emerging and replacing the original Wuhan strain [68]. During the COVID-19 pandemic, our understanding of SARS-CoV-2 greatly advanced, thanks to extensive research into the virus's antigenicity, pathogenicity, and transmissibility [42]. This progress facilitated the development and implementation of effective COVID-19 vaccination strategies and robust public health measures in many countries. By November 26, 2023, nearly 13.6 billion doses of COVID-19 vaccines had been administered globally [68], saving tens of millions of lives and profoundly altering the course of the pandemic [69]. Vaccination efforts have significantly reduced the severity of symptoms following infection with various strains of SARS-CoV-2, including the original Wuhan-1 strain [70]. However, certain mutations, particularly those found in the Omicron variant, have been shown to diminish the neutralization efficacy of existing vaccines [70,71]. There remains a possibility that an undetected variant or sublineage could emerge with high transmissibility and novel antigenic properties [72].

2.2.1. SARS-CoV-2 evolution

The ancestral Wuhan-1 strain of SARS-CoV-2, first identified in 2019 [45], has continuously evolved, leading to the emergence of new variants that have driven multiple pandemic waves [68]. Significant variants to date include the Alpha lineage (primarily B.1.1.7), the Delta lineage (B.1.617.2 and all AY sublineages), and the most recent Omicron lineage (B.1.1.529 and all BA sublineages). The Alpha variant, characterized by a single spike protein substitution, demonstrated a roughly 20% [73]growth advantage over earlier variants [74]. Subsequent variants exhibited increased transmissibility. Starting in October 2020, new variants, particularly Omicron, were marked by a higher number of mutations in the spike (S) protein, affecting both transmissibility and antigenicity [72]. Since February 2022, the Omicron variant has predominated, accounting for over 98% of publicly available sequences [68].

The epidemiology of COVID-19 has evolved significantly since the start of the pandemic, with a notable decrease in COVID-19-related deaths and hospitalizations and high levels of population immunity to SARS-CoV-2 [75]. Each successive SARS-CoV-2 variant has shown a selective advantage over its predecessor [76]. While Omicron infections have generally resulted in milder disease compared to earlier variants [77,78], some studies report comparable

severity [79] . This variance suggests that immunity from previous infections and vaccinations may help mitigate disease severity [80]. Despite the continued emergence of Omicron sublineages that can evade vaccine-induced antibodies, widespread population immunity has contributed to reducing severe outcomes [81]. Mutations in the Omicron lineage have reduced the virus's replication in the lower respiratory tract, which partly explains the milder clinical severity seen with initial Omicron strains, even in regions with low vaccine coverage [10,73,81,82]. However, some later Omicron sublineages have shown increased fusogenicity, though this does not necessarily correlate with greater severity, underscoring the protective effect of prior immunity [81]. Predicting novel mutations remains challenging but crucial for anticipating changes in epitope loss and potential vaccination targets [83-85]. The global efforts in SARS-CoV-2 genome sequencing and modeling have been instrumental in identifying advantageous mutations and guiding laboratory research, thereby enhancing our understanding of the virus-host interactions [85,86]. These studies have identified numerous advantageous mutations in the SARS-CoV-2 spike protein, affecting transmissibility, infectivity, and associated mortality [71,86,87]. However, less is known about mutations outside the spike protein that affect viral replication and infectivity, highlighting the need for further research [72]. Recent research using a systems approach has illustrated how different variants have evolved convergently to enhance viral replication and transmission by modulating host and viral protein expression and interactions [88]. This convergent evolution has resulted in recurrent mutations across various lineages [89]. The predominant Omicron variant, known for its high transmissibility and immune evasion, has continued to evolve, with several new lineages emerging over time. One study observed that five recurrent substitutions in younger Omicron lineages increased viral fitness [90]. Importantly, mutations in the spike (S) protein have compromised the efficacy of existing vaccines, impacting vaccination strategies [71,81,89,91,92]. Understanding how SARS-CoV-2 evolves to bypass immune defenses is crucial for identifying future vaccination targets. Significant knowledge gaps remain, particularly concerning mutations that affect viral infectivity, replication, and structural proteins other than the S protein. Additionally, there is limited understanding of SARS-CoV-2 evolution in asymptomatic individuals. Continued research and modeling are vital for predicting future variants and mitigating potential COVID-19 waves. On May 5, 2023, the WHO Director-General declared that COVID-19 is now an established and ongoing health issue, no longer constituting a public health emergency of international concern [75]. Despite this progress, COVID-19 remains a global threat. The slowdown in national surveillance, reporting systems, and testing capabilities [93] suggests a misconception that the pandemic is over. However,

several uncertainties about the epidemiology of COVID-19 persist. These include unclear definitions of the end of the pandemic and the transition to endemicity, accurate rates of SARS-CoV-2 infections and transmissions, and the potential seasonality of COVID-19, which could lead to predictable, recurring patterns. Defining the endpoint of the COVID-19 pandemic remains contentious, with no single agreed-upon definition. Some epidemiologists suggest that the pandemic may end when herd immunity or endemicity is achieved. Endemicity is characterized by a non-zero level of incidence determined by factors such as waning immunity, variant transmission rates, vaccination coverage, seasonality, and human behavior. This endemic 'equilibrium' represents a balance between the effectiveness of mitigation measures and the level of disease incidence that societies will tolerate [94]. Additionally, the persistence and immunity levels of COVID-19 vary globally, influenced by regional COVID-19 policies and guidance [95]. The transition to endemicity may not be simultaneous across all regions, with the risk of pandemic reactivation in areas with high population density and limited healthcare access. Endemicity does not necessarily equate to lower severity, as seen with endemic malaria, which remains a significant health threat. The historical inconsistency in the severity of successive SARS-CoV-2 variants further complicates predictions about future variant severity [96]. While high population immunity to SARS-CoV-2 may indicate the end of the pandemic's acute phase, ongoing disease management measures are essential. Unbiased monitoring of SARS-CoV-2 transmission and infection rates should continue [68], as periodic 'wavelets' of milder infections may occur approximately every 3–4 months. The magnitude of these wavelets is expected to decrease over time as baseline immunity is established, with seasonal peaks likely during colder weather. Variant surveillance must be maintained to identify SARS-CoV-2 mutations and variants capable of evading vaccine-induced immunity, which could drive new waves [68]. Given the decline in clinical testing, wastewater-based genomic surveillance is a promising strategy for monitoring mutations and lineages, although it presents its own challenges [97]. Public online dashboards that compile the latest COVID-19 data and information can also be valuable tools [98].

2.3. SARS-COV-2 RNA monitoring in wastewater

SARS-CoV-2, the virus responsible for the global COVID-19 pandemic [99], has underscored the critical need for innovative and robust surveillance methods. The scale of the crisis has swiftly emphasized the importance of tracking the prevalence of SARS-CoV-2 in communities and developing dynamic models for monitoring infections [11]. The complexity of the problem

is exacerbated by the fact that SARS-CoV-2 symptoms are often nonspecific, and the infection can frequently be asymptomatic, especially among younger individuals. Initially, epidemic monitoring depended on hospital indicators (such as the number of hospitalizations, emergency room visits, and deaths), followed by individual testing campaigns, which only the wealthiest countries could afford to conduct on a large scale. Both approaches have significant biases, primarily due to the size of the asymptomatic population capable of transmitting the virus and the selective targeting of monitoring campaigns [11].

Groundbreaking research conducted by G. Medema in the Netherlands [10,100] and the OBEPINE consortium in France (Observatoire Epidémiologique dans les eaux usées) [101] has shown that measuring the viral genome in WWTP serves as a valuable early warning and dynamic monitoring system for epidemics. WBE has emerged as an invaluable tool for proactive and scalable monitoring of viral presence within populations. Additionally, wastewater surveillance has played a crucial role in identifying the dominant variant(s) within a community. Metagenomic analysis of sewage is an economically viable approach to global surveillance, and the COVID-19 pandemic has highlighted its importance in public health response efforts [102]. Several countries have successfully integrated WBE into their surveillance systems to complement clinical testing. Hundreds of research groups and governments globally established local, regional, and national COVID-19 surveillance programs. These programs generated data on SARS-CoV-2 abundance and prevalence in a manner that was more cost-effective, faster, less biased, and more detailed than traditional epidemiological surveillance methods. This method aids in early detection and prevention of outbreaks and enables timely public health interventions, such as targeted testing, contact tracing, and resource allocation [103-105]. Multiple studies have underscored the importance of environmental surveillance as an early warning system, detecting viral levels in populations and identifying outbreaks before they are reported to healthcare systems[106,107]. Wastewater surveillance serves as an early detection mechanism for identifying novel coronavirus disease (COVID-19) due to the presence of SARS-CoV-2 RNA in wastewater [20]. This approach is notably more cost-effective compared to individual testing campaigns. The expectation of detecting signals in wastewater monitoring is enhanced by its structured sampling method, which mimics the characteristics of a random survey. This approach helps to reduce biases and difficulties linked to gathering single samples [11]. While SARS-CoV-2 primarily causes respiratory infections, it's noteworthy that human coronaviruses can also reproduce in the digestive system. Consequently, infected individuals, whether symptomatic or asymptomatic, can shed the virus into wastewater [108]. Wastewater acts as a collective reservoir of genetic

material shed by an entire community, offering insights into the prevalence of SARS-CoV-2, including asymptomatic and presymptomatic cases [103,104,109]. Advances in molecular techniques, particularly quantitative reverse transcription-polymerase chain reaction (qRT-PCR), have been crucial in accurately quantifying viral RNA in wastewater [103,105]. Including asymptomatic infected individuals is crucial because research indicates that the viral loads of SARS-CoV-2 in asymptomatic individuals are often similar to those in symptomatic patients [110,111]. For SARS-CoV-2, it is estimated that 18–32% of infections are asymptomatic, a proportion comparable to that observed with norovirus [112]. Furthermore, it is estimated that 48–67% of infected individuals excrete SARS-CoV-2 in their feces [113-115]. Importantly, the extent of fecal RNA excretion is not correlated with the severity of COVID-19 [116]. Moreover, SARS-CoV-2 RNA has been detected in stool samples despite negative laboratory results from nasopharyngeal swabs [117,118], and it persists in patient stool for a longer duration (9–16 days after symptom onset compared to 6–11 days for nasopharyngeal swabs). Consequently, monitoring defecation may be beneficial in cases where nasopharyngeal swabs yield negative results [119]. Numerous studies have reported the detection of SARS-CoV-2 RNA in wastewater samples from several cities before the first COVID-19 cases were officially reported by public health authorities. Recent evidence from several high-income countries, including Australia, Germany, France, Israel, Italy, the Netherlands, Spain, and the USA, indicates that SARS-CoV-2 RNA detected in untreated municipal wastewater predicted COVID-19 outbreaks before the first officially confirmed cases [9,10,18-20,100,101]. For example, La Rosa et al. (2020) identified SARS-CoV-2 RNA in wastewater samples from Milan and Turin in December 2019 and Bologna in January 2020, despite Italy recording its first COVID-19 case in February 2020 [120]. These findings indicate that WBE offers a symptom-independent approach for monitoring SARS-CoV-2 transmission trends within communities over extended periods compared to individual laboratory tests [81]. Additionally, SARS-CoV-2 RNA exhibits high stability in untreated wastewater at temperatures between 4 and 37 °C, ensuring reliable detection post-sample collection and processing [121]. In Italy, one of the countries hardest hit by the pandemic, urban sewage monitoring began in July 2020 as part of the “SARI project” (Epidemiological Surveillance for SARS-CoV-2 in Urban Sewage in Italy). This pilot study, coordinated by the National Institute for Public Health, involves a network of regional wastewater service providers, environmental protection agencies, and local health authorities [122]. On March 17, 2021, the European Commission recommended that EU member states begin monitoring SARS-CoV-2 in wastewater by October 1, 2021 [123]. Consequently, since October 2021, Italy has transformed the SARI project into a full-fledged

surveillance system. This system focuses on analyzing SARS-CoV-2 trends in urban wastewater to predict epidemiological trends and studying the spread of SARS-CoV-2 variants over time [122]. Access to real-time data on COVID-19 cases and SARS-CoV-2 RNA levels in wastewater can significantly inform personal decisions about managing potential exposures, such as whether to wear a mask or avoid crowded areas. As we transition to a post-pandemic era where COVID-19 is treated as an endemic disease, wastewater surveillance is poised to remain future-ready by integrating monitoring for additional public health priorities, emerging SARS-CoV-2 variants, and new or unknown pathogens under a 'disease X' framework as highlighted by the WHO. To this end, efforts are underway to adopt broader approaches for detecting untargeted agents, such as next-generation sequencing. These initiatives include evaluating protocols for detecting RNA shed during arboviral infections (e.g., Dengue, Zika, and Yellow Fever), demonstrating the proof of concept for applying wastewater surveillance to track these diseases [124].

3. WBE: A Tool for Monitoring and Combating Antimicrobial Resistance

3.1. Antimicrobial Resistance

Antimicrobial Resistance (AMR) represents a major global Public Health threat and is one of the most urgent challenges facing modern medicine. According to the U.S. Centers for Disease Control and Prevention (CDC), ARB now account for over 2.8 million infections and 35,000 deaths annually in the United States. This marks a significant increase from the 2 million infections and 23,000 deaths reported in 2013 [125,126]. In the European Union alone, AMR is responsible for approximately 670,000 infections annually, leading to nearly 33,000 deaths. The growing prevalence of AMR undermines the effectiveness of life-saving treatments, making it a critical issue that demands immediate and coordinated action worldwide [127]. Since their introduction into medical practice in the 1940s, antibiotics have been essential in treating bacterial infections, preventing infections in surgical patients, and protecting immune-compromised individuals and cancer patients. Moreover, antibiotics play a crucial role and are extensively used not only in human medicine but also in livestock to promote growth and prevent diseases [128]. However, the rise of antibiotic resistance is undermining the effectiveness of these treatments, leading to increased risks of infectious disease outbreaks and higher mortality rates [129]. The growing threat of ARB endangers the future effectiveness of antibiotics, potentially transforming once-manageable diseases and routine medical procedures into significantly more dangerous challenges [130,131]. Infections caused by ARB are already associated with higher mortality rates, prolonged hospital stays, and escalating healthcare costs [132]. Globally, AMR contributes to longer durations of illness, increased death rates, and a surge in healthcare expenses due to ineffective antibiotic treatments. Patients with AMR infections face significantly higher treatment costs compared to those with drug-susceptible infections [133,134]. The economic burden of AMR is further compounded by the inability to safely perform surgeries or administer therapies such as chemotherapy without effective antibiotics [135]. Particularly concerning is the global spread of multidrug-resistant and pan-resistant bacteria, or "superbugs," which cause infections that are untreatable with existing antibiotics. This situation is exacerbated by the lack of access to novel and effective antibiotics in many low- and middle-income countries, where people often rely on over-the-counter antibiotics without prescriptions. Newer antibiotics, when available, tend to be more expensive and out of reach for many in these regions [136]. While the full social and economic impact of AMR is still unfolding, projections indicate that by 2050, drug-resistant infections could become the leading cause of death worldwide [137]. This looming crisis underscores the urgent need for coordinated global efforts to curb the spread of AMR and to develop innovative

treatments. The spread of ARB is driven by several factors, including the overprescription of antibiotics, inadequate disposal practices, the use of antibiotics in agriculture and livestock, and the slowdown in the development of new antibiotics [138]. Antimicrobial use exerts ecological pressure on bacteria, promoting the development of resistance, while shortcomings in infection prevention and control (IPC) practices further facilitate their spread. These bacteria can acquire multiple resistance mechanisms, severely restricting treatment options and accelerating the spread of multidrug-resistant (MDR) organisms in the environment. To combat this, the responsible use of antimicrobials is crucial, along with strengthening IPC practices, particularly in healthcare settings, to reduce transmission risks [139]. A coordinated, global effort is essential to address the growing threat of AMR and mitigate its impact. Comprehensive strategies are needed to manage AMR effectively. Key interventions include monitoring antibiotic usage, detecting resistance in both humans and animals, raising public awareness about the proper use of antibiotics, and implementing stewardship programs within healthcare facilities. Collaboration among healthcare providers, pharmacists, and patients is vital to achieve significant reductions in AMR [140,141]. It is important to note that the extensive use of antimicrobials in both human and veterinary medicine has not only facilitated the spread of ARB between humans and animals but has also led to environmental contamination, affecting surface water, soil, and ecosystems [142-145] . Wastewater treatment plants (WWTPs) are major sources of antibiotic resistance genes (ARGs) from human activities, while manure applications contribute to their spread from animal sources. Although natural selection can lead to low-level antibiotic resistance, the high prevalence of ARB and ARGs in the environment is predominantly driven by human activities, with ARGs entering ecosystems from various human and animal sources [146,147]. The term "genetic reactor" refers to locations where genetic evolution happens frequently, potentially leading to the development of antibiotic resistance. There are four main areas where this genetic evolution and antibiotic resistance commonly occur: 1. Human and animal microbiota, 2. Hospitals and long-term care facilities, 3. Wastewater and various types of biological waste, and 4. Soil, as well as surface and groundwater environments [145]. Therefore, numerous national and international reports emphasize the urgent need for a comprehensive approach to combat antibiotic resistance. This includes increased investment in research to better understand how environmental sources and pathways contribute to the evolution and spread of resistance to human pathogens [148-150]. Both the World Health Organization (WHO) and the EU's Global Action Plans advocate for a One Health approach—integrating human, animal, and environmental health—to address antibiotic resistance. These plans highlight critical connections with water sanitation and stress

the importance of establishing surveillance programs to monitor these linkages [148,150,151]. Similarly, the updated U.S. National Action Plan acknowledges the necessity of environmental monitoring [152]. To support this effort, the CDC recently launched a multi-year initiative to fund research on antibiotic resistance, focusing on sources related to water and wastewater [126].

3.1.1. Spread of Antimicrobial resistance

While AMR is a natural phenomenon, its rapid development and spread are largely driven by human activities, including the misuse and overuse of antimicrobials in humans, animals, and agriculture [153]. AMR is a complex, multifaceted issue that affects not only human health but also animal health and the environment [154]. Studies utilizing whole genome sequencing have confirmed the transfer of resistance genes from animals to humans, underscoring the need for coordinated international efforts to combat AMR across sectors such as healthcare, agriculture, and environmental management [155]. A key factor driving AMR is the exposure of bacteria to subinhibitory concentrations of antimicrobials. Under this selective pressure, microorganisms enhance their survival by acquiring and expressing resistance genes, which can then be transferred to other bacteria. These resistant bacteria, along with their genes, can easily move between humans, animals, and the environment, further complicating efforts to control AMR [154]. Numerous pathways facilitate the spread of AMR microorganisms and their genes across different ecosystems, including hospital effluent, wastewater treatment facilities, and agricultural waste [156]. These routes enable the dissemination of AMR into water, soil, and surrounding ecosystems. Given that AMR genes can be transferred both vertically and horizontally, various sources such as hospitals, community environments, animals, farms, and aquaculture settings serve as significant reservoirs of resistance and residual antimicrobials [154,156]. Research into the spread of AMR has identified several key global drivers: misuse and overuse of antimicrobials in humans and animals, environmental contamination (including sewage and heavy metals), healthcare-associated transmission, inadequate rapid diagnostics, insufficient preventative medicine and vaccination, improper dosing (including from substandard and falsified medications), travel, and mass drug administration in human health [157]. Climate change (CC) has also been identified as a potential indirect contributor to AMR [158]. CC can alter ecosystems and environmental conditions, facilitating the spread of resistant bacteria. The link between global warming and infectious pathogens has been observed for many years [159], as demonstrated by the zoonotic microorganisms that have crossed into the

human population [160]. For example, bacteria such as *Campylobacter*, *Salmonella*, and *Vibrio cholerae* have adapted to warmer temperatures, leading to the re-emergence of infectious diseases as water systems heat up [161,162]. Rising temperatures are intimately linked to AMR, as they accelerate bacterial growth rates [163] and promote Horizontal Gene Transfer (HGT) [164], both of which contribute to the spread of resistance. The climate crisis may also drive the emergence and re-emergence of pathogens like *Candida auris* [165,166] and *Plasmodium falciparum* [167], which may harbor new resistance mechanisms, increasing hospital admissions and straining healthcare systems. Furthermore, higher temperatures are associated with extreme weather events such as flooding, population displacement, and overcrowding caused by storms and heavy precipitation. These severe weather conditions can damage wastewater and sewage systems, increasing the risk of floodwater contamination [168] . These factors increase the risk of waterborne infections [169,170], putting additional pressure on healthcare systems and leading to the collapse of sanitation infrastructures [171]. For instance, outbreaks of cholera in Haiti [172]and Nepal [173] following earthquakes are well-documented examples. Extreme weather events can also trigger droughts, which, combined with food shortages and weakened healthcare systems, heighten the risk of contracting antibiotic-resistant enteric pathogens [174]. Diarrheal diseases like *Campylobacter*, *Salmonella*, and cholera thrive in warmer temperatures, explaining their recent resurgence in many regions [161,162]. Given that the factors contributing to AMR span humans, animals, plants, food, and the environment, a comprehensive One Health approach is essential. This approach unites all stakeholders—healthcare providers, veterinarians, agricultural professionals, environmental scientists, and policymakers—around a shared vision and common goals to effectively combat AMR [153]. The US National Action Plan to Control Antibiotic Resistance [175] underscores the importance of gaining a deeper understanding of how ARBs and ARGs spread among humans, plants, and animals via the environment. A significant challenge lies in the fact that ARGs can be exchanged between bacteria through mobile genetic elements (MGEs), necessitating an examination of their movement alongside ARBs and ARGs in environmental settings. These ARGs build up in the environment and can be transferred to clinical pathogens via HGT, potentially leading to the failure of future antibiotic treatments [176]. HGT is a critical mechanism for the distribution of antibiotic resistance in the environment. This transfer can occur from donor bacteria, phages, free DNA, or even from dead cells to living cells [177,178]. There are four main mechanisms of HGT:

A. Conjugation: DNA is transferred from the donor cell to the recipient cell via a sexual pilus, requiring cell-to-cell contact. The recipient cell, previously susceptible, becomes resistant through the newly acquired resistance genes.

B. Transformation: This involves the uptake, integration, and functional expression of naked DNA by naturally transformable bacteria.

C. Transduction: DNA is transferred from one bacterium to another through bacteriophages.

D. Gene Transfer Agents (GTAs): These are bacteriophage-like elements produced by several bacteria. GTAs carry random DNA segments from the host bacterium, which can be transduced to a recipient cell. GTA particles can be released through cell lysis and spread to a recipient cell [179].

Notably, genes encoding resistance to different antibiotics are often located in the same regions of chromosomes or MGEs, resulting in multiple resistances[180]. Therefore, MGEs like plasmids, transposons, and integrons are crucial in the emergence and dissemination of ARGs [181]. For example, significant amounts of specific ARGs, such as the class 1 integron gene (intI1), can promote subsequent HGT in WWTPs, leading to the spread and occurrence of ARGs and multi-resistant microorganisms [182]. Transposases, primarily part of a family of insertion sequences, are typically found flanking clusters of resistance genes. Integrons carry resistance cassettes that encode various ARGs, including those for aminoglycoside and sulfonamide resistance, as well as qacEΔ1 efflux pump genes [181]. These multi-gene cassettes can express different ARGs under a shared promoter, facilitating the co-selection of ARGs. Consequently, the selection pressure from one antibiotic can select for ARGs associated with various antibiotics within the integron's gene cassette [183-185]. An example of this is MRSA, which acquires a gene cassette that transfers multiple ARGs simultaneously [178]. Another example is the mechanism of trimethoprim resistance, which involves replacing a trimethoprim-sensitive dihydrofolate reductase with a plasmid-, transposon-, or cassette-borne trimethoprim-resistant version [144]. Multidrug-resistant bacteria (MRB) are often released into sewage systems and end up in WWTPs, making these facilities hotspots for ARB and ARGs. Despite treatment, some pathogens like methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant enterococci (VRE) [186,187] can persist and potentially spread to other bacteria through mechanisms like HGT [188]. Studies have shown that treated wastewater used for irrigation can support the survival of harmful bacteria, including coliforms and various pathogens like Clostridium and Salmonella, which can contaminate crops [189,190]. The WHO

has highlighted the critical threat posed by the ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species), which are known for their significant resistance to multiple antibiotics, including those used as last-resort treatments such as carbapenems and glycopeptides [191-195]. Furthermore, the widespread use of antibiotics in agriculture and the limitations of WWTPs lead to the release of various resistant bacteria, such as those resistant to tetracyclines, sulfonamides, and vancomycin, into natural water bodies. This contamination can cause the accumulation of ARGs in crops irrigated with this water, posing a risk to human health [196].

3.1.2. Overview of Antibiotic Resistance Mechanisms and Major Resistance Genes (ARGs)

ARGs are genetic elements, or DNA sequences, carried by ARB that enable them to survive and thrive in the presence of antibiotics [197]. A key concern with ARGs is their ability to spread between microbial populations through HGT, facilitating the evolution of new resistant strains. This characteristic makes ARGs distinct as contaminants of emerging concern [144,198]. ARGs can proliferate under the selective pressure of antibiotics and other antimicrobials in the environment, highlighting potential "hot spots" where targeted mitigation efforts could be most effective [199] . Recognized increasingly as significant contaminants of emerging concern [200], ARGs have become the focus of extensive research, particularly in WWTP effluents and their downstream environments [146,147,201,202]. The studies, which predominantly employ molecular biology techniques, provide valuable insights into the occurrence and distribution of ARGs [203]. The ARGs *sul1* and *tetA* are frequently detected in aquatic environments, often showing increased prevalence in response to anthropogenic activities and environmental stressors [200,204]. Similarly, the *intI1* gene, which encodes the class 1 integron-integrase, serves as a strong indicator of human-related contamination [205] . This gene not only reflects anthropogenic inputs but also provides a broader metric for multi-antibiotic resistance, as it can carry cassettes harboring multiple ARGs and facilitate their mobility [206]. The CDC recently highlighted five clinically significant ARGs frequently found in pathogenic ARB that pose serious public health risks. These genes encode extended spectrum beta-lactamases (ESBLs) and include blaCTX-M, blaKPC, blaOXA-48, blaNDM, and blaVIM [126]. In wastewater treatment plants, a variety of bla genes are frequently detected, including blaCTX-M, blaSHV, blaTEM, blaOXA-48, blaNDM-1, and blaVIM-2, as well as the ermB

gene, a commonly acquired resistance gene [207,208]. Clinically significant ARGs, such as *blaCTX-M* and *vanA*, are particularly monitored due to their association with resistance to last-resort antibiotics. β-Lactams are the most extensively used class of antibiotics, vital in both human and veterinary medicine[209]. This broad class includes penicillins, cephalosporins, cephamycins, monobactams, and carbapenems, all characterized by the presence of a beta-lactam ring essential for their antibacterial activity. Penicillins and cephalosporins, in particular, are among the most commonly prescribed antibiotics. These antibiotics are bactericidal, interrupting bacterial cell-wall synthesis by covalently binding to penicillin-binding proteins (PBPs). PBPs are enzymes critical for the final stages of peptidoglycan cross-linking in the cell walls of both Gram-negative and Gram-positive bacteria. The chemical diversity among β-lactams is due to various side groups attached to the beta-lactam ring, enabling them to target different bacterial species and combat specific resistance mechanisms. Resistance to β-lactams has become a significant clinical challenge, primarily driven by the production of β-lactamase enzymes by bacteria. These enzymes hydrolyze the beta-lactam ring, rendering the antibiotic ineffective. Resistance mechanisms include altered bacterial outer membrane permeability, modification of PBPs, and most commonly in Gram-negative bacteria, inactivation by beta-lactamases [210]. The genes responsible for these enzymes, such as amp, bla, and oxa, are located on various genetic elements. For instance, ampC is typically found on the chromosome, while ampR, blaTEM, blaFOX, blaCTX-M, oxa-2, and blaSHV are frequently plasmid-borne, facilitating the rapid spread of resistance. To combat this growing resistance, new broad-spectrum β-lactamase inhibitors have been developed. These inhibitors are designed to counteract a wide range of β-lactamases, including cephalosporinases and serine-based carbapenemases, which have significantly limited therapeutic options. This ongoing development is crucial to preserving the efficacy of β-lactam antibiotics in the face of evolving bacterial resistance [209,211]. The emergence and global spread of extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae from animals has become a significant public health concern [212]. ESBL-producing strains synthesize beta-lactamase enzymes that confer resistance to many beta-lactam antibiotics, particularly cephalosporins, posing a serious threat to antibiotic efficacy [213]. While clinical pathogens are a well-known source of these resistant bacteria, ESBL producers can also colonize the gut microbiota of humans and animals, serving as a reservoir for resistance. Additionally, other environmental sources, including aquatic environments serve as critical mixing points where pathogenic bacteria from diverse sources—such as humans, livestock, industry, and soil—can converge [214]. This convergence facilitates the exchange of genetic material, including plasmids, transposons, and integrons, which harbor

ARGs [215]. This gene sharing can transform non-pathogenic bacteria into reservoirs of resistance, thereby increasing the prevalence of resistant organisms in natural bacterial ecosystems. The most common ESBL-encoding genes include blaCTX-M, blaTEM, blaSHV, and blaOXA [216]. The TEM and SHV families of ESBL enzymes are mutant forms of earlier beta-lactamases, while the CTX-M family originated from environmental bacteria. Unlike TEM- and SHV-derived ESBLs, the epidemiology of CTX-M-producing organisms is markedly different. CTX-M enzymes are not confined to nosocomial infections caused by Klebsiella spp.; instead, they are increasingly found in a variety of settings, including the community and various environmental sources. This broad distribution significantly amplifies public health concerns, as CTX-M-producing bacteria have the potential to spread rapidly beyond hospital environments, complicating efforts to control antibiotic resistance. SHV enzymes, named after their "sulfhydryl variable" active site, are most commonly associated with *Klebsiella pneumoniae*. Initially, strains of this bacterium typically carried a single ESBL gene, but over time, multiple ESBL genes have become common within a single strain, making detection and treatment more challenging. The TEM-type ESBLs are derived from the original TEM-1 and TEM-2 enzymes. A notable variant, TEM-3, has emerged, differing from TEM-2 by just two amino acid substitutions. The TEM enzyme family was first identified in *Escherichia coli* in Greece, but it rapidly spread to other members of the Enterobacteriaceae family, including *Citrobacter freundii* and *Acinetobacter* species. This widespread dissemination has significantly contributed to the growing problem of antibiotic resistance. Instead, the name "CTX" stands for cefotaximase, highlighting the enzyme's potent ability to hydrolyze cefotaxime, a third-generation cephalosporin [217]. Numerous variants of the blaCTX-M gene have emerged due to point mutations, contributing to the diversity of ESBL enzymes. The CTX-M family of β-lactamases is highly diverse, comprising over 50 distinct types, categorized based on their amino acid sequences into five major groups: CTX-M1(includes CTX-M-1, -3, -10, -12, -15, -28, -30, and FEC-1), CTX-M2 (includes CTX-M-2, -4, -5, -6, -7, -20, and Toho-1), CTX-M8, CTX-M9 (includes CTX-M-9, -13, -14, -16, -17, -19, -21, -24, -27, and Toho-2), and CTX-M25 (includes CTX-M-25 and -26). ESBL-producing bacteria can secrete a range of enzymes, including CTX-M, TEM, and SHV, with more than 450 variants identified to date [218]. Until 2000, the SHV and TEM variants were the most prevalent ESBLs. However, over the last few decades, CTX-M enzymes have become the dominant type, reflecting the dynamic and evolving nature of antibiotic resistance [219]. Instead, carbapenem resistance is primarily driven by the production of carbapenemases, enzymes that belong to three of the four β-lactamase classes: A, B, and D. Over time, numerous new variants of carbapenemases have

emerged across different classes, including Class A (e.g., KPC and GES enzymes), Class B metallo- β -lactamases (e.g., IMP, VIM, and NDM), and Class D (e.g., OXA-23) [220-224]. Numerous NDM-1 variants have emerged in Enterobacteriaceae, Vibrionaceae, and various non-fermenting bacteria due to single and double amino acid substitutions at different positions [225]. These include NDM-1 (the primary variant), along with NDM-2, NDM-3, NDM-4, and NDM-5 (minor variants), which have been reported globally [226-228]. Class D carbapenemases, known as OXA-β-lactamases, are further subdivided into several subgroups, including blaOXA-23, blaOXA-24/40, blaOXA-58, blaOXA-48, blaOXA-51, and blaOXA-143[220,223]. These OXA-type β-lactamases are particularly widespread in Acinetobacter species, with blaOXA-51 being the most prevalent. This enzyme is chromosomally encoded, making it intrinsic to these bacteria, but it can confer carbapenem resistance when its expression is upregulated through genetic reorganization [220,223,229]. Class B carbapenemases, also known as metallo-β-lactamases (MBLs), are typically encoded by integron-borne mobile gene cassettes, allowing them to be transferred between different bacteria through HGT, particularly conjugation. MBLs are particularly concerning as they can hydrolyze nearly all clinically available β-lactam antibiotics. These enzymes are characterized by the distinctive αβ/βα sandwich fold of the metallo-hydrolase/oxidoreductase superfamily. The blaNDM-1 gene encodes NDM, an enzyme capable of hydrolyzing a broad spectrum of antibiotics, including carbapenems, which are often considered the last line of defense against multi-resistant bacterial infections. The rise of resistance markers like New Delhi metallo-beta-lactamase (NDM-1) has severely compromised the effectiveness of the entire beta-lactam antibiotic group, rendering infections caused by NDM-1-producing microorganisms extremely difficult to treat [222,230]. New Delhi metallo-beta-lactamase (NDM) enzymes, first identified in bacterial isolates from the Indian subcontinent, represent the latest class of carbapenemases. These enzymes can hydrolyze all beta-lactam antibiotics, including broad-spectrum carbapenems, except aztreonam, leading to severe challenges in both hospitals and the community [231]. The gene encoding NDM-1 is frequently plasmid-borne, facilitating its transfer to other microorganisms via horizontal gene transfer. This greatly increases the risk of the emergence and spread of drug-resistant pathogenic strains [232]. Finally, Class A carbapenemases include the Klebsiella pneumoniae carbapenemase (KPC) family, which can be either plasmid-encoded or chromosomally encoded, further contributing to the spread of carbapenem resistance [221,224]. Bacterial resistance to antimicrobial agents often stems from a reduction in the antibiotic's affinity for its target. This can occur through enzymatic drug detoxification, target modification, or decreased target access due to active efflux or reduced uptake of the antibiotic.

Macrolides, a class of antibiotics extensively used in pharmaceutical treatments as well as in the livestock and poultry industries, are known for their environmental persistence. Their therapeutic effects are achieved by inhibiting bacterial protein synthesis, specifically by narrowing the nascent protein exit tunnel (NPET) in ribosomes [233-235]. However, these antibiotics can also induce the expression of resistance genes [236,237]. For example, macrolides cause ribosome stalling on regulatory leader peptides ermCL or ermBL, which subsequently activates the expression of ermC or ermB genes [237]. These genes encode ribosomal methylases that dimethylate a specific adenine in 23S rRNA, markedly reducing macrolide affinity for the ribosome. Erythromycin resistance often results from ribosomal target modification via methylation or mutation, alongside active drug efflux. The earliest and most enduring mechanism of erythromycin resistance involves ribosomal methylation, driven by the acquisition of the *erm* (erythromycin ribosome methylase) gene, usually located on transposable elements in pneumococci. This gene encodes a ribosomal methylase that dimethylates adenine 2058 in 23S rRNA, a critical site for erythromycin binding. This modification significantly reduces erythromycin's binding affinity, likely by blocking direct access to the binding site or altering its conformation, leading to high levels of macrolide resistance and enhanced bacterial survival [238].

3.2. Antimicrobial Resistance in the Environment

Over the past two decades, bacterial resistance has surged dramatically, leading to the widespread development and dissemination of ARB globally [211,239]. This emergence is driven by the increased presence of antibiotics in the environment, largely due to their improper use during human activities [240,241]. In fact, anthropogenic activities continually release substantial amounts of antibiotics into sewage systems, which then disperse through various aquatic and terrestrial environments via streams, rivers, and lakes [242-244]. These substances persist in environmental compartments for extended periods. Antibiotics not only act as chemical pollutants with potential toxic effects in the environment but also exert selective pressure. This process entails the elimination of susceptible microorganisms and the survival of resistant cells with attributes enabling them to withstand the adverse effects of antibiotics [245]. Furthermore, the environment is increasingly recognized not only as a pathway for the spread of antibiotic resistance but also as a reservoir for the acquisition of new ARGs by pathogens [204,246-248]. Current knowledge suggests that most ARGs found in pathogens originally stem from the extensive and diverse gene pools of environmental bacteria [249]. Therefore, pollution

serves as a direct source of ARBs, ARGs and MGEs in the environment, with contamination also introducing antimicrobials that induce selective pressure, thereby perpetuating and amplifying ARBs and ARGs while fostering the emergence of novel forms of resistance. One of the primary objectives outlined by the WHO and JPIAMR (Joint Program Initiative on Antimicrobial Resistance) in the fight against AMR is to ascertain the environmental role in the selection and prevalence of AMR [250]. In addition, MGEs pose a particular concern as they harbor multiple ARGs and may contribute to the development of resistance in pathogens. Given that the emergence of ARBs is recognized to diminish the effectiveness of antibiotic treatment [251], the identification of hotspots and reservoirs of resistant bacteria is crucial to effectively address this challenge. Traditionally, antibiotic resistance has been viewed as a concern confined to clinical environments, where exposure of natural bacterial communities to low doses of antibiotics was believed to have negligible effects on ecosystem ecology. These imprudent practices in antibiotic use and misuse contribute to the widespread presence of antibiotic residues, their metabolites, numerous ARB, and their genetic elements in human and animal waste, landfills and their effluents, water bodies, soil and sediments, as well as water-dependent food crops such as seafood and produce. The hospitals and other healthcare facilities are major sources of antimicrobial waste, either indirectly through patient secretions or directly as discarded medications. Therefore, investigations into the prevalence of ARGs within environmental bacterial communities are imperative to comprehend the true impact of human activities on the dissemination of resistant bacteria [252].

3.2.1. The aquatic environment and AMR

The WHO has identified ARB and ARGs as two major public health threats in the twenty-first century, highlighting the growing concern about the spread of ARGs in aquatic ecosystems [253]. AMR arises from ARBs and is mediated by ARGs, with urban water cycles serving as pathways for their dissemination through wastewater discharges, agricultural applications, and runoff from farmlands [254]. Therefore, aquatic ecosystems are prime locations for the occurrence and spread of ARGs because they are continuously contaminated by antimicrobial compounds from human activities [253].

AMR poses a profound societal threat, with the aquatic environment being a key factor in its development and spread. This environment is instrumental in various stages of AMR dynamics, including:

- Serving as a reservoir for ARB originating from animals and humans treated with antibiotics (i.e., anthropogenic resistance). Aquatic ecosystems, such as rivers, lakes, and oceans, often receive effluents containing antibiotics from sources like agricultural runoff, industrial discharge, and untreated wastewater. These pollutants create selective pressure, fostering the emergence of resistant microbial strains.
- Providing direct and indirect exposure routes for humans and animals to ARB and ARGs. Water used for irrigation or in aquaculture can carry ARB to crops and seafood, introducing them into the food chain. This can lead to the indirect consumption of ARB and ARG, further amplifying the public health challenge of AMR. Direct exposure, on the other hand, can happen during recreational activities in contaminated water or through the consumption of tainted drinking water. This enhances the potential for resistant infections, complicating treatment options and increasing health risks.
- Facilitating the transmission of antimicrobial-resistant bacteria and resistance markers to other locations via surface water, groundwater, air, dust, and wildlife.
- Acting as a natural reservoir for resistance markers and facilitating the exchange of resistance markers among different bacterial species. Once resistance emerges, the aquatic environment acts as a reservoir and conduit for the transmission of antimicrobial resistance genes. These genes can be exchanged among bacteria through HGT, facilitated by the close contact within dense microbial communities in water bodies.

Therefore, environmental monitoring plays a crucial role in mitigating the spread of antimicrobial resistance, encompassing assessments of circulating ARGs in human populations, identification of major resistance hotspots, development of epidemiological and human health risk assessment models, and evaluation of removal efficiency by domestic wastewater infrastructure. Evidence increasingly links clinical and environmental ARGs [200,255], suggesting WWTPs as reservoirs where AMR 'superbugs' can accumulate, recombine, and proliferate, posing threats to the environment and public health [229].

3.2.1.1. AMR in wastewater

Antibiotics are widely utilized today, not only in treating human diseases but also across various sectors such as poultry farming, livestock management, biochemical industries, and agriculture. This extensive and often improper use has significantly contributed to the rise of ARB, which harbor numerous ARGs [180,256-258]. These ARB frequently enter the environment through

sewage systems, ultimately accumulating in WWTPs [199]. Wastewater serves as a repository for a wide range of substances from diverse sources, creating a dynamic environment for numerous biological processes. This complex mixture includes antibiotics, ARBs, ARGs, MGEs and human bacterial pathogens originating from clinical, household, and industrial activities [145,259]. Compounds and biological bonds formed in wastewater can enter the environment, impacting human and animal populations. Consequently, antibiotics, ARB, and ARGs, identified as emerging pollutants, are now recognized as a novel and hazardous form of environmental contamination. This threat is often underestimated in the context of sewage system management [198,260,261]. Several studies have highlighted the significance of wastewater, particularly from healthcare facilities, as a reservoir and transporter of multidrug-resistant bacteria (MDR) [262,263]. It is estimated that 30–90% of antibiotics consumed by humans and animals are excreted through urine and feces [264]. Antibiotic residues, stemming from their widespread use and overuse, incomplete drug metabolism, and inadequate removal in WWTPs, persist in the aquatic environment at concentrations ranging from nanograms to low micrograms per liter [142,254,265]. The presence of antibiotic residues in WWTPs significantly contributes to the persistence and dissemination of ARBs and their genes. This phenomenon not only favors the selection of resistant phenotypes but also disrupts indigenous bacterial communities [146,147]. In particular, in WWTPs the most commonly detected antibiotics are macrolides (such as clarithromycin, erythromycin, azithromycin, and roxithromycin), quinolones (including ofloxacin, ciprofloxacin, and norfloxacin), sulfonamides (like sulfamethoxazole), trimethoprim, and tetracycline. As previously mentioned, the ARGs frequently reported in these plants include bla genes (blaCTX-M, blaTEM, blaOXA-48, blaNDM-1, blaVIM-2), sul genes (sul1, sul2), tet genes (tetO, tetQ, tetW), and the ermB gene [207,208]. Recent studies indicate that even low concentrations of antimicrobials can exert sustained selective pressure on bacterial populations, driving the development of antibiotic resistance through mutation or HGT [252,254].

3.2.1.2. WWTPs as potential hotspots for ARBs and ARGs dissemination

In recent decades, antibiotic resistance has proliferated globally, reaching alarming levels and ushering in what experts term the "post-antibiotic era" [266]. Traditionally associated with healthcare-acquired infections [267,268], AMR is now increasingly linked to animal farming, aquaculture, and urban WWTPs. These sectors contribute significantly to the spread of ARB, ARGs, and residual antibiotics into surrounding aquatic and terrestrial environments [255,258].

The detection and characterization of AMR in various environments have been greatly advanced by molecular techniques, including quantitative PCR and metagenomic analysis [269-272]. Currently, environmental resistome analysis enables the quantification and assessment of ARGs, with particular focus on sewage and WWTPs, which are the focal points of numerous international monitoring efforts. Studies by Cully (2014), Cars (2014), and McCullough et al. (2016) highlight the widespread presence of AMR across various societal sectors, emphasizing the significant impact on wastewater treatment facilities as demonstrated by Amador et al. (2015) [273-276]. WWTPs are significant contributors to the spread of antibiotics, ARBs, and ARGs into various environmental compartments [199]. Although these plants are designed to reduce bacterial loads and contaminants, they often struggle to eliminate multidrug-resistant strains. Treated wastewater is discharged into environments such as rivers, lakes, seas, and agricultural soils through irrigation, facilitating the transfer of bacterial resistance [277,278]. Consequently, these plants inadvertently act as reservoirs and conduits for the spread of antibiotic resistance, posing significant threats to public health and environmental safety. Recognized as hotspots for antibiotic resistance, WWTPs are critical points where resistance epidemics concentrate [145,259].

Wastewater significantly contributes to the dissemination of ARB due to two main factors:

- Presence of Antibiotic Residues: During wastewater treatment, antibiotic residues are not fully eliminated and eventually make their way into surface waters. Although the concentrations of these antibiotics in water may be too low to be lethal to bacteria, they can still reach levels (up to several hundred μg L−1) that encourage the development of resistance[279,280];
- Antibiotic-Resistant Bacteria and Resistance Genes: Wastewater contains ARB and ARGs. It has been shown that the conditions under which wastewater treatment operates, along with the specific technologies employed, can influence the presence and subsequent release of ARGs and ARB into the environment [146,147].

The continuous exposure to sub-inhibitory concentrations of antibiotic residues creates selective pressure that promotes resistance. This pressure, combined with the high density and diversity of microorganisms—including pathogenic, commensal, environmental, and indigenous strains—in the nutrient-rich environment of wastewater, significantly fosters the spread of antibiotic resistance [147]. Furthermore, wastewater microbes are exposed not only to antibiotic residues but also to a variety of other pollutants (e.g., metals, biocides, organics)

and various physical and oxidative treatment processes (e.g., chlorine, ozone, ultraviolet radiation). These factors can potentially stimulate the selection, propagation, and HGT of emerging organic pollutants (EOPs) and ARGs [255,281]. Research has consistently shown that a variety of EOPs and ARGs are present in both municipal wastewater and treated effluents [277,282,283]. This persistent presence has led to the designation of WWTPs as hotspots for AMR [259]. Since the 1980s, global efforts have aimed to mitigate the impact of effluent discharge by implementing regulations on sludge and sewage emission standards [284]. Some researchers suggest that pre-treating hospital sewage with disinfection methods before it enters the sewage system could help reduce the spread of ARB into the environment [285]. However, only a few countries, such as France and Italy, have enacted legal provisions requiring the treatment of hospital wastewater (HWW) before its release [286,287]. Unfortunately, current emission standards for wastewater do not include the assessment of biological safety related to ARGs [288]. This oversight poses a significant biosafety risk due to ARGs in HWW, a risk largely neglected in current wastewater treatment practices and regulatory frameworks. Unfortunately, current WWTP technologies are ineffective at removing many biorecalcitrant compounds, including contaminants of emerging concern (CECs) like antibiotics. These compounds resist degradation through conventional biological treatment methods [8,289,290]. Numerous studies have indicated that biological treatment processes can inadvertently contribute to the proliferation of ARB and the transfer of ARGs [291-293]. Additionally, the final disinfection step in wastewater treatment often fails to completely eliminate resistant bacteria and may even promote the selection of ARGs [184,185]. This disinfection phase mainly targets fecal bacteria, particularly coliforms like E. coli, while neglecting the broader spectrum of the pathobiota, including potential human pathogenic bacteria [294-296]. Moreover, current wastewater emission standards do not assess the biological safety related to ARGs [288,297]. This omission represents a significant biosafety risk due to the presence of ARGs in hospital wastewater (HWW), a risk largely overlooked in existing wastewater treatment practices and regulatory frameworks. The behavior of ARGs in wastewater treatment processes differs significantly from that of bacteria. While these processes can reduce bacterial numbers by up to 99%, there has been an observed increase in the abundance of ARGs in the effluent [265,298,299]. WWTPs and their disinfection processes are critical in mitigating the environmental transfer of AMR [259,282]. However, there is limited understanding of the diversity, abundance, and survival of EOPs and ARGs in highly treated recycled waters. Treatment processes such as conventional activated sludge, membrane ultrafiltration, UV treatment, and chlorination vary significantly in their effectiveness at removing EOPs and

ARGs, with results ranging from highly effective to minimal or even adverse effects [281,300,301]. WWTP sludge has been identified as a primary source of bacteria and genes resistant to tetracycline and sulfonamides being released into the aquatic environment [302]. MGEs such as plasmids, transposons, and integrons carrying ARGs have been detected in wastewater and activated sludge samples from WWTPs [259,303,304]. Recent research suggests that phages and phage-derived particles may also contribute to the spread of ARGs in the environment. The high prevalence of ARGs, particularly in the bacteriophage fraction, raises the risk of these genes spreading and being incorporated into new bacterial hosts, potentially leading to the emergence of new resistant strains [305]. Effective wastewater treatment is crucial for safeguarding public health, as the spread of multidrug-resistant strains through WWTPs poses significant threats. With limited treatment options and serious risks to human health, addressing environmental antibiotic resistance becomes an urgent priority requiring coordinated and sustained efforts [145].

3.3. Monitoring AMR through Wastewater Surveillance

AMR surveillance plays a crucial role in tracking geographical and seasonal patterns of resistance, identifying new or rare resistance traits, and monitoring emerging trends. This information is vital for mitigating AMR infections, prioritizing interventions, evaluating the effectiveness of previous measures, guiding empirical treatment guidelines, reducing adverse impacts, and informing the development of new antimicrobial drugs [306,307]. The insights gained from surveillance are invaluable to policymakers, healthcare providers, pharmaceutical developers, and the scientific community. Numerous surveillance mechanisms and institutions operate at national, regional, and global levels to monitor AMR [308]. At the global level, the World Health Organization's Global Antimicrobial Resistance and Use Surveillance System (GLASS) spearheads efforts to standardize AMR surveillance and enhance knowledge through collaborative research. In the European Union (EU), the European Antimicrobial Resistance Surveillance Network (EARS-Net) and the European Surveillance of Antimicrobial Consumption Network (ESAC-Net) cover 30 countries in the European Economic Area. Additionally, the Central Asian and European Surveillance of Antimicrobial Resistance (CAESAR) network includes 19 countries across Eastern Europe and Central Asia. Despite these efforts, most national and international AMR surveillance initiatives focus primarily on a limited number of pathogens in clinical settings. Clinical isolate-based surveillance, which relies on passive reporting of laboratory results, often requires large patient samples to yield

meaningful data. While effective in identifying multidrug-resistant pathogens, this approach is inherently biased. It predominantly captures data from diseases with clear clinical symptoms and hospitalized patients, overlooking AMR in the commensal microbiota of healthy individuals. Consequently, it fails to provide a comprehensive picture of AMR prevalence in the general population [309-311]. WBE offers a valuable method for environmental surveillance of AMR, facilitating the identification of epidemiological connections between humans, animals, and the environment [312]. Addressing the global spread of AMR requires coordinated international efforts, yet a significant challenge lies in the scarcity of surveillance data in many regions [313]. Unlike traditional clinical surveillance systems, WBE presents a promising, resource-efficient alternative, particularly for low- and middle-income countries [246]. Municipal sewage serves as an optimal medium for monitoring ARB from a surveillance standpoint. It collects bacterial biomass from the entire community, including pathogens, through contributions such as feces, urine, nasal mucus, skin cells, and sputum. This encompasses bacterial contributions at every stage of colonization and infection—whether symptomatic, asymptomatic, pre-symptomatic, or post-symptomatic—originating from a wide range of sources, including households, hospitals, and nursing homes [314,315]. Recent studies have successfully leveraged WBE to assess AMR at both regional and national levels. For instance, quinolones and their associated resistance genes have been tracked in wastewater across seven European countries [316]. In Beijing, China, WBE has been used to estimate antibiotic use during a flu season in eight densely populated areas [317]. Additionally, WBE combined with local prescription data has been utilized in Southwest England to quantify antimicrobial usage [318]. Typically, parent compounds, or active antimicrobial agents (AAs), are selected as biomarkers in WBE analyses; however, incorporating AA metabolites can further enhance the assessment of AMR exposure. Building on a national study that monitored ARGs across U.S. rivers and streams, the U.S. Environmental Protection Agency (EPA) [319], in collaboration with the U.S. Food & Drug Administration, the U.S. Department of Agriculture, and the CDC, has expanded efforts to monitor antimicrobial resistance in the environment. This initiative complements existing clinical and foodborne monitoring programs, advancing a more integrated One Health approach to understanding and addressing antibiotic resistance. At the state level, California and other regions have proactively explored monitoring ARB and ARGs in recycled water, alongside other emerging contaminants of concern [320]. Standardizing methods across these efforts would be highly beneficial, allowing for the synthesis and comparison of results on both local and global scales. This would enhance the ability to track trends in antibiotic resistance, quantify anthropogenic inputs, assess ARGs escaping wastewater

treatment, identify clinically relevant ARGs, and inform human health risk assessments [255,321].

4. VALORIZING WASTEWATER: A CIRCULAR ECONOMY STRATEGY TO MITIGATE THE WATER CRISIS AND ENHANCE ENVIRONMENTAL SUSTAINABILITY

4.1. The Global Water Crisis: Limited Availability and Growing Demand

Water is an essential resource for human life and the entire ecosystem. Its quality, safety, and availability are crucial for human health, acting through both short- and long-term effects. Water covers about 71% of the Earth's surface [322], with oceans containing roughly 97% of the planet's water in the form of saline water. This leaves only 3% as freshwater. Of this small fraction, a mere 0.5% is in liquid form, accessible in rivers, lakes, ponds, and groundwater. The remaining 2.5% is locked in glaciers [323]. Since ancient civilizations flourished along riverbanks, water has been a crucial resource for domestic use and agriculture. The modern era has seen an exponential increase in water demand due to rapid urbanization, industrialization, and evolving lifestyles [324]. Today, vast quantities of water are extracted from natural sources to satisfy this growing need. This escalating demand is driven by factors such as industrial expansion, rising per capita water use, climate change-induced weather shifts, population growth, and migration from drought-stricken areas [325]. Over the past several decades, the relentless increase in demand for water resources—coupled with their widespread mismanagement—has significantly heightened the risk of pollution and severe water stress in many regions around the world. Localized water crises have become more frequent and intense, with profound consequences for public health, environmental sustainability, food and energy security, and economic development. Rapid demographic shifts and unsustainable economic practices are further straining the availability and quality of water, making it an ever-scarcer and more costly resource—especially for the poor, marginalized, and vulnerable populations. In response to the global water and sanitation challenges, the United Nations established UN-Water in 2003, a coordination mechanism to streamline efforts among UN entities and international organizations. This initiative aimed to enhance collaboration on all freshwater and sanitation-related issues. On 28 July 2010, the United Nations General Assembly officially recognized the human right to safe drinking water and sanitation through Resolution 64/292. Further reinforcing this commitment, the UN adopted the 2030 Agenda for Sustainable Development in September 2015, which includes Sustainable Development Goal (SDG) 6, dedicated to ensuring access to water and sanitation for all. In December 2016, the UN General Assembly unanimously passed the resolution for the International Decade for Action – Water

for Sustainable Development (2018–2028), further emphasizing the importance of water-related targets within the SDG framework. The eight targets of SDG 6 focus on various critical aspects of water and sanitation. These include ensuring access to safe drinking water (target 6.1), improving sanitation and hygiene (6.2), enhancing wastewater treatment and water quality (6.3), increasing water-use efficiency and reducing water stress (6.4), promoting integrated water resources management (IWRM) and transboundary cooperation (6.5), protecting water-related ecosystems (6.6), fostering international water cooperation (6.a), and encouraging community participation in water management (6.b). To facilitate the achievement of SDG 6, the UN General Assembly designated 2018–2028 as the International Decade for Action, "Water for Sustainable Development," also known as the Water Action Decade. This initiative seeks to break down silos, fostering collaboration and coordination among global efforts to tackle water-related challenges. Water is also central to other major global agreements, such as the Sendai Framework for Disaster Risk Reduction and the 2015 Paris Agreement on climate change. Ensuring the availability and sustainable management of water and sanitation has long been a key priority for the United Nations. The focus now is on turning the ambitious water-related SDGs of the 2030 Agenda into actionable results, driven by national leadership and strengthened by global partnerships. Water and sanitation lie at the heart of sustainable development, underpinning efforts to reduce poverty, foster economic growth, and protect the environment. Today, 2.2 billion people still lack access to safe drinking water, while over 4.2 billion are without proper sanitation services. Climate change is intensifying this crisis, driving an increase in extreme events like floods and droughts [326]. Globally, 80% of wastewater is discharged into ecosystems untreated or unreused, and 70% of the world's natural wetlands have disappeared, leading to a significant decline in freshwater biodiversity. To meet the needs of billions, the world must urgently transform how it manages water resources and delivers essential water and sanitation services [327]. The extensive use of natural resources, coupled with economic and population growth, threatens to exceed the Earth's carrying capacity and its ability to regenerate these resources. The growing population in urban areas increases water demand, exacerbating the water footprint of major cities. After several decades of relentless technological and population growth, the resulting surge in demand for energy and natural resources such as water and nutrients has highlighted the finite nature of these resources. The negative consequences of past and current overexploitation are becoming increasingly evident, leading to a global recognition of the need for sustainable resource management [309]. Moreover, the sharp increase in water consumption has resulted in a corresponding rise in wastewater production [328]. The quantity and composition of wastewater produced by

households, hospitals, and industries present significant concerns for both human and environmental health. Historically, small populations managed their sewage by diverting it into nearby natural water bodies, relying on the waters' ability to dilute and dissipate waste. Unfortunately, due to inadequate sewage conveyance and treatment infrastructure, much of this untreated sewage is still directly discharged into streams, ponds, and lakes. This practice has severely impacted vital water resources, undermining countless livelihoods and hindering economic growth in many regions [329]. Efficient wastewater management, encompassing collection, treatment, and discharge with minimal environmental impact, is crucial. Despite advancements, wastewater management remains a pressing issue, necessitating evolving strategies to protect public health while minimizing ecological damage to receiving waters. Treatment and discharge of wastewater are increasingly regulated at national and international levels, such as within the European Union (EU), where the first common wastewater regulation was established in the 1990s [330]. However, compliance with EU legislation varies among member states [331], highlighting the need for periodic enforcement and updates to ensure environmental protection and prevent social conflicts. Today, the most critical challenges are the development of renewable energy, water conservation, and environmental protection. Numerous studies confirm that our current consumption and production systems are unsustainable for the planet and pose a significant threat to our well-being [309]. In response, the concept of a circular economy is gaining traction worldwide. The circular economy seeks to minimize the extraction of natural resources and the production of waste by promoting the reuse, remanufacture, and recycling of materials, as opposed to the traditional linear economy model of "take, make, use, dispose, and pollute." This shift towards a circular economy, coupled with growing social awareness and environmental concerns, has prompted significant paradigm shifts in wastewater management. By emphasizing the full valorization of residuals, the circular economy aims to transform wastewater from a waste product into a valuable resource, thereby reducing the human footprint on the Earth. Transitioning to a circular economy, an alternative model to the traditional linear economy, presents a significant opportunity to achieve substantial health benefits. These include direct improvements to healthcare systems and indirect benefits from reducing negative environmental impacts. The concept of a circular economy provides a pathway to sustainable growth and improved health by promoting the efficient use of resources, minimizing waste, and preventing resource depletion and environmental degradation [332,333]. This holistic approach encourages innovative solutions and sustainable practices in wastewater management, aligning with the broader goals of environmental protection and resource conservation [334].

4.2. The Role of Circular Economy in Addressing Water Scarcity

The circular economy is a sustainable economic model aimed at the wise and efficient utilization of multiple resources, with the goal of reducing environmental impacts. The concept gained momentum in the late 1970s and has since been championed by researchers, practitioners, and policymakers. It was formally integrated into the European Union's strategy with the adoption of the Circular Economy Strategy in 2015 [332,333]. The circular economy is typically described as a shift from a linear model (take, make, use, dispose) to a circular model (restorative and regenerative) [335]. While there isn't a single, universally accepted definition in the literature, there is a general agreement on the core concepts and objectives of a circular economy. Definitions tend to fall into two categories: those that focus on resources, emphasizing the need for closed material loops and reduced use of virgin resources, and those that extend beyond material resource management to include other aspects, such as changing consumption patterns [336].

Pearce and Turner were pioneers in introducing the concept of the circular economy within environmental and natural resource economics [337]. In 2010, the Ellen MacArthur Foundation significantly advanced the practice of the circular economy across various sectors [338]. Despite substantial efforts to develop and apply the circular economy concept in multiple countries and industries, the strategies for implementing circular economy practices are still not well-defined [337]. According to Saidani et al., [339] there is no consensus on how to measure the practical and large-scale implementation of the circular economy. Sekulić et al. [340] noted that the requirements for implementing a circular economy in developing countries make it a slow, complex, challenging, and often intangible process. To better understand and agree upon a definition of the circular economy, Geissdoerfer et al. conducted a study in 2017 [240], compiling various existing definitions. They proposed a comprehensive definition of the circular economy as “a regenerative system in which resource input and waste, emission, and energy leakage are minimized by slowing, closing, and narrowing material and energy loops. This can be achieved through long-lasting design, maintenance, repair, reuse, remanufacturing, refurbishing, and recycling” [341]. For example, water scarcity is currently a serious problem worldwide, as the annual water demand is rapidly increasing due to the growing global population, industrialization, and climate change [342,343]. A circular economy promotes sustainable interactions between markets, consumers, and natural resources, enhancing water use efficiency and encouraging resilience in water availability. However, challenges remain, such as improving public perception, overcoming technological barriers, and addressing regulatory limitations. Current documents on the circular economy reveal a lack of focus on the

water sector, including the benefits and legal framework associated with the circular economy. Addressing this gap requires a thorough review of the circular economy within the water and wastewater sectors [337]. Del Borghi et al. [344] pointed out that transitioning from theoretical concepts to practical applications of the circular economy remains challenging [240]. It is recommended that water supply management entities adopt circular economy principles by eliminating waste through recycling and intentional reuse of secondary waste [345].

4.2.1. Political Incentives for Implementing Circular Economy Solutions

A critical factor in shifting from a linear to a circular economy is the political will and awareness of decision-makers. Changing the existing system requires updating the legal framework to facilitate necessary changes while considering the political advancements made in the water sector over recent decades [346]. Various planning policies must be evaluated to determine the appropriate levels of water reuse planning for each system. For effective integration at regional and municipal levels, it is essential to adopt holistic approaches that incorporate water reuse sources and potential application areas [347]. However, these approaches are often lacking in most countries. The European Union advocates that achieving an efficient, climate-neutral economy necessitates specific policies for wastewater reuse. In Iberian countries, European water policy is perceived as a means to overcome the entrenched hydraulic paradigm. In contrast, Australia's increase in wastewater reuse has been largely driven by involving all stakeholders in creating incentive policies for wastewater reuse. In the United States, the "California Recycled Water Policy" has significantly advanced water recycling efforts in California, despite its shortcomings in fostering a shift to a circular economy [346]. There is broad agreement among authors that clear public policies are vital to advancing wastewater reuse projects [348]. These policies must be embedded within a legal and institutional framework to create an environment conducive to investment in wastewater reuse. This requires political, regulatory, and legislative support across key sectors, including health, agriculture, and energy [339].

4.2.2. Economic Drivers for Water Recycling and Reuse

From a circular economy viewpoint, wastewater reuse represents a sound investment [349]. The core principles of circular economy emphasize waste avoidance and resource optimization [350]. Water reuse becomes viable in a specific region only when the cost of reuse is less than that of other water management methods [351]. In Portugal and Spain, various taxes and fees apply to different types of water usage [352]. In contrast, the United Arab Emirates promotes water reuse through price discounts and tax incentives, while in Germany, municipal water reuse projects rely on subsidies [353]. However, according to Maaß et al., the economic sustainability of wastewater reuse is highly sensitive to regulations, particularly those stringent about end-user protection [345].

4.3. Water Recycling: A Key Component of Circular Economy

Water scarcity is a critical global issue, increasingly aggravated by the impacts of climate change. The swift depletion of freshwater resources, coupled with soaring demand, underscores the urgent necessity for innovative solutions like wastewater reclamation and reuse. This escalating water demand is driven by factors such as rapid population growth and industrial expansion [342,343], making water recycling not just beneficial but essential. Agriculture is the dominant consumer, accounting for an overwhelming 92% of the world's water usage [354]. A significant portion of this, about 70%, is allocated to irrigation [355], predominantly sourced from rivers and groundwater reserves [356]. As freshwater supplies dwindle, the imperative for sustainable water management practices in agriculture and other sectors becomes increasingly clear. These statistics underscore the severe concern for countries facing water scarcity. Shen et al. (2014) reported that 40% of the global population resides in highly water-stressed basins, indicating a significant water crisis for irrigation. In this context, the reuse of treated wastewater has become increasingly vital. Widely recognized as an environmentally friendly strategy and a viable alternative water source at both international and national levels, this practice has expanded significantly [357]. Annual growth rates range from 10% to 29% in regions such as Europe, the USA, and China, and reach up to 41% in Australia [358]. Through meticulous treatment, monitoring, and management, water recycling offers a solution to ensure safe, dependable, and sustainable water resources for diverse purposes. For instance, agriculture extensively utilizes human wastewater and excrement as sources of water and nutrients for plants, encouraged by management practices such as ecological sanitation, reuse utilities, and water reclamation. Globally, the use of treated wastewater for irrigation plays a crucial role in

supporting agricultural productivity and sustaining the livelihoods of millions of smallholder farmers [359]. The extent of treated wastewater reuse for agriculture varies significantly worldwide, with estimates ranging from 1.5% to 6.6% of total agricultural water use [359,360]. Over 10% of the global population consumes agricultural products grown using wastewater irrigation [360]. Domestic water and treated wastewater are rich in various nutrients, including phosphorus, nitrogen, potassium, and sulfur. Among these, the high levels of nitrogen and phosphorus in wastewater are particularly beneficial for plant absorption, making it a popular choice for irrigation [361]. The nutrient-rich composition of reclaimed wastewater can reduce the need for synthetic fertilizers, enhance crop productivity, and improve soil fertility. Consequently, this not only boosts agricultural yield but also lowers the cost of crop production [362]. Furthermore, treated wastewater is typically utilized for non-potable applications, including groundwater recharge, golf course maintenance, vehicle washing, toilet flushing, firefighting, and construction activities. Additionally, it serves as a valuable resource for cooling processes in thermal power plants [363] To realize these benefits and fulfill duty of care obligations concerning public health, water managers and regulators must proactively identify and assess risks related to emerging contaminants [364].

4.3.1. Wastewater Characteristics and Composition

Domestic wastewater (DW) is composed of three main parts: blackwater (BW) from toilets, which often includes yellow water (YW, urine), and greywater (GW) from sources like showers, dishwashers, and laundry machines. The quality of unsegregated DW can vary significantly based on local conditions, with Chemical Oxygen Demand (COD),ranging from 50 to 250 mg/L, Total Nitrogen (N) between 20 and 100 mg/L, Total Phosphorus (P) from 6 to 30 mg/L, and Total Suspended Solids (TSS) between 300 and 1,500 mg/L. However, the individual components of DW show distinct characteristics [365]. BW is high in organic content (COD≈5,000–93,000 mg/L) and nutrients (N ≈ 1,500–16,000 mg/L, P ≈ 500–3,000 mg/L) and has a high solids content (TSS ≈ 16,000–125,000 mg/L). In contrast, GW has lower levels of COD (200–450 mg/L), N (6–22 mg/L), P (0.4–8 mg/L), and solids and pathogens. However, GW tends to have higher concentrations of metals and micropollutants than BW. YW has high levels of COD (4,000–11,000 mg/L), N (4,000–11,000 mg/L), and P (200–4,000 mg/L) [365]. Black and yellow water components are significantly diluted by grey and storm water before being discharged to WWTPs. Numerous strategies have been proposed for nutrient removal during WWTP operations [366]. Typically, effluents from WWTPs, such as those from

conventional activated sludge (CAS) processes, still contain valuable nutrient concentrations (e.g., TN = 5–30 mg/L, TP = 0.2–3 mg/L, K = 0.6–18 mg/L), which can potentially replace traditional fertilizing methods [367]. The relative nutrient ratios in reclaimed water (RW) show a higher nitrogen content compared to phosphorus and potassium, often necessitating the addition of P and K. RW can also provide plants and soils with micronutrients such as, Calcium (Ca), Magnesium (Mg), Boron (B); Iron (Fe), Manganese (Mn) and Zinc (Zn). However, irrigation with RW can lead to soil salinization, increasing the concentration of soluble salts, or soil modification due to an excess of exchangeable sodium compared to other cations [368]. Additionally, an excess of ammonium in RW can interfere with calcium absorption because of competition between NH_4^+ and Ca^{2+} ions [367]. The higher amounts of solids and salts in RW compared to freshwater can also cause irrigation devices to clog and increase soil salinity, reaching up to 42.0 dS/m in the topsoil layer [369].

4.3.2 Public Health Risks and Safety Considerations in Wastewater Reuse

While using wastewater offers numerous advantages, it also poses significant health risks. Target 6.3 of SDG 6 aims to improve water quality by 2030 through reducing pollution, eliminating dumping, minimizing the release of hazardous chemicals and materials, halving the proportion of untreated wastewater, and significantly increasing recycling and safe reuse globally. One of the most critical barriers to water reuse is microbiological contamination. Contaminants in wastewater can threaten human health and the environment by harboring disease-causing microorganisms and harmful substances, and by causing eutrophication due to excessive nutrient content [370]. In fact, a growing body of scientific evidence indicates the widespread global presence of antibiotics, other antimicrobial agents, ARB, and AMR in wastewater and aquaculture systems. Therefore, over the past decade, the reuse of wastewater has emerged as a significant global health concern, impacting both public health and the environment [371,372]. In 1973, the WHO established guidelines to safeguard public health by promoting safe conditions for the use of wastewater and excreta in agriculture and aquaculture [373]. These initial guidelines were formulated without the support of comprehensive epidemiological studies and adopted a minimal risk approach. In 2005, WHO revisited and updated these guidelines to reflect better the evolving understanding of risks and the importance of safety in wastewater [374]. Adegoke et al. (2018) [375] conducted a comprehensive review of the epidemiological evidence and health risks linked to the reuse of wastewater for irrigation. They identified significant health concerns associated with microbial

hazards, such as infectious pathogens, and exposure to chemicals and pharmaceuticals present in wastewater or graywater [375-378]. Researchers have found that exposure to wastewater can lead to infectious diseases, such as helminth infections, which are linked to anemia and can severely impair physical and cognitive development [379,380]. Ensuring global access to safe, high-quality water, particularly in terms of reducing microbial contamination, remains a pressing challenge [381]. According to the Sustainable Development Goal (SDG) Synthesis Report on Water and Sanitation, the targets for SDG 6, which aim to ensure access to clean water and sanitation by 2030, are far from being met. As a result, it is critical to identify strategic measures to reduce risks associated with waterborne diseases, such as diarrheal infections [382]. Epidemiological studies have helped establish threshold values for E. coli concentrations, commonly used as indicators of fecal contamination, to monitor water quality [383]. However, more comprehensive efforts are required to further improve water quality and curb the spread of infections. Global data on total wastewater flows—both domestic and industrial—that are safely treated according to national or local standards remains insufficient. In 2022, only 58% of household wastewater worldwide was safely treated before being released into the environment, a modest improvement from 56% in 2020 [384]. Regional disparities in wastewater treatment are stark, with rates ranging from just 24% in Central and Southern Asia to 86% in Europe and North America. This uneven progress highlights significant challenges in achieving global targets. Data for 2022 on the proportion of safely treated domestic wastewater were available from 129 Member States, covering 89% of the world's population (UN-Water, n.d.e) [140,385]. As of 2020, just 96 countries, representing 35% of the global population, reported data on water bodies with good ambient water quality. Many low-income countries lack the capacity to routinely monitor water quality, putting over 3 billion people at risk due to the unknown health status of their freshwater ecosystems [386]. This underscores the need for greater efforts to improve water monitoring and management, especially in vulnerable regions. To mitigate health risks from wastewater reuse, stringent safety measures and effective treatment processes are essential, emphasizing the need for proper wastewater management to prevent adverse health outcomes. While using treated wastewater for crop irrigation can offer substantial agricultural benefits, it also poses significant health risks if not carefully managed [360]. Irrigating crops with industrial wastewater, either alone or mixed with domestic water, poses a higher risk due to potential contamination with heavy metals and pathogens [387]. These heavy metals, including copper (Cu), chromium (Cr), manganese (Mn), iron (Fe), lead (Pb), zinc (Zn), and nickel (Ni), are particularly worrisome because they are non-biodegradable and have long biological half-lives [388]. These toxic elements tend to

accumulate in the topsoil (up to a depth of 20 cm) and are absorbed by plant roots. They can then enter the human and animal food chain through the consumption of contaminated leafy vegetables or inhalation of dust from contaminated soils. Consequently, assessing the health risks associated with wastewater irrigation is crucial, particularly for adults who may be more susceptible to the adverse effects of these contaminants [389-391]. Furthermore, one significant concern is the introduction of antibiotics into agroecosystems, which can lead to the proliferation of ARB and ARGs. This contamination has been highlighted by numerous studies [392,393]. Unfortunately, in many countries, equipment for treating municipal, community, and household waste, which may contain antimicrobial drugs, ARB, and AMR genes, is either insufficient or nonexistent. Conventional WWTPs are not equipped to completely eliminate antibiotics, along with ARB and ARGs, from wastewater. The primary reasons are the high costs and technical challenges associated with treating such complex and diverse waste mixtures. Consequently, these contaminants are directly released into the environment, where human exposure is likely, facilitating the persistence and spread of ARB and ARGs. Municipal wastewater, in particular, serves as the primary reservoir for antimicrobial agents, ARB and ARGs in the environment, creating a conducive setting for gene transmission among bacteria before or after treatment [146,147]. Using reclaimed wastewater for crop irrigation can inadvertently introduce antibiotics into the soil, which may lead to soil bacteria acquiring antibiotic resistance [394]. This resistance can then potentially enter the food chain, posing a serious threat to public health [277]. Additionally, the escalating public health concern is that ARB and ARGs from human waste may infiltrate water sources via untreated or treated wastewater, posing a risk to human populations that utilize the water for activities such as recreation and irrigation. To mitigate these health risks, advanced wastewater treatment methods must be implemented before wastewater is released into rivers, agricultural land, or soils.

5. WASTEWATER TREATMENT TECHNIQUES

Wastewater treatment is crucial for safeguarding public health and preserving ecosystems. Initially, WWTPs were designed primarily to meet specific effluent criteria, focusing on the reduction of organic pollutants, measured as biological oxygen demand (BOD) or chemical oxygen demand (COD), and nutrients. However, energy efficiency has become increasingly significant. Over the past decade, WWTPs have evolved into water resource recovery facilities, emphasizing the recovery of energy and other valuable resources from wastewater [395]. Ensuring the quality of our water resources hinges on the effective monitoring and control of pollution sources and discharges. Contaminated water bodies threaten human health and disrupt ecosystems. Uncontrolled discharges can contaminate drinking water, lead to eutrophication from organic matter overload, and cause the accumulation of heavy metals and other pollutants. There is growing concern about emerging pollutants in aquatic environments, including pharmaceuticals (such as anti-inflammatory drugs, analgesics, antibiotics, hormones) and microplastics [329]. Enhancing wastewater monitoring and management should be seen as a vital component of sustainable solutions to both quantitative and qualitative aspects of the ongoing water crisis. Despite this, the willingness to invest in wastewater collection, treatment, and monitoring remains low compared to drinking water services, particularly in countries with lower health and environmental standards. Additionally, treated wastewater is not widely recognized as a renewable resource for use in agriculture, industry, and energy generation. A significant paradigm shift in wastewater management is necessary to better protect drinking water resources and aquatic ecosystems, and to contribute to sustainable development and climate change mitigation and adaptation. Effective wastewater management and reuse can mitigate climate change impacts, as sanitation and wastewater systems contribute to greenhouse gas emissions both directly (through the breakdown of excreta) and indirectly (through energy-intensive treatment processes). Treated wastewater should also be integrated into river basin water balances to reduce financial burdens on treatment plants and increase environmental benefits. Current estimates suggest that over 80% of wastewater is released into the environment without adequate treatment [396]. However, more recent and thorough analyses indicate that just under 50% of global wastewater is released untreated [108]. In 2017, the United Nations General Assembly approved the global indicator monitoring framework developed by the Inter-Agency and Expert Group on Sustainable Development Goal Indicators (IAEG-SDG), putting wastewater management on the global development agenda for the first time. Sustainable Development Goal (SDG) 6 aims to ensure the availability and sustainability

of water and sanitation for all by 2030, covering the entire sanitation chain—from the safe management of household sanitation services (indicator 6.2.1a) to the safe treatment and discharge of domestic and industrial wastewater flows (indicator 6.3.1). Beyond the public health benefits, safe wastewater treatment provides social, environmental, and economic advantages. Unlike the Millennium Development Goals (MDGs), which primarily targeted low- and middle-income countries, the SDG framework on sanitation applies universally. All countries are challenged to improve service levels and enhance their capacities to measure and monitor these improvements.

5.1. Background of Wastewater treatment (WWT)

WWT inherently employs a variety of physicochemical and microbial processes to effectively separate and neutralize complex organic and inorganic contaminants present in wastewater. These methods play a crucial role in significantly diminishing the toxicity of untreated sewage, as highlighted by Dhote et al. (2012) [397]. The primary objective of WWT is to mitigate the pollutants responsible for elevating Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD), since untreated wastewater can drastically reduce dissolved oxygen levels in freshwater ecosystems. Additionally, it is essential to remove Total Suspended Solids (TSS) and Total Dissolved Solids (TDS) found in typical wastewater to preserve the clarity and natural composition of freshwater bodies, as noted by Tchobanoglous [398]. The overarching goal is to reduce the pollution load of raw wastewater to a level that allows for its safe release into surface waters such as rivers, wetlands, and ponds [399]. Over time, a range of techniques and technologies has been developed to address the challenges associated with wastewater disposal and to ensure the effective treatment of wastewater. Since the dawn of human civilization, the management and treatment of wastewater have been integral to societal development. Early wastewater systems primarily focused on collection and conveyance. Notably, advanced wastewater infrastructure existed as early as 2500 BC in ancient cities like Harappa and Mohenjo-Daro, with similar systems recently unearthed at Rakhigarhi [400]. It wasn't until the late 19th century that modern scientific WWT began to take shape. This period marked the transition from rudimentary collection to systematic treatment, initiating what can be described as the age of sanitary enlightenment after centuries of minimal progress in public sanitation. The mid-20th century witnessed groundbreaking advancements with the development of aerobic and anaerobic treatment technologies [401]. The first significant implementation of anaerobic treatment for municipal wastewater dates back to 1860. According to the French

journal Cosmos, this method utilized an airtight septic tank, a precursor to modern anaerobic processes [402]. Over the 19th and 20th centuries, various WWT technologies evolved. A pivotal innovation was the introduction of the Activated Sludge Process (ASP) in 1914, which established a fully aerobic system for wastewater treatment [403]. This method revolutionized WWT by integrating additional stages such as filtration, contact beds, trickling filters (TFs), and septic tanks, thereby significantly enhancing the efficacy and reliability of wastewater treatment systems. The evolution of these technologies underscores the continuous efforts to refine and improve how we handle wastewater, paving the way for the sophisticated systems in use today. In the late 20th century, advancements in WWT processes accelerated as countries adopted increasingly stringent environmental standards. This period marked a significant shift in societal attitudes towards wastewater management, driven by heightened awareness of environmental impacts and the evolution of environmental analytical chemistry. Consequently, WWT technologies advanced rapidly, incorporating primary, secondary, and nutrient removal methods as essential components of comprehensive treatment schemes. Aerobic and anaerobic treatment processes are energy-efficient and environmentally friendly methods commonly used to treat Chemical Oxygen Demand (COD) [404]. The choice between aerobic and anaerobic treatment systems depends largely on the characteristics of the wastewater. Aerobic systems are generally more effective for treating low-strength wastewaters, with biodegradable COD concentrations less than 1000 mg/L, due to their efficiency in breaking down organic matter. On the other hand, anaerobic systems are better suited for high-strength wastewaters with higher organic loading (COD > 4000 mg/L), as they can handle the greater volume of biodegradable material [405]. Aerobic treatments are particularly noted for their higher removal rates of biologically degradable matter compared to anaerobic techniques [406]. Given the unique advantages and limitations of both aerobic and anaerobic systems, hybrid approaches that combine these methods are often employed to maximize treatment efficiency and effectiveness.

5.2. Emerging contaminants in wastewater treatment plants

In recent decades, the presence of numerous newly identified compounds, both anthropogenic and natural, in aquatic environments has emerged as a significant global environmental concern. These contaminants, primarily organic, are typically found in trace concentrations ranging from parts per trillion (ppt or ng/L) to parts per billion (ppb or μg/L) [407]. Known as "emerging contaminants (ECs)" [408,409], they are also referred to as micropollutants (MPs), emerging pollutants (EPs), contaminants of emerging concern (CECs), or trace organic compounds

(TrOCs) by various researchers [410-412]. The United States Geological Survey (USGS) defines ECs as "any chemical of synthetic or natural origin or any microorganism that is not commonly monitored in the environment but has the potential to cause adverse ecological and/or human health effects"[413]. This definition also encompasses microbial contaminants, including ARGs and bacteria, often mediated by antibacterial drugs. These specific contaminants can be classified as emerging microbial contaminants [25,408,414]. The United States Environmental Protection Agency (USEPA) defines ECs as relatively new compounds that pose harmful, though not yet fully understood, impacts on the health of living beings and the environment [415]. The occurrence of these chemical compounds in the environment is not a new phenomenon. However, it is only within the last two decades that extensive research has focused on the detection and analysis of emerging contaminants (ECs) in various environmental media. This delay is largely due to the progressive advancement of sensitive analytical techniques and innovative detection methods, which have made it possible to identify the extremely low concentrations of ECs in environmental samples [416].

5.3. Primary Treatment

Primary treatment, serving as a pretreatment stage, primarily involves physical processes and basic chemical reactions. This initial phase of wastewater treatment aims to remove large, suspended, and floating solids from raw sewage, thereby reducing the organic load that enters the biological treatment plant. This process begins with grille and screening processes, where physical barriers trap substantial solid debris. Following this, sedimentation tanks allow gravity to settle and remove smaller suspended particles. While this phase primarily relies on mechanical separation techniques, chemical additives are occasionally employed to enhance sedimentation efficiency. As a result, this initial treatment phase effectively lowers the BOD of the incoming wastewater by 20–30% and reduces total TSS by approximately 50–60% [364].

5.3.1. Grille: screening processes

Screening and grille are essential steps in the primary treatment of wastewater. Screening is designed to remove large solids and debris from the wastewater using various types of screens, tailored to meet the specific needs of the treatment plant. Upon entering the treatment facility, wastewater first encounters a bar screen, which captures larger solids. Alternatively, a grinder or comminutor may be used to break down these large particles into smaller sizes. The grille process is designed to remove large, coarse solids from wastewater, including items such as

sticks, leaves, and plastic debris. These large solids can potentially damage equipment or interfere with subsequent treatment stages. Grilles are constructed with bars or plates that create gaps, allowing water to flow through while capturing larger objects. The spacing of these bars or plates is optimized to trap sizable debris while ensuring efficient water flow. Screening, on the other hand, targets smaller solids that are not captured by the grille, such as sand, grit, and finer organic matter. Screening systems include:

- Coarse Screens: These have smaller openings than grilles and are used to remove medium-sized solids that may pass through the grille but are still too large for further treatment stages.

- Fine Screens: These screens feature very small openings designed to capture fine particles and small debris. They are typically used following coarse screening to ensure that only very fine solids remain in the wastewater.

Following this initial stage, the wastewater moves to a grit chamber where heavier, undesirable solids are separated. At this point, the wastewater velocity has kept these solids suspended, but in the grit chamber, the flow speed is reduced to approximately 0.3 meters per second (1.0 feet per second). This slower velocity allows the inorganic solids, or grit, to settle out, while lighter organic materials remain suspended in the water. After the grit removal process, the water moves on to the oil removal stage, where floating oils and fats are separated, as they could cause issues in the subsequent biological and chemical treatment phases. These processes effectively prepare the wastewater for further treatment stages by removing both large debris and heavier particles [417].

5.3.2. Sedimentation

When wastewater velocity is reduced to below 0.3 meters per second (1.0 feet per second), heavier materials will settle to the bottom, while lighter materials will rise to the surface. This separation of solids and liquids, facilitated by reduced velocity and gravity, is known as sedimentation. Settling tanks are designed to distribute flow evenly throughout the tank, which helps achieve consistent reduction in velocity and minimizes mixing. Under these conditions, suspended solids settle to the bottom as sludge, while lighter materials float to the surface as scum. In primary sedimentation tanks, settleable solids—those that can easily settle out when the wastewater slows to a fraction of a meter or foot per second—typically account for 90 to 95% of the suspended solids.Primary sedimentation tanks effectively lower the wastewater velocity to less than 0.3 meters per second (1.0 feet per second), enabling the separation of

settleable solids from the wastewater stream. This process also reduces a portion of suspended solids and the associated BOD. Despite variations in tank design and location, all settling tanks share the same purpose: to slow down the wastewater flow and minimize mixing to allow settling and flotation. It's important to note that settling tanks primarily remove settleable solids. Lighter materials either remain suspended or float to the surface, requiring additional removal methods. Primary sedimentation tanks are typically situated immediately after preliminary treatment. Colloidal solids, which are finer and remain suspended even in reduced velocities, do not settle out within the detention times of primary sedimentation tanks. These finer particles pass through the primary treatment stage and are addressed in secondary treatment processes. If a primary tank fails to remove enough settleable solids, it can lead to increased oxygen demand, adversely affecting subsequent biological processes. Conversely, excessive removal of settleable solids can reduce the organic matter available for effective biological treatment [417].

5.3.3. Flotation

Flotation is a vital process in wastewater treatment, especially for the removal of suspended solids, oils, and greases. This technique leverages the buoyancy of bubbles, which attach to particles and lift them to the surface for removal. Flotation systems, such as Dissolved Air Flotation (DAF), Induced Air Flotation (IAF), and vacuum flotation, offer compact and efficient solutions for various wastewater treatment challenges. Typically used alongside primary or secondary treatment processes, flotation significantly enhances the overall efficiency of wastewater treatment. DAF specifically focuses on removing suspended solids by utilizing the lifting force created when low-density bubble-floc aggregates are formed. The DAF process occurs in two distinct zones, each with a specific function:

- Contact Zone: In this zone, bubbles come into contact with flocs, resulting in the formation of bubble-floc aggregates. This aggregation is crucial for the flotation process as it prepares the particles for separation.

- Separation Zone: Here, the bubble-floc aggregates are separated from the water phase. The aggregates rise to the surface, forming a froth layer that is subsequently removed, leaving behind clearer water.

This dual-zone approach ensures efficient particle removal and enhances the performance of the wastewater treatment system [418].

5.4. Secondary (Biological) Treatment

Secondary treatment is a critical phase in wastewater management, aimed at removing dissolved organic matter that primary treatment cannot eliminate. It revolves around biological processes, predominantly aerobic and anaerobic treatments. During this stage, microorganisms play a vital role by consuming organic pollutants, converting them into carbon dioxide, water, and energy to support their growth. This biological process is followed by additional settling to extract more suspended solids. This stage is highly effective, typically removing about 85% of the remaining suspended solids and reducing BOD to similar levels. It also targets carbonaceous pollutants, which settle in secondary settling tanks, allowing for the separation of biological sludge from the clearer water. This biological sludge can be repurposed as a co-substrate in biogas plants, where it produces biogas—a mixture of methane (CH_4) and carbon dioxide (CO_2). This biogas can then be utilized to generate heat and electricity. While existing literature generally agrees that the abundance of ARGs decreases during secondary wastewater treatment, the reported prevalence of ARGs varies significantly. Different studies, considering diverse plant configurations, have observed decreases, no change, or even increases in ARG prevalence [241,302,419,420]. For example, some studies have found no significant change in the relative abundance of the sul1 gene after secondary treatment [302,419], whereas others have noted an increase [241,253]. Secondary treatments can be aerobic, anaerobic, or anoxic.

5.4.1. Aerobic Wastewater Treatment (WWT)

Aerobic Wastewater Treatment (WWT) leverages microorganisms that thrive in the presence of oxygen to break down and decompose organic matter in wastewater. These microorganisms use the organic compounds as a rich source of carbon and energy, facilitating the purification process.

Classification of Aerobic Treatment Systems:

1. **Suspended Growth Systems**: in these systems, both the microorganisms and the organic substrates are suspended in the wastewater, forming what is known as mixed liquor. A prominent example of this approach is the Activated Sludge Process (ASP), which is widely employed in wastewater treatment. In the ASP, air or oxygen is introduced into a combination of wastewater and biological sludge, promoting microbial growth and facilitating the digestion of organic matter. This technique utilizes a bacterial biomass suspension to effectively remove impurities from the wastewater. It is one of the most extensively employed technologies in wastewater treatment [421]. This

technology, pioneered by Lockett in 1914, contributed significantly to the widespread adoption of full-scale aerobic WWT systems [422]. Activated sludge is a complex liquid mixture primarily composed of phosphates, nitrates, and carbon derived from wastewater, along with residual flocs featuring adaptable surfaces like bacteria and protozoa [423,424]. These flocs can undergo continuous restructuring through deflocculation and flocculation processes, which are influenced by the shear forces they experience. In basic active sludge systems, the aeration tank must consistently provide sufficient oxygen for complete waste stabilization. Typically, a dissolved oxygen (D.O) concentration of 1.0 mg L−1 is maintained in the air circulation tank [425]. Activated sludge systems are cost-effective to install, offering a favorable return on initial investment. They require minimal space for installation and operation, making them suitable for facilities with limited space availability. These systems operate hygienically, with minimal odors or pest issues, ensuring safe and convenient operation [426]. However, despite these advantages, activated sludge processes can be resource-intensive, with significant costs associated with their operation, maintenance, and waste-activated sludge management. In sewage treatment plants, these costs can constitute 50–60% of overall maintenance expenses [427]. Other drawbacks include limited flexibility in handling varying loads, high initial capital outlay, and the necessity for skilled supervision during mass sludge removal operations. An alternative technology is the Sequencing Batch Reactor (SBR), which operates in cycles by alternating between aeration and settling phases within a single tank. This system employs a fill-and-drain activated sludge technology. An SBR operates based on time rather than space, essentially functioning as an activated sludge plant [428]. The SBR method, with its single reaction tank, minimizes the need for extensive construction materials. Additionally, its compact floor space requirement makes this method more flexible compared to the traditional activated sludge process. Typically, an SBR system progresses through five stages: Fill, React, Settle, Decant, and Idle [429]. These cycles are noted for reducing operational costs by more than 60% compared to traditional ASP methods, while achieving high-quality effluent within a shorter aeration period [430]. The SBR cycle effectively removes up to 90% of carbonaceous components such as BOD5, COD, and TSS. This treatment method achieves a COD removal efficiency exceeding 94.90%, capable of eliminating organic carbon and offering robust performance under varying load conditions [431]. VSS (volatile suspended solids) and TSS are individually removed at rates of 80% and 70%, respectively. Despite these

strengths, the SBR system shows limited effectiveness in pathogen removal, necessitates substantial aeration costs, and requires skilled supervision for plant operation and maintenance. SBR systems are evolving with advancements like baffles near the inlet to create a separate reaction chamber from the aeration basin, aiming to reduce the discharge of floating solids and settled sludge [432]. Recent modifications include continuous flow SBRs, anaerobic-aerobic SBRs, and membrane SBRs (MSBRs), reflecting innovations in SBR technology [230].

2. **Attached Growth Systems**: here, microorganisms form a biofilm on the surfaces within the treatment unit. Examples include:

    - **Trickling Filters**: wastewater passes over a bed of media (such as rocks or plastic), where the biofilm develops and degrades the organic matter. Trickling filters are primarily utilized to remove both organic and inorganic substances from wastewater. The United States pioneered their use in 1901, followed by the installation of the first commercial trickling filter in West Germany in 1960. The filter media within a trickling filter typically consist of various materials such as gravel, boulders, slag, plastic, and polyurethane foam, which are colonized by biological sludge. These media can be arranged in different configurations, including cross-flow, vertical flow, tubular flow, or randomly packed formats. Compared to other wastewater treatment methods, biological trickling filters are much more space-efficient. They are highly effective at quickly reducing BOD in wastewater streams with moderate to high organic loads. The primary energy requirement for trickling filters is to pump water to the distribution system, making them more energy-efficient than many other aerobic treatment technologies [433]. At temperatures ranging from 5 to 15 °C, different filter media like rubber, plastic, stone, and polystyrene achieve average BOD removal rates of 81.9%, 89.4%, 93.4%, and 86.3%, respectively [434]. When used in combination with ASP or Upflow Anaerobic Sludge Blanket (UASB) systems, the overall efficiency of trickling filters can reach 80-94% [435]. However, trickling filters have some drawbacks, including a relatively low loading capacity depending on the type of filter media used and a higher tendency for media blockage.

Additionally, the sludge generated by trickling filters requires further treatment before it can be disposed of.

- **Rotating Biological Contactors (RBCs)**: these systems comprise large, rotating disks partially submerged in wastewater, providing a surface for microorganisms to attach and treat the water as it flows by Lee 2000. The disks are arranged sequentially and rotate around a horizontal axis, ensuring continuous contact with the wastewater [436]. This design facilitates efficient biological treatment by maintaining a consistent environment for microbial growth and activity. The fundamental principle resembles that of a trickling filtration system. However, with a Hydraulic Retention Time (HRT) of 32 hours, average COD removal efficiencies of 74–80% can be achieved [437]. One of the main drawbacks of operating RBCs is their reliance on a complex mechanical system, which increases the potential for mechanical failures during operation.

5.4.2. Anaerobic Wastewater Treatment (WWT)

Anaerobic WWT is a biological process that operates without the use of oxygen or air. The sequential aerobic and anaerobic technology is predominantly utilized in municipal wastewater treatment, offering greater energy efficiency and producing less sludge compared to other domestic WWT alternatives [397]. Anaerobic treatment technology offers several advantages: minimal sludge production, low energy consumption, reduced floor space requirements, minimal monitoring needs, high resistance to shock loads, and overall stability. Consequently, it is an excellent choice for treating large volumes of wastewater. During anaerobic treatment, microorganisms decompose organic compounds, generating hydrogen, carbon dioxide, and methane. Methane can constitute up to 80% of biogas production, with the remainder being carbon dioxide. Additionally, the methane produced during the process can be recycled, offering benefits similar to those of aerobic treatment technology [438].

Key Anaerobic Wastewater Treatment Technologies:

Upflow anaerobic sludge blanket reactor (UASB): is widely adopted in anaerobic WWT, first implemented by the Netherlands in 1971. Within the UASB system, a dense sludge bed forms at the bottom of the reactor, where all biological processes take place. Above this sludge bed, a

layer of finely suspended flocs forms, characterized by a slow settling velocity [439,440]. As biogas is generated within the reactor, a mixture of methane and carbon dioxide accumulates and is released through the dome [441]. Granules in the quiescent zone, which have been lifted from the tank bed due to gas formation and possess adequate settling rates, return to the bed [439]. A UASB reactor consists of four primary components: (1) a sludge/mud bed, (2) a sludge/mud cover, (3) a solid-gas separator, and (4) a resettlement compartment. Compared to ASP-based plants, the overall operation and overhead costs of UASB plants are approximately 30% lower [442]. However, a drawback of this biological treatment method is that treated effluent often requires additional treatment. Moreover, controlling the granulation process can be challenging as it depends on the characteristics of the wastewater [443]. Robust sludge bed reactors, such as upflow anaerobic sludge bed reactors, expanded granular sludge bed reactors, and their variants, constitute roughly 90% of the technologies adopted for anaerobic wastewater treatment in industrial settings [444].

- Anaerobic Baffled Reactor (ABR): The anaerobic baffled reactor (ABR) featuring horizontal flow conditions, serves as an alternative to the UASB system. Originally developed by Bachmann et al. (1985) [445], the ABR can be conceptualized as a series of UASB reactors [446]. This technology evolved from the concept of anaerobic rotating biological contactors, where McCarty (1981) [447] and Tait and Freidman (1980) [448] eliminated rotating disks and utilized suspended biomass. In the ABR, wastewater is directed from inlet to outlet, passing beneath and above a series of vertical baffle walls [449]. The ABR is capable of treating both lower and higher strength effluents, ranging from 0.3 g COD L−1 to 115 g COD L−1. It achieves a reduction of 95% COD in 10 hours Hydraulic Retention Time (HRT) for lower strength effluents and 94% COD in 6 hours HRT for effluents with 5 g COD L−1 [450]. However, a significant drawback of the anaerobic baffled reactor is its lack of supplemental systems to manage biomass accumulation during substantial fluctuations and high inlet flow peaks [451]

- Expanded granular sludge bed reactor (EGSB): part of the UASB reactor family, designed to enhance substrate-biomass interaction within the treatment system by extending the sludge bed with a high flow velocity (> 4 m h−1). This promotes hydraulic mixing and results in improved efficiency and stability compared to UASB systems [405]. According to Mmontshi et al. (2019), when treating high-strength wastewater with a COD content of 80,000 mg L−1, an average COD removal efficiency of 91% was achieved with a hydraulic retention time of 48 hours using EGSB. However, the EGSB reactor has several drawbacks,

including a slow rate of nutrient and pathogen removal and the potential for generating unpleasant odors due to methane production [452].

5.4.3. Further Methods in Secondary Treatment

Following biological treatment, the water undergoes further processes for nutrient removal, including nitrification and denitrification to manage carbon and nitrogen levels. Beyond the conventional activated sludge process, several alternative methods for secondary treatment are used in full-scale reactors. These include:

- **Membrane Bioreactors (MBRs)**: MBRs combine biological treatment with advanced membrane filtration, resulting in high-quality effluent and efficient solids separation. These systems integrate membrane processes such as ultrafiltration or microfiltration with a suspended growth bioreactor, ensuring superior removal of contaminants and improved overall treatment performance [453]. Dorr-Oliver Inc. pioneered this method in the late 1960s by combining an activated sludge bioreactor with a cross-flow membrane filtration loop. The membranes used typically consist of polymeric flat sheets with porosities ranging from 0.003 to 0.01 [454]. A non-aerobic MBR system with a relatively short Hydraulic Retention Time (HRT) of 24 hours can effectively treat high-strength organic wastewater (COD substrate ranging from 6000 to 14,500 mg L^{-1}), achieving impressive COD removal rates of 98–99% [455]. However, one of the primary drawbacks of MBR technology is its higher capital and operating costs compared to traditional wastewater treatment technologies. Regular membrane cleaning is essential to prevent fouling, necessitating skilled labor for proper operation.

- **Pond Systems:** Utilized in different configurations such as aerobic, anaerobic, facultative, and maturation ponds. An aerobic oxidation pond is a straightforward and scientifically designed pond, typically 2 to 6 feet deep, where wastewater's BOD is diminished through the action of algae and bacteria [456]. These ponds are particularly effective in warm climates and provide an economical and straightforward method for treating sewage before it is discharged into natural water bodies. The performance of these ponds is largely influenced by weather conditions such as sunlight, temperature, rainfall, and wind, as well as the quality of the incoming wastewater. Aerobic oxidation ponds are notable for their ability to significantly reduce BOD levels. Typically, they have a retention time of 2 to 6 days, handle BOD loads between 112 and 225 kg per 1,000 cubic meters per day, and can achieve BOD removal efficiencies of up to 95%. This treatment method is economical regarding its construction, upkeep, and energy

consumption. Anaerobic ponds are deep treatment facilities that operate in the absence of oxygen, providing an environment conducive for bacteria to decompose effluent. Typically, anaerobic oxidation ponds have depths ranging from 2 to 5 meters and a residence time of 1 to 2 days. They are most effective under conditions where pH is ideally below 6.2, temperatures exceed 15 °C, and organic loading capacity reaches 3000 kg ha day−1, allowing for the removal of approximately 60% of BOD [457]. These efficiencies, however, can vary depending on local climatic conditions.

- **Biofilm technology:** Biofilm-based technologies underscore the importance of microorganisms adhering to a surface [458]. Similar to human community-building, bacteria form microcolonies within biofilms, representing neighborhoods where bacteria settle after transient attachment, adaptation, and final establishment phases. Once a biofilm is established, bacteria associated with it may detach irregularly from the biofilm surface. In response to limitations of conventional anaerobic-aerobic wastewater treatment systems, new technologies such as high-rate bioreactors based on biofilm technology have been developed [452]. Below are examples of various high-rate bioreactors that utilize biofilm technology for wastewater treatment: i. **Moving bed biofilm reactor (MBBR):** MBBRs are continuous biofilm reactors similar to ASP, but they incorporate small carrier modules within the reactor that move with the water flow [453]. Compared to traditional activated sludge systems, MBBRs offer several advantages, including enhanced oxygen transfer efficiency, reduced Hydraulic Retention Time (HRT), and higher organic loading rates [405]. However, drawbacks include the need for manual monitoring and expert supervision. Additionally, MBBRs are often susceptible to pest issues, such as sewer flies, mosquitoes, and red worms, which are attracted to the reactor and consume the biofilm; ii. **Fixed-film bioreactor (FFB):** this technology integrates anaerobic and aerobic fixed-film bioreactors with structured media connected in series, optionally with recirculation. By utilizing inactive cells, it introduces additional bacterial populations arranged in a distributed framework, which offers advantages such as reduced susceptibility to environmental fluctuations and enhanced growth rates [459]. In non-aerobic fixed-film bioreactors, alongside COD removal, improved denitrification is observed as the distribution ratio increases from 1 to 6. For instance, in a study where wastewater was treated with an Organic Loading Rate (OLR) of 9 kg COD m−3 day−1, an 88.3% COD removal efficiency was achieved. However, with an 8-hour Hydraulic Retention Time (HRT), the COD removal rate slightly decreased to 84.9% [460]. A primary drawback of Fixed-Film Bioreactors

(FFBs) is their requirement for pre-treatment of suspended particles, oil, and grease. Depending on the discharge characteristics, post-treatment may also be necessary; iii. **Fluidized bed bioreactor (FBB):** operates by recirculating wastewater at a flow velocity high enough to fluidize particles that are coated with biofilm. This approach effectively overcomes the substrate diffusion limitations found in fixed-bed processes [452]. By integrating aerobic and anaerobic processes, FBBs can efficiently remove nitrogen and organic carbon from municipal sewage [461]. However, FBBs have several drawbacks. They are expensive to construct and maintain and tend to experience significant pressure drops during operation. Additionally, the fixed vessel size can lead to challenges when there are fluctuations in organic loading.

These diverse methods enhance the efficiency and sustainability of wastewater treatment, catering to different scales and types of wastewater management needs [364].

Solid wastes generated during both primary and secondary treatment stages undergo additional processing. Initially, they are transferred to a gravity-thickening tank where air is continuously supplied. This thickened sludge is then dewatered in a centrifuge and stabilized with lime, yielding treated solid waste. This byproduct can be further processed for various applications, including landfilling, fertilizer production, or as a construction material. Despite these treatments, the water may still harbor various microbial, chemical, and metal contaminants. To ensure the water is safe for reuse, such as in irrigation, it undergoes filtration and disinfection using sodium hypochlorite. Once disinfected, the water is deemed safe for agricultural use.

5.5. Tertiary or Advanced Treatment Processes

Tertiary treatment is the final and most advanced phase in wastewater treatment, designed to eliminate specific contaminants that secondary treatment may not fully address. This stage is critical for achieving the highest levels of water purity, aiming to remove up to 99% of remaining impurities. Employing sophisticated physical and chemical methods, tertiary treatment ensures near-total purification of wastewater. This process is essential for applications requiring exceptional water quality, such as discharge into environmentally sensitive areas.

5.5.1. Coagulation

Coagulation is an effective method for removing colloidal particles from water and addressing turbidity, color, natural organic matter, and heavy metals [462]. Colloidal particles typically

carry negative electrical charges, while coagulants carry positive charges. When coagulants are added, they neutralize the particles, causing them to clump together. This process is widely used in WWTPs as a tertiary treatment to improve water quality and remove contaminants. Coagulation can also remove phosphorus contamination, which is crucial since residual phosphorus in WWTP effluent can lead to eutrophication and environmental harm. Research using electrochemical coagulation demonstrated a 97% removal of phosphorus, irrespective of its initial concentration [463]. A study on the coagulation behavior of polyferric chloride showed that it can influence the density and stability of flocs, enhancing the removal of algal cells, turbidity, color, and humic acid in eutrophicated water [464]. Additionally, persistent organic pollutants in surface water, such as perfluorooctane sulfonate and perfluorooctanoate, can be removed from drinking water using alum and ferric chloride as coagulants. Factors such as solution pH, coagulant dosage, coagulant type, natural organic matter concentration, turbidity, and flocculation time can affect removal efficiency [465]. Recently, the effectiveness of coagulation technology in removing ARGs from treated wastewater has been investigated. Coagulation has proven to be an effective method for ARG removal from WWTP effluent. Li N. et al. (2017) used inorganic coagulant FeCl3 and inorganic polymer coagulant polyferric chloride (PFC) to examine the removal of sul genes, tet genes, and integrase genes. They found significant correlations between the removal of dissolved NH3-N, DOC1, and ARGs, with removal efficiencies ranging from 0.5-log to 3.1-log reductions [466].

5.5.2. In-Situ Treatment technology utilizing Plant Species

This green emerging technology employs constructed wetlands to break down organic matter in wastewater. Constructed wetlands are small semi-aquatic ecosystems that support a diverse microbial community and facilitate various physical and chemical reactions. Wetlands containing beneficial plants, such as reeds (Phragmites australis), cattails (Typha latifolia), bulrushes (Scirpus), Canna indica, and Cyperus rotundus, have become increasingly popular for WWT and are being utilized more frequently today [467]. These plants, along with associated microbial rhizobia, naturally degrade and absorb both organic and inorganic contaminants [468]. The metabolism of these plants can aid in the remediation of polluted waterways or ponds. Pollutants typically enter the plant through its roots. Plants provide unique cleansing mechanisms and offer a surface area for the adsorption and retention of water and nutrients needed for their growth. Plants can absorb these pollutants through their roots, stems, or leaves, where they may be transformed into less harmful compounds or converted into gases

that are released into the air. Phytoremediation can occur in several ways: pollutants can be stored in the harvestable parts of the plant (phytoextraction/phytoaccumulation), volatilized by the plant (phytovolatilization), metabolized by the plant (phytodegradation), or through any combination of these processes [469]. This approach is often implemented at the community level to treat domestic wastewater. It is primarily designed to promote low-cost wastewater treatment while revitalizing abundant water bodies, such as streams, lakes, and ponds. In recent years, these man-made wetlands have been developed and are now recognized as effective, simple, and cost-efficient methods for treating municipal, industrial, and agricultural wastewater, including the removal of ARGs [470]. The efficiency of ARG and antibiotic ARB removal in constructed wetlands is influenced by their characteristics, such as flow configuration, plant species, and flow types (surface flow, horizontal subsurface flow, and vertical subsurface flow). Key processes like biodegradation, substrate adsorption, and plant uptake all contribute to reducing nutrients, antibiotics, and ARGs, with biodegradation being the most crucial [297]. Constructed wetlands can effectively remove aqueous ARGs but can also act as reservoirs for specific ARGs. A study by Fang et al. (2017) recommended constructed wetlands for domestic sewage treatment, achieving removal rates of 77.8% in winter and 59.5% in summer for 14 targeted ARGs in integrated surface flow wetlands. This study also found strong positive correlations between intI1 concentrations and ARGs, indicating the role of mobile genetic elements in ARG dissemination in constructed wetlands [470]. Research on the removal of ARB and ARGs from raw domestic wastewater using different types of constructed wetlands examined 8 antibiotics, 12 genes, and 16S rRNA (bacteria) across various matrices. The removal efficiencies of total antibiotics ranged from 75.8% to 98.6%, while total ARG removal varied between 63.9% and 84.0%. The presence of plants improved pollutant removal, and subsurface flow wetlands were more effective than surface flow wetlands, especially for antibiotics [297]. Certain naturally growing plants can be highly effective for wastewater treatment by utilizing pollutants and contaminants as nutrients [471]. Phytoremediation, a method renowned for its cost-effectiveness and energy efficiency, can be conveniently implemented on-site where wastewater is generated [472]. One form of phytoremediation, the constructed wetland method, offers numerous advantages: minimal monitoring, low operational costs, low energy consumption, easy maintenance, and high pollutant removal efficiency. Compared to traditional secondary biological treatment technology, it is more economical in both construction and operation [473]. Nizam et al. (2020) studied five plant species—Centella asiatica, Ipomoea aquatica, Salvinia molesta, Eichhornia crassipes, and Pistia stratiotes—and found them remarkably effective in reducing pollutants. These plants significantly decreased

TSS, ammoniacal nitrogen (NH_3-N), and phosphate levels by 63.9-98% [474]. Coleman et al. (2001) examined the effectiveness of three Appalachian plant species: common rush (Juncus effusus L.), gray club-rush (Scirpus validus L.), and broadleaf cattail (Typha latifolia L.) [475]. Their research showed a reduction of about 70% in TSS and BOD, and a 50-60% reduction in nitrogen, ammonia, and phosphate levels. Additionally, these plants significantly reduced fecal coliform populations. Zamora et al. (2019) explored the use of ornamental plants—Canna indica, Cyperus papyrus, and Hedychium coronarium—for wastewater treatment [476]. They reported up to 20–60% higher removal efficiency for COD, TSS, nitrogen in the forms of ammonium (N-NH_4) and nitrate (N-NO_3), and phosphate (P-PO_4) compared to other methods. Moreover, microalgae have proven to be an effective and economical method for wastewater treatment. In substantial quantities, microalgae can remove nitrogen and phosphorus, degrade refractory organics, and absorb heavy metal ions such as cobalt, manganese, and mercury [477]. Utilizing plants for wastewater treatment is generally less expensive than conventional methods, as it requires fewer resources and less energy. Furthermore, this method relies on the natural biological processes of plants, reducing the need for energy-intensive mechanical or chemical treatments.

5.5.3. Disinfection

Disinfection of water and wastewater involves processes that eliminate a significant proportion of pathogenic organisms responsible for bacterial, viral, or parasitic diseases. Chlorination is the most commonly used method in wastewater treatment, although ozone and UV radiation are also employed [478]. Chlorine, in particular, is extensively used as a disinfectant in municipal sewage treatment plants because of its high efficacy, low cost, and ease of application [479]. Chlorination involves adding chlorine or chlorine-containing compounds to treated wastewater to eliminate or reduce pathogenic microorganisms. This process works by breaking down the cell walls and interfering with the metabolic processes of bacteria, viruses, and other harmful organisms present in the water. While chlorine effectively inactivates pathogenic microorganisms, it also reacts with organic matter in sewage effluents, resulting in the formation of unintended disinfection byproducts (DBPs) [480-482]. Common methods of chlorination in wastewater treatment include gaseous chlorination, where chlorine gas is directly bubbled into the water, and liquid chlorination, where chlorine solutions are added in measured amounts to achieve the desired disinfection levels. It is crucial to carefully monitor and control the dosage of chlorine to prevent the formation of harmful disinfection by-products

(DBPs), which can have environmental and health implications. Chlorination is the predominant disinfection method in wastewater treatment plants before discharge. However, there are indications that chlorination within these plants might facilitate the transfer of ARGs and increase the concentration of ARB, raising concerns about the potential selection of pathogens and the rates of regrowth among indigenous bacteria [483]. To make the treated water safe for drinking or other high-standard uses, advanced methods are applied, either individually or in combination. These methods include ultrasonication (US), ultraviolet light treatment (UV), and ozone exposure (O_3), which are highly effective in eradicating bacteria and heavy metal contaminants. In the tertiary process, the secondarily treated water first undergoes ultrasonication. This technique employs high-frequency sound waves to create cavitation bubbles that collapse, generating powerful shear forces and free radicals. These forces disrupt the cell membranes of microorganisms and enhance the efficiency of subsequent treatments. Following ultrasonication, the water is exposed to ultraviolet light, which penetrates and destroys the DNA of any remaining pathogens, rendering them harmless. Finally, the water passes through an ozone chamber where ozone gas acts as a potent oxidant, breaking down organic molecules and neutralizing any residual contaminants [484,485]. The combined application of US, UV, and O_3 treatments synergistically enhances the purification process. Ultrasonication alone can cause cell membrane disruption through free-radical attacks and mechanical forces, facilitating the penetration of chemical disinfectants and enhancing their effectiveness. When paired with UV and ozone treatments, the process generates additional free radicals that attack the biological contaminants' cell membranes, allowing chemical oxidants to infiltrate and dismantle the internal cellular structures. This comprehensive approach not only deagglomerates microorganisms but also increases the overall efficiency of the disinfection process [484,485]. Studies have demonstrated the efficacy of these combined treatments across various applications. For example, ultrasound pre-treatment followed by UV irradiation has proven effective for textile wastewater treatment, improving the breakdown of complex pollutants [486,487]. Moreover, integrating UV radiation with TiO_2 photocatalysis [488] or combining ultrasound with ozone has shown to optimize the disinfection process, ensuring that the treated water meets the highest standards of safety and purity for reuse or discharge [489]. Effective disinfection is crucial and routinely practiced to disrupt ARB and ARGs and to inactivate harmful microorganisms. Studies have examined the impact of chlorination on the removal of various antibiotics such as cephalexin, ciprofloxacin, chloramphenicol, erythromycin, gentamicin, rifampicin, sulfadiazine, tetracycline, and vancomycin, as well as its effectiveness in inactivating ARB and ARGs. Advanced oxidation processes, including ozone,

UV irradiation, Fenton reagent, and photocatalytic systems, have also been applied to enhance disinfection. Some research has compared the efficacy and mechanisms of two or more of these processes [178,490].

6. MATERIALS AND METHODS

This research project has redefined wastewater from an unwanted by-product into a valuable resource for assessing and monitoring public health within a One Health framework. Through advanced wastewater analysis, known as WBE, we can obtain a dynamic snapshot of community health, including microbial contamination and exposure levels. This innovative approach positions WBE as a green strategy for public health by enabling early detection and sustainable monitoring of environmental and health threats. WBE not only provides insights into the overall health status of a population but also serves as an effective tool for the indirect monitoring of epidemics, offering real-time data for public health interventions. Over this three-year project, a scoping review was conducted to investigate the role of wastewater and treatment plants in the development and spread of antimicrobial resistance in contaminated environments. In parallel, a scoping review was carried out to map the current research on biosensors for detecting microorganisms ARGs and antibiotics in clinical and environmental samples. Additionally, from February 2022 to September 2024, wastewater samples were systematically collected from three treatment plants: Pantano D'Arci in Catania, Mascali in Giarre, and a plant in Siracusa. Starting in July 2024, monthly wastewater samples, both pre- and post-treatment, were also collected at Catania Fontanarossa International Airport. These samples were analyzed using protocols designed to monitor ARGs and to detect and quantify the presence of SARS-CoV-2. Therefore, in the following sections, I present the results of the scoping review, which explore how wastewater and treatment plants contribute to the evolution and spread of AMR in contaminated environments. The scoping review examines the role of wastewater in the dissemination of AMR, with a particular focus on the impact of antibiotic residues, heavy metals, and microbial community composition on resistance development. It also discusses how various wastewater treatment methods and water quality parameters can either mitigate or exacerbate the spread of AMR. The mechanisms by which resistance genes are transferred from the environment to humans are also outlined. Furthermore, this thesis describes the results of the scoping review, which assess the current understanding of biosensors as cutting-edge technologies, highlighting their potential applications for detecting microorganisms, ARGs, and antibiotics across different matrices. Instead, the subsequent sections describe the laboratory work carried out to monitor the COVID-19 pandemic, track the presence of antimicrobial resistance genes, and assess the environmental benefits and risks associated with wastewater reuse within a circular economy framework. These sections will outline the methodologies

employed and present the results obtained, offering a comprehensive overview of the work carried out.

6.1. First Section: Scoping Review "The Impact of Wastewater on Antimicrobial Resistance: A Scoping Review of Transmission Pathways and Contributing Factors"

Study design

The scoping review "Impact of Wastewater on Antimicrobial Resistance: A Scoping Review of Transmission Pathways and Contributing Factors" was conducted according to the Preferred Reporting Items for Systematic reviews and Meta-Analyses extension for Scoping Reviews (PRISMA-ScR) protocol. This approach is particularly suitable for mapping the broad issue of AMR spread via wastewater, allowing for the identification of key concepts, gaps, and a wide range of evidence types. To ensure comprehensive coverage of relevant studies, the review includes research that examines wastewater as a source of AMR, encompassing hospital effluents, industrial wastewater, urban sewage, and agricultural runoff. The review focuses on studies that explore the pathways through which AMR spreads and the factors that influence this process. There are no geographical restrictions, and both quantitative and qualitative empirical research will be included. Studies that focus solely on AMR without any mention of wastewater and/or WWTPs are excluded, as are articles not available in English.

Search strategy

The search for relevant literature published up to June 2024 was conducted across PubMed and Web of Science databases. Additional studies were identified through citation chasing, using the reference lists of selected articles to find further relevant research. The search strategy was designed with the following combination of key terms: ((Drug Resistance) OR (Antibiotic Resistance) OR (Antimicrobial resistance) OR (Antibacterial Resistance)) AND (Wastewater OR (Waste Water*)).

Study selection

All retrieved studies were imported into reference management software (e.g., EndNote) to remove duplicates. Two independent reviewers then screened the titles and abstracts to evaluate

their relevance according to the predefined inclusion and exclusion criteria. In cases of disagreement, a third reviewer was consulted to resolve any inconsistencies.

Data extraction and synthesis

A data extraction form was developed to collect key information, including study characteristics (authors, year, location, study design), identified AMR pathways, influencing factors, proposed or studied interventions, and study outcomes related to the spread of AMR. The data were then synthesized thematically to provide a structured understanding of the findings.

6.2. Second Section: Scoping Review "Updates on developing and applying biosensors for the detection of microorganisms, antimicrobial resistance genes and antibiotics: a scoping review"

For the present scoping review, the Preferred Reporting Items for Systematic Reviews and Meta-Analyses extension for Scoping Reviews (PRISMA-ScR) checklist was employed. To ensure comprehensive coverage of relevant literature, a search was conducted across the following databases, covering the period from 1999 to November 2022: MEDLINE, EMBASE, Web of Science, BIOSIS Citation index, Derwent Innovations index, and KCI-Korean Journal database. The search strategy consisted of the following combination of terms: (Biosensors OR Biosensor OR Bioprobes OR Bioprobe) AND (Drug Resistances, Microbial OR Antimicrobial Drug Resistance OR Antimicrobial Drug Resistances OR Antibiotic Resistance OR Antibiotic Resistances). To be included in the scoping review, articles needed to report studies using biosensors to collect data about the presence of microorganisms, ARGs and antibiotics in clinical and/or environmental samples. Studies were selected only if they: (i) were written in English, (ii) included the development and validation of biosensors for the detection of microorganisms and/or genes of resistance and/or antibiotics, and (iii) using clinical and/or bacterial and/or environmental samples. Articles that did not align with the primary focus of the scoping review were excluded. Additionally, abstracts, editorials, commentaries, reviews, systematic reviews, and meta-analyses were also excluded from the study. The study selection and data charting processes were conducted independently by two authors using a standardized data abstraction tool specifically developed for this scoping review. The tool systematically captured essential details about the articles, including: (i) author information, (ii) publication year, (iii) country of origin, (iv) comprehensive information about the sensors employed (e.g., sensor type, target, and sample types), and (v) the key findings obtained. In the event of any disagreements, they were resolved through discussion between the two authors or, if necessary,

by involving a third author for further adjudication. Studies were grouped by the type of target considered (i.e., detection of microorganism, antimicrobial resistance genes and/or antibiotics), and summarized describing the development and application of these biosensors in clinical, bacterial and environmental samples.

6.3. Third Section: Biological Samples and Laboratory Activities

This research includes 316 untreated waste-water samples collected from three Wastewater treatment plants (WWTPs) of Sicilian municipalities between February 2022 and September 2024, located in Catania (Pantano D'Arci), Giarre (Mascali) and in Siracusa respectively. Additional details regarding the location and characteristics of the WWTPs can be found in Figure 1 and Table 1. In particular, from February 2022 to March 2023, weekly sampling led to the collection of 262 wastewater samples. Following this period, from April 2023 to September 2024, the sampling frequency shifted to a monthly schedule, conducted during the first week of each month, resulting in the collection of an additional 54 wastewater samples. Starting from May 2024, to evaluate the environmental benefits and risks associated with the reuse of wastewater within a circular economy framework, samples were collected both before and after water treatment. This approach allowed for the analysis of the effectiveness of treatment systems in removing or reducing SARS-CoV-2. Therefore, a total of 26 samples were collected, with 13 taken at the entrance of the treatment plant, before any treatment, and 13 at the exit, following treatment. In addition, since July 2024, wastewater samples have been collected from Catania Fontanarossa International Airport, alongside the three existing sites (Pantano D'Arci, Giarre, and Siracusa). The airport's treatment facilities process wastewater that reflects the prevalence of microorganisms, antimicrobial agents, and ARGs from travelers worldwide, with treated water being discharged into the local environment. Notably, samples were taken both before and after treatment, with four samples collected so far—one pre-treatment and one post-treatment for both July and August.

In general, the sampling was carried out by means of automatic samplers in order to obtain the composite average of the 24 hours. Wastewater samples (500 mL) were collected in 500–1000 mL polyethylene bottles equipped with safety caps. The bottles were labeled with location and date and disinfected externally before storage. All samples were stored at 4°C and processed whenever possible within 6-24 hours of collection (Ahmed et al., 2020; Hutinel et al., 2022). Specifically, of the 316 samples collected from Catania (Pantano D'Arci), Giarre (Mascali) and in Siracusa, 188 were analyzed fresh, while 128 were frozen at -20°C and analyzed a few days

later. In contrast, all samples collected from the airport were analyzed fresh within 6-24 hours of collection. The laboratory analyses related to sample concentration, nucleic acid extraction, and SARS-CoV-2 detection were conducted in accordance with the national protocol established for the SARI network (Istituto Superiore di Sanità - Surveillance Protocol of SARS-CoV-2 in Municipal Wastewater (SARI) - Rev.3). The relative abundance of various ARGs in wastewater samples was determined using the TaqPath™ qPCR Master Mix protocol.

6.3.1. Concentration of the waste-water samples

A 50 ml aliquot was immediately frozen for potential confirmatory tests or further analyses. The second 45 ml aliquot was promptly processed.

The concentration of urban wastewater samples was performed starting with a volume of 45 ml, using a PEG/NaCl protocol based on the method published by Wu et al. (2020) [41]. The procedure involves the following steps:

1. **Thermal Pretreatment:** The sample undergoes thermal pretreatment in a water bath at 56°C for 30 minutes to inactivate any infectious viral particles present.
2. **Cooling:** The sample is then allowed to cool.
3. **Centrifugation:** The sample is centrifuged at 4500 x g for 30 minutes at +4°C to remove larger particles and debris.
4. **Addition of Reagents:** After centrifugation, 40 mL of sample was mixed with Polyethylene glycol (PEG 8.000) at 8% (wt/vol) and NaCl at 0.3 M) (both supplied by Sigma-Aldrich, St. Louis, MO, USA) and spiked with a known amount of Murine Norovirus, used as a process control.
5. **Final Centrifugation:** The mixture is centrifuged at 12000 x g for 2 hours.
6. **Pellet Collection:** The resulting pellet is collected for the subsequent extraction phase.

6.3.2. Nucleic acid extraction

The final pellet was resuspended in 2 mL of NucliSENS Lysis Buffer (bioMérieux, Marcy-l'Étoile, France) for nucleic acid extraction. The extraction process started with a cell lysis step using guanidine isothiocyanate, which degrades and inactivates proteins and cellular components, including RNases and DNases. Nucleic acid extraction was then performed using

magnetic silica beads on the eGeneUP platform (bioMerieux Italia Spa, Via di Campigliano, 58 | Loc. Ponte a Ema-50012 Bagno a Ripoli (FI), Italy), following the manufacturer's instructions. This platform is based on the adhesion of nucleic acids onto magnetic silica. Specifically, after a brief 20-minute incubation with the NucliSENS Lysis Buffer reagent, 50 µL of magnetic silica was added to the mixture. The sample was then allowed to sit at room temperature for 10 minutes. Subsequently, a series of washes were performed to remove residual impurities. The sample was then eluted with TE buffer (pH 8.0), yielding a final volume of 100 µL. The eluted nucleic acids were further purified using the OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA) and stored at −80 °C until molecular analysis. The concentration of the extracted DNA was measured using a Qubit fluorometer and the dsDNA HS Assay Kit (Life Technologies) [491].

6.3.3. Detection of SARS-COV-2 by Real-time RT-PCR

To assess the concentration and extraction efficiency of the method, during the concentration process, 100 µL of a process control virus solution (Murine Norovirus at 2.0×10^3 genomic copies/µL) was added to each wastewater sample.

Quantitative detection of SARS-CoV-2 was performed via real-time RT-qPCR in accordance with the EU 2021/472 Recommendation [123]. The quantitative determination of SARS-CoV-2 targeted the ORF-1**a**b (nsp14) region of the genome (with an assay described in La Rosa et al. 2021), with Murine Norovirus used as a control virus during the analysis [120]. The qPCR was performed on a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Carlsbad, CA, USA), according to the manufacturer's protocol, while used to prepare the mixture for RT-qPCR were AgPath-ID One-Step RT-PCR (Life Technologies), primer 2297-CoV-2-F, primer 2298-CoV-2-R and probe 2299-CoV-2-P. For the preparation of the reaction mix in a 96-well Fast (0.1-mL) plate, a total volume of 20 µL per reaction was used, consisting of: 12.5 µL of 2× RT-PCR Buffer to achieve a final concentration of 1×, 1 µL of Primer F 2297 (12.5 µM) to reach a final concentration of 500 nM, 1 µL of Primer R 2298 (22.5 µM) for a final concentration of 900 nM, 1 µL of Probe 2299 (6.25 µM) to achieve a final concentration of 250 nM, 1 µL of 25× RT-PCR Enzyme Mix, 1.67 µL of Detection Enhancer (optional), and 1.83 µL of Nuclease-free Water to bring the final volume to 20 µL. Additionally, 5 µL of sample was added, bringing the total reaction volume to 25.0 µL. For the preparation of the reaction mix for the detection of Murine Norovirus (MNV), a total volume of 20 µL per reaction was used. The components and their respective volumes per reaction are as follows: 12.5 µL of 2×

RT-PCR Buffer to reach a final concentration of 1×, 1 µL of Primer F MNV (12.5 µM) for a final concentration of 500 nM,1 µL of Primer R MNV (22.5 µM) reaching a final concentration of 900 nM, 1 µL of Probe MNV (5 µM) for a final concentration of 200 nM, 1 µL of 25× RT-PCR Enzyme Mix and 3.5 µL of Nuclease-free Water to bring the final reaction volume to 20 µL ± 0.2 µL. All samples were analysed in duplicate in qPCR. Each PCR run included RNA from the SARS-CoV-2 Alpha variant, kindly provided by the Infectious Diseases Department of the National Institute of Health-ISS, as a positive control, and molecular grade water as a negative control. The synthetic RNA standard containing the SARS-CoV-2 target sequence used in real-time RT-qPCR was included in three serial dilutions, each tested in triplicate. This was done to achieve the following objectives: a) serve as a positive control for the PCR; b) assess PCR efficiency through the analysis of serial RNA standard dilutions; c) function as an external control for PCR inhibition (RNA-EC). The amplification conditions were: reverse transcription for 30 minutes at 50 °C, inactivation for 10 minutes at 95 °C, and 45 cycles of 15 seconds at 95 °C and 45 seconds at 60 °C. Analyses were considered acceptable if the standard curves for the quantification of SARS-CoV-2 provided a slope close to −3.32 (minimum −3.1, maximum −3.6) and a regression coefficient equal to or above 0.98 ($R^2 \geq 0.98$), and if the other quality controls were within acceptability limits (recovery ≥ 1% and PCR inhibition ≤ 50%). Indeed, the sample was considered acceptable if the concentration/extraction efficiency was ≥1%, as suggested by the protocol of National Institute of Health (NIH). To verify inhibition, the Ct value of the reaction containing the sample supplemented with 1 µl of control RNA was compared to the Ct value of the reaction containing molecular biology-grade water supplemented with 1 µl of RNA, according to the following formula:

ΔCt = Ct (sample + control RNA) - Ct (water + control RNA)

The sample was considered acceptable if ΔCt≤ 2 (corresponding to a 75% inhibition of PCR).

To verify the efficiency of concentration/extraction of the process control virus, the Ct value of the reaction containing the sample was compared with the Ct value of the reaction containing the undiluted RNA of the process control virus, according to the following formula:

Efficiency of concentration/extraction (%) = $10^{(\Delta Ct/m)} \times F \times 100$

where

ΔCt= Ct (sample) - Ct (undiluted RNA of the process control virus)

m = slope of the standard curve of the process control virus

F = fraction of the initial sample subjected to the concentration and extraction process

The sample was considered acceptable if the concentration/extraction efficiency was ≥1%.
For each sample and for each replicate of each sample, the concentration of SARS-CoV-2 was calculated in genomic copies /µL of RNA, referring to the calibration curve produced by the serial ten-fold dilutions of the dsDNA standard containing the sequence target of the virus and provided by the National Institute of Health (Italy). Samples with a Ct value ≤40 were considered positive. In particular, the concentration of SARS-CoV-2 in genomic copies /L of wastewater was calculated as follows:

SARS-CoV-2(c.g./L wastewater) = C × 100 × 25
where
C = genomic copies /µL of RNA
100 = total volume of extracted RNA sample
25 = proportional factor between the analyzed quantity and the reference volume (1 L).
See the table 2.

6.3.4. Preparation of composite wastewater samples and Real-Time PCR for detecting the relative abundance of ARGs in wastewater samples

For each of the three WWTPs, a monthly composite DNA extract was prepared to eliminate daily fluctuations. This was done by combining the DNA extracts from samples collected weekly over each month at each WWTP to achieve an equal volume of DNA. Covering the period from February 2022 to March 2023, a total of 14 samples were obtained for each WWTP. Therefore, 42 composite wastewater samples were analyzed: 14 samples from the Pantano D'Arci site, 14 samples from the Siracusa site, and 14 samples from the Giarre site, each representing one month from February 2022 to March 2023.
The relative abundance of various ARGs in wastewater samples was determined using real-time PCR (QuantStudio™ 7 Flex Real-Time PCR System, Applied Biosystems, Foster City, CA, USA), following the TaqPath™ qPCR Master Mix protocol. For the preparation of the reaction mix for a 96-well Fast (0.1-mL) plate, 10.0 µL of TaqPath™ qPCR Master Mix, CG, was used per reaction. This mix, at a 2X concentration, provided all the necessary components for the PCR reaction, including the enzyme, buffer, and dNTPs.In addition, the TaqMan® Gene Expression Assay, which contains the specific primer and probe set required for gene expression analysis, was added. The volume of this component varied depending on the assay used, but it did not exceed 8.0 µL per reaction. Then, nuclease-free water was added in a variable volume to bring the total reaction volume to 18.0 µL. The amount of water added depended on the

volume of the TaqMan® Gene Expression Assay used. After mixing all the reaction components, 18.0 µL of the prepared reaction mix was dispensed into each well of the 96-well Fast PCR plate. Additionally, 2 µL of template DNA was added, bringing the total reaction volume to 20.0 µL. All samples were analysed in triplicate.

Each sample was analyzed for the following ARGs:

- SHV class A beta-lactamases (blaSHV)
- OXA-48 family OXA class D beta-lactamases (blaOXA)
- Subclass B1 metallo-beta-lactamases (blaNDM)
- Subclass B1 metallo-beta-lactamases VIM (blaVIM)
- Class A extended-spectrum beta-lactamases (blaTEM)
- Class A extended-spectrum beta-lactamases CTX-M (blaCTX-M)
- 23S rRNA (adenine(2058)-N(6))-methyltransferase Erm(A) (erm(A))
- 23S rRNA (adenine(2058)-N(6))-methyltransferase Erm(B) (erm(B))

Specifically, for the blaNDM and blaCTX-M genes, more than one probe was used, capable of identifying different sequences within the same gene.
The 16S rRNA gene was used as a reference for qPCR measurements to normalize the relative abundances of ARGs in the wastewater samples.
The amplification conditions are described in Table 3.
The mean cycle threshold (CT) from three technical replicates of each qPCR reaction was used to calculate ΔCT values. If a gene was detected in only one of the three replicates, it was excluded from analysis. Relative gene abundances were determined using the $2^{-\Delta CT}$ method, where ΔCT is defined as the difference between the CT of the target gene and the CT of the 16S rRNA gene (ΔCT = CT detected gene - CT 16S rRNA gene). This approach allowed for the calculation of gene abundances relative to the 16S rRNA gene in each sample.
It's important to emphasize that qPCR provides a key advantage in monitoring antibiotic resistance by enabling the analysis of DNA from the entire microbial community within a water sample. This comprehensive approach allows for a more accurate and sensitive detection of resistance genes across diverse bacterial populations. Unlike culture-based methods, qPCR avoids biases such as the need to focus on specific bacterial species or genera and the challenges posed by selective media, which often struggle to isolate and quantify targets amidst non-target

environmental bacteria. Moreover, culture-based approaches fail to detect viable but non-culturable organisms [492]. However, a major challenge in qPCR-based monitoring of antibiotic resistance is selecting the most appropriate target ARG(s), given the vast number of gene variants available [493]. The clinical relevance, mobility, and sensitivity of individual ARGs to anthropogenic inputs, as well as to selective and co-selective agents like antibiotics and metals, can vary considerably [494,495]. qPCR also has inherent limitations. For instance, it cannot differentiate between DNA from viable versus non-viable bacteria or detect extracellular DNA. Additionally, it relies on primers designed for previously known ARG variants, which may not capture all gene variants present in the environment [496].

6.3.5. Statistical analysis

Descriptive analyses were performed to characterize the collected data and provide an overview of the main variables' distributions. Continuous variables were described using means and standard deviations for normally distributed data, while medians and interquartile ranges (IQR) were used for non-normally distributed data. Categorical variables were summarized through frequencies and percentages. To visually represent the data in a clear and intuitive manner, bar plots were used to display the distributions of categorical variables, and line plots were employed for temporal trends. A correlation analysis was conducted to explore linear relationships between variables, utilizing Pearson's correlation coefficient for normally distributed variables or Spearman's correlation coefficient for non-normally distributed ones. Joinpoint regression, conducted using the Joinpoint Regression Program (version 4.3.1.0) provided by the Surveillance, Epidemiology, and End Results Program (National Cancer Institute; http://surveillance.cancer.gov/joinpoint/), is a powerful analytical method for assessing trends over time. This approach is particularly useful for analyzing rates, proportions, or any time-dependent measure. Its primary objectives are twofold: first, to identify potential time points where significant changes in trends occur (known as joinpoints), and second, to estimate the regression function with these joinpoints. What distinguishes joinpoint regression from other similar models, such as piecewise or segmented regressions, is its emphasis on continuity at the change-points. Unlike these models, joinpoint regression does not abruptly transition between segments but maintains smooth transitions. Furthermore, joinpoint regression automatically determines the number and placement of joinpoints within the model, rather than requiring a predetermined specification. The process of determining joinpoints involves a grid search method. This method entails creating a grid representing all potential

positions for joinpoints and fitting the model for each combination. The optimal number and placement of joinpoints are then determined by minimizing the sum of squared errors (SSE). This selection process involves hypothesis testing, wherein simpler null models are compared against more complex alternative models until an optimal fit is achieved. To account for multiple comparisons, an approximate permutation Monte Carlo method is employed to calculate p-values under the null hypothesis. Additionally, Bonferroni adjustment is applied to control the overall type I error rate. Ultimately, the selected joinpoint regression model is chosen for its superior fit compared to alternative models within the specified range of potential joinpoints. We applied the joinpoint regression model on the logarithmically transformed outcome variable, employing 5,000 permutations and assuming uncorrelated errors. We computed the Weekly Percent Changes (WPCs) and their 95% Confidence Intervals (95%CIs), revealing the average weekly percentage change between identified joinpoints.

7. RESULTS

7.1. Results of the Scoping Review on "The Impact of Wastewater on Antimicrobial Resistance: Transmission Pathways and Contributing Factors"

Figure 2 provides a detailed summary of the study selection process. After removing duplicates, a total of 3,367 articles were identified through the literature search. During the initial screening of titles and abstracts, 3133, articles were excluded, leaving 234 full-text articles for a more in-depth evaluation. Of these, 180 were excluded for the following reasons: 6 lacked access to full-text, 57 were reviews, 30 were not in English,
and 87 were not aligned with the objectives of the scoping review. As a result, 54 studies were deemed eligible and included in this scoping review. Additionally, 16 studies were identified and included through manual bibliographic searches.

In the following sections, we summarize key studies investigating the role of wastewater in the spread of AMR. We begin by examining the presence of antibiotics in wastewater, highlighting how antibiotic residues and heavy metals contribute to the development and amplification of resistance. Next, we analyze the composition of microbial communities within wastewater ecosystems and their role in promoting AMR dissemination. We then evaluate how various wastewater treatment methods and water quality parameters either curb or facilitate the spread of AMR. Finally, we outline the primary mechanisms through which resistance genes are transferred from environmental reservoirs to humans, emphasizing the critical pathways involved in this process.

Role of Hospital and Industrial Wastewater in the Spread of Antimicrobial Resistance

Clinical wastewater is a major contributor to the dissemination and development of ARGs. Hospital effluents, which are recognized as key reservoirs of these resistance genes, release both bacteria and ARGs into natural water bodies. Hospital wastewater consistently showed higher concentrations of ARGs compared to the raw wastewater entering WWTPs. In qualitative screenings, researchers identified 23 distinct ARGs in hospital wastewater, with 16 of these genes found in more than 75% of the samples. This highlights the significant and concerning role of hospital wastewater in the propagation of antibiotic resistance [497]. Specifically, members of the genus Aeromonas carrying quinolone resistance genes and beta-lactamases, aac(6′)-Ib-cr and blaOXA, have been highlighted as significant markers for tracking

antibiotic resistance in wastewater habitats (these genes were found in 58% and 56% of isolates, equally prevalent in both hospital effluents (HE) and urban wastewater treatment plants (UWTP)). This exposure can lead to the integration of these resistance elements into local microbial communities, particularly under conditions that favor selection pressure, such as the presence of antibiotic residues. In contrast, the qnrS2 gene, which facilitates tracking resistance from non-clinical sources, is mainly found in UWTP isolates (51%) and rarely in HE isolates (3%), suggesting a non-clinical origin [498]. Specifically, a high frequency of E. coli FosR has been found in wastewater samples from clinical sources. The persistence of FosR bacteria in hospital and municipal wastewater also depends on water properties, such as antimicrobial residues or other substances (e.g., biocides) that can certainly influence bacterial persistence due to selection pressure and co-selection. In particular, ESKAPE bacteria and E. coli have been commonly identified and reported to efficiently adapt their genomes based on prevailing conditions, exchanging MGEs with the environment or other bacteria. Indeed, the results suggest that the spread of fosA4 and fosA3 genes mediated by plasmids is one of the main mechanisms of fosfomycin resistance dissemination[499]. An intriguing discovery was made in February 2022 when two bacterial isolates were found in treated wastewater that were almost identical to two clinical isolates from a patient hospitalized at the same facility three months later, in May 2022. These four ST919 strains of Acinetobacter baumannii—two from wastewater and two from the patient—were clonal, exhibiting only four to nine single nucleotide polymorphisms (SNPs) differences, and all harbored both the blaNDM-1 and blaOXA-23 antibiotic resistance genes. Although the current data do not clarify the direction or connection of transmission between these strains, the findings suggest a probable transmission link between the hospital environment and the human population. The close genetic relationships and clustering patterns observed between the clinical and wastewater isolates, even in the final treated effluent, point to possible transmission chains from the hospital environment—or even directly from patients—to the surrounding environment that receives the treated wastewater. Additionally, the presence of carbapenemase genes, which were significantly associated with the presence of multiple antimicrobial resistance determinants, was remarkably high among the wastewater isolates. This emphasizes the potential role of hospital wastewater as a reservoir and a conduit for the spread of highly resistant bacterial strains [500]. Furthermore, the release of industrial effluents also leads to elevated levels of resistance genes in the environment. Notably, the transferable resistome appears to be the primary mechanism contributing to the persistence of macrolide resistance genes in downstream sediments following the discharge of inadequately treated effluents from

azithromycin production into receiving rivers. All targeted resistance genes persisted in sediments up to 11 kilometers downstream. It was revealed that among the five macrolide-resistance genes studied, three specific subtypes—encoding macrolide phosphotransferase (mphG), ribosome protection protein (msrE), and ribosomal methylase (ermB)—were predominant in the examined effluents. Their relative abundance was notably increased in sediments receiving the effluents. Additionally, although the mefC and mphE genes, which encode efflux and macrolide phosphotransferase, respectively, were less prevalent in effluents compared to msrE, mphG, and ermB genes, they showed significant increases in abundance in exposed sediments [501].

Marathe NP et al. have pointed out that WWTPs serving antibiotic manufacturing industries could serve as breeding grounds for such events due to the high bacterial densities and intense, persistent selection pressures from antibiotic-contaminated waste. Their qPCR analysis of community DNA revealed an exceptionally high prevalence of integrons, indicating that bacteria in these high-pressure environments have a significant potential for the genetic exchange of resistance genes via mobile gene cassettes [502]. It was revealed that the total abundance of ARGs in sludge from a treatment facility receiving wastewater from pharmaceutical manufacturing was three times higher compared to municipal sludge from a sewage treatment plant in Zagreb, Croatia. Specifically, the overall quantity of ARGs per 16S rRNA gene copy was approximately three times greater in the industrial sludge than in the municipal sludge ($p = 3.24 \times 10^{-5}$). A significantly higher total abundance of integrases and transposases was observed in the industrial samples ($p = 1.32 \times 10^{-6}$), suggesting an enrichment of mobile antibiotic resistance genes. Additionally, the relative abundance of plasmids in the industrial samples was elevated ($p = 1.95 \times 10^{-6}$). Moreover, the diversity of detected plasmids was also greater in the industrial samples compared to the municipal ones, with an average of 1,497 versus 926 plasmid types ($p = 0.00067$). The collective results imply that in environments with high levels of antibiotic contamination, the selection pressure predominantly favors taxonomic changes towards inherently resistant communities or strains carrying resistance mutations. However, the total count of distinct ARGs, known as resistance gene richness, was lower in the industrial sludge compared to the municipal sludge ($p = 0.00136$). Significantly, although the total abundance of macrolide resistance genes did not surpass that of a municipal WWTP, the presence of resistance genes typically linked with MGEs like integrons was higher. This indicates that heightened antibiotic exposure leads to augmented genetic mobility within microbial communities. The absence of elevated levels of macrolide resistance genes suggests that the intense selection pressure exerted by macrolide antibiotics has prompted taxonomic

shifts towards inherently resistant species or strains harboring chromosomal resistance mutations, rather than the acquisition of mobile resistance determinants for macrolides [503]. Khanal S et al. found that E. coli isolates producing Extended-Spectrum Beta-Lactamases (ESBL), with an average of more than 55 ARGs, were predominantly found in municipal wastewater and river sites where treated wastewater is discharged. In contrast, Klebsiella pneumoniae was more prevalent in wastewater from pharmaceutical and hospital sources, but these isolates contained an average of only 40 ARGs. The data indicated that municipal and hospital wastewaters are significant contributors to environmental antibiotic resistance. This was evidenced by the higher presence of ESBL phenotypes and bacteria carrying the blaCTX-M gene in river waters compared to the originating wastewater sources. The plasmid replicons and MGEs found in these isolates demonstrated a high potential for spreading resistance genes across multiple antibiotics. Although the incidence of ESBL bacteria was notably reduced in treated hospital and municipal wastewater effluents, this level of reduction was not observed in treated effluents from pharmaceutical wastewater treatment plants [504]. Anthropogenic pollution significantly influences both the microbial community and the antimicrobial resistome. It was found that the diversity of ARGs was notably higher in post-disinfected wastewater (TWW) compared to lake water, even when considering high-risk ARGs alone. This suggests that pollution from human activities boosts the variety of ARGs in treated wastewater. Additionally, regardless of the specific ARG-positive plasmids tested, pollution positively affected the rates at which extracellular antimicrobial-resistant plasmids transformed and were selected in the microbial community [505].

Occurrence of antibiotics in wastewater

Antimicrobial substances are released into the environment through human-derived wastewater from various sources, including households (domestic), hospitals (clinical), veterinary and animal farming, as well as pharmaceutical factories (industrial) [506]. Indeed, studies indicate that a significant portion of antimicrobial substances is not completely metabolized during their therapeutic use. Approximately 30-90% of these substances are believed to enter wastewater in their active forms. Consequently, various classes of antibiotics are extensively detected in wastewater treatment facilities and their surrounding environments globally [507,508]. In particular, antimicrobial substances and their resulting by-products are identified in the environment at concentrations ranging from nanograms per liter (ng L-1) to micrograms per liter (μg L-1) upon their introduction into aquatic systems [509]. These substances are identified as emerging micropollutants, and a concern associated with their presence in the environment

is the increase in ARGs and the development of ARB [510,511]. The level of environmental pollution is influenced by the patterns of their usage. A rise in consumption, particularly during the colder seasons when infection rates are higher, leads to an elevated presence of these substances in environmental systems [512]. This is associated with the emergence of bacteria resistant to multiple antibiotics and their rapid proliferation [513].

The antibiotic resistance of environmental bacteria could be significantly impacted by antibiotic selection pressure in the environment. For example, the emergence of antibiotic resistance in a non-pathogenic environmental bacterium, Pseudoxanthomonas mexicana, isolated from a laboratory-scale bioreactor treating streptomycin-containing wastewater, has been evaluated. Streptomycin pressure has triggered genetic events and influenced transcriptional and translational regulations in the streptomycin-resistant strain (SR) [514]. It was also observed that the tetracycline concentration gradient influenced the development patterns of tet genes under anaerobic conditions. In the anaerobic hybrid reactor (AHR), the abundance of intI1 genes increased with rising tetracycline dosages, while they decreased in the completely mixed aerobic reactor (CMAR). In the AHR, tetracycline showed significant positive correlations with tet genes such as tet(C), tet(G), tet(X), tet(O), and tet(M) ($r=0.904$, 0.446, 0.649, 0.573, and 0.898, respectively, $p<0.05$). Conversely, a significant negative correlation was found between tetracycline and tet(W) ($r=-0.667$, $p<0.01$), indicating that tetracycline exerted selection pressure on the bacterial hosts carrying these tet genes. In the CMAR, tetracycline exhibited significant correlations with tet(A), tet(C), tet(G), tet(X), and tet(O) ($r=-0.415$, 0.583, 0.790, 0.746, and -0.767, respectively, $p<0.05$). These findings suggest a close relationship between the presence of tetracycline and the occurrence of tet genes. Additionally, a notable correlation between tetracycline and intI1 genes was found in both the AHR ($r=0.698$, $p<0.01$) and CMAR ($r=-0.477$, $p<0.05$). This suggests that the presence of tetracycline in wastewater may exert selection pressures for bacterial resistance and influence the evolutionary pathways of tet genes mediated by intI1 genes. The variations in the gene distribution between the AHR and CMAR could be due to differences in bacterial growth rates, oxygen availability, and nutrient levels. Moreover, strong correlations were observed between intI1 genes and tet genes in anaerobic-aerobic sequential (AAS) bioreactors [e.g., tet(C), tet(G), tet(X), tet(O), and tet(M) in the AHR; tet(A), tet(W), tet(O), and tet(M) in the CMAR], highlighting their significant role in the horizontal spread of tetracycline resistance. The AAS system could be a viable and cost-effective alternative for simultaneously reducing nutrients, antibiotics, and antibiotic resistance in practical applications[515]. The simultaneous detection of meropenem, ceftazidime, and ciprofloxacin with Pseudomonas aeruginosa exhibiting resistance to third-generation

cephalosporins (3GCR) has demonstrated notable clinical relevance. This observation is supported by the calculated odds ratios. In particular, the likelihood of encountering P. aeruginosa 3GCR isolates increased by a factor of 2,8 when ciprofloxacin residues were detected concurrently (5,0 for meropenem and 3,2 for ceftazidime). In this scenario, the elevated levels of ciprofloxacin and the presence of P. aeruginosa may contribute to further dissemination and increased selection pressure favoring resistance development. The positive identification of residual concentrations of carbapenems, specifically meropenem, is correlated with corresponding carbapenemases such as blaNDM, blaVIM2, and blaOXA48. Additionally, notably high odds ratios were identified between antibiotics such as linezolid, vancomycin, and ampicillin, and the genes blaOXA48, blaCTX-M, and blaVIM2 [516]. Similarly, it has been observed that the prolonged presence of the antibiotic erythromycin (ERY) in wastewater, even at low concentrations, has the potential to exert selective pressure on resistant bacteria, contributing to the development of antibiotic resistance [517]. Significant and positive correlations ($0,883 < r < 0,929$, $P < 0,05$) have been identified between the ERY antibiotic and the ere(A), ere(B), and mef(A)/mef(E) genes. This suggests that the selection pressure on these resistance genes may be linked to the presence of ERY, despite its very low concentration in wastewater. Additionally, a significant correlation ($r = 0,829$, $P < 0,05$) was observed between erm(B) genes and Tetracycline (TC), while other identified ERY-ARGs did not show such a correlation [518]. In contrast, in 2003, Ohlsen K et al. conducted experiments on sewage agar plates using gentamicin, tetracycline, erythromycin, and ciprofloxacin at concentrations ranging from 0.001 to 0.1 mg/L. Their aim was to evaluate how antibiotics in sewage contribute to the horizontal transmission of antibiotic resistance genes in Gram-positive pathogens, specifically using conjugative antibiotic resistance plasmids derived from S. aureus. They found that the presence of these antibiotics at a concentration of 0.1 mg/L did not significantly impact the transfer rates. The results suggest that while resistance gene transfer in Gram-positive pathogens can occur in environmental settings, the introduction of antibiotics into sewage does not enhance the spread of conjugative antibiotic resistance plasmids through increased transfer rates. This is particularly true for the low-level concentrations of antibiotics typically found in the environment[519]. In 2019, it was emphasized that the primary mechanism driving the emergence of tetracycline resistance genes under prolonged exposure to low levels of tetracycline stress was horizontal gene transfer. The increase in tetracycline concentrations rapidly drove the evolution of tetracycline resistance. Specifically, at concentrations of 20 and 50 μg/L, the efflux pump became the primary resistance mechanism. Notably, tet(A) and tet(G) showed positive correlations with class 1 integrons ($p < 0.05$). Consequently, prolonged

exposure to low levels of tetracycline tended to increase the relative abundance of class 1 integrons as a defensive response, potentially further promoting the prevalence of efflux pump genes [520]. Brown CL et al. confirmed that varying antibiotic levels in sewage are linked to the HGT of ARGs, as well as to changes in microbial communities and the diversity of resistance gene behavior within activated sludge systems. Their findings indicate that the impact of antibiotics on the selection of resistant bacteria is influenced by the existing interactions within the microbial community and may be facilitated by HGT [521]. Sutradhar I et al. conducted research to address the lack of quantitative understanding regarding how antibiotics interact in environments with constant flow. They focused on experimentally monitoring populations of E. coli exposed to subinhibitory concentrations of antibiotic combinations with different interaction types: synergistic, antagonistic, and additive. Their findings revealed that E. coli populations subjected to synergistic and antagonistic antibiotic conditions exhibited behaviors that deviated significantly from expected outcomes. Specifically, E. coli populations exposed to synergistically interacting antibiotics developed less resistance than predicted, suggesting that these combinations might suppress resistance development. Conversely, E. coli populations exposed to antagonistically interacting antibiotics showed resistance development that was dependent on the specific ratio of antibiotics used. This implies that not only the type of antibiotic interaction but also the relative concentrations of the antibiotics play crucial roles in predicting how resistance develops [522].

The role of antibacterial residues and heavy metals in promoting the development of antibiotic resistance

Antibiotic resistance can arise even in the absence of direct antibiotic pressure in the environment. Studies have shown that not only antibiotics but also other factors, such as antibacterial residues and heavy metals, can contribute significantly to the development and proliferation of antibiotic resistance [523]. In contrast to antibiotics, heavy metals are not subject to degradation. Consequently, they serve as a persistent and prolonged selective force in the sustenance and amplification of antibiotic resistance. Zinc (Zn) exhibited a significant correlation with ERY-ARGs, including ere(A), ere(B), mef(A)/mef(E), and erm(B) genes ($0.823 < r < 0.866$, $P < 0.05$). In contrast, the correlations between Chromium (Cr) and ERY-ARGs were weaker ($-0.192 < r < 0.582$, $P > 0.05$). Additionally, similar patterns ($0.852 < r < 0.871$, $P < 0.05$) were observed between Lead (Pb) and specific resistance genes, namely ere(B) and mef(A)/mef(E) genes. However, no significant correlation was identified between Copper (Cu) and the detected ERY-ARGs. Furthermore, there are noteworthy concerns

about the robust correlations between a non-therapeutic antibacterial agent, triclosan (TCS), and ERY-ARGs, including ere(A), ere(B), mef(A)/mef(E), and erm(B) genes ($0.859 < r < 0.956$, $P < 0.05$). This suggests that the presence of TCS in wastewater holds significant importance as a selecting agent influencing the evolution of ERY-ARGs [518]. Research indicates that AMR in wastewater can respond to regulatory changes. The study shows that cross-resistance to clinically significant antibiotics in bacteria, influenced by non-therapeutic antimicrobials, varies depending on the type of antimicrobial used, bacterial taxonomy, and temporal factors. This highlights the complexity of AMR patterns and underscores the need for nuanced regulatory approaches to manage and mitigate the spread of resistance in diverse microbial communities. Notably, it identified numerous clinically relevant bacteria, including opportunistic pathogens capable of causing multidrug-resistant (MDR) infections. The research underscores significant differences in the taxa and cross-resistance patterns influenced by various antimicrobials within a community relevant to the human microbiome. Although bacteria resistant to benzalkonium chloride (BC) currently represent a small portion of the culturable community, the extensive use of BC could lead to an increased proportion of resistant microorganisms.

A key finding is the clear association between BC resistance and cross-resistance to colistin, a last-resort antibiotic, indicating a need for further investigation. As BC replaces TCS in many consumer products, understanding its impact on the spread of resistance to clinically important antibiotics is crucial, particularly concerning the cross-resistance to critical, last-resort treatments [524]. Moreover, microorganisms also play a crucial role in the HGT of ARGs. A metagenomic approach was utilized to simultaneously identify ARGs and antibacterial biocide and metal resistance genes (BMRGs), along with their corresponding microbial hosts with high mobility during the aerobic granular sludge (AGS) formation process. The results indicated that the relative abundance of BMRGs was 88-123 times higher than that of ARGs. The AGS process more readily enriched BMRGs, posing a greater risk of drug resistance from BMRGs compared to ARGs. The enrichment of ARGs and BMRGs in AGS was closely linked to several enhanced microbial metabolisms, including cell motility, transposase activity, and ATP-binding cassette transporters, along with their regulatory genes [525].

Impact of microbial community composition on ARB and ARG Fate

Wastewater treatment alters the composition of bacterial populations, inevitably influencing the destiny of antibiotic-resistant bacteria and ARGs. This reassessment is crucial to mitigate the environmental and health hazards associated with wastewater management, reuse, and disposal.

The water treatment process frequently removes antibiotics, but it is ineffective in eliminating bacteria, ARGs, and factors contributing to antimicrobial resistance. Throughout the wastewater treatment steps, there was an overall shift from Gram-negative bacteria to Gram-positive bacteria, although the overall bacterial biomass remained unchanged in the WWTP samples. Despite the effluent wastewater sample (EW) containing the lowest quantity of antimicrobial resistance determinants, the number of these determinants was still substantial (n = 20), only marginally lower than that in the influent water (IW) sample (n = 25). Additionally, the assessment of ARG and antibiotic levels in the water and active sludge (AS) samples revealed significant correlations among floR, intI1, sul1, and ermB, suggesting a coordinated regulation of these ARGs, possibly through co-expression in integron-type MGEs. A notably strong correlation was found between the chloramphenicol resistance gene cmlA and the β-lactam resistance gene blaTEM. However, no significant associations were observed between antimicrobial resistance determinants, antibiotic residue levels, and ARGs[526]. The composition and diversity of bacterial communities emerged as the main determinants driving resistance selection and propagation within biofilms, outweighing the influence of antibiotic exposure. Antibiotic exposure did not visibly alter the structure of planktonic bacterial communities [527]. Biofilms could potentially increase the stability of plasmids, which in turn could broaden the range of hosts for MGEs engaged in horizontal transfer. In vitro, the transfer of plasmids carrying cefotaxime resistance genes (blaCTX-M gene) through conjugation was significantly higher in the biofilm growth mode compared to planktonic cultures, as clearly demonstrated (P = 0.0346) [528]. Their conclusion, aligning with the findings of Auguet et al. (2017)[529], suggests that antibiotic exposure is not the primary driver of resistance selection and propagation. The condition that favors the dissemination of ARGs, whether in planktonic or biofilm communities, occurs when wastewater is sterilized and biofilm is present. In sterilized wastewater, although biofilms reduced planktonic 16S abundance, ARG absolute abundance remained similar, resulting in a higher relative abundance. This underscores that when activity, competition, and diversity are diminished between planktonic and biofilm communities, the dissemination of ARGs is favored [527].

Both biofilm-based and suspended growth-based WWTPs may promote the genetic mobilization of persistent ARGs, which are then released into effluent and spread into receiving water bodies. There was a significant increase in the relative abundance of plasmid- and integron-associated ARGs between influent and effluent. In the biofilm system, the overall levels of ARGs in the effluent were reduced by 91.1% compared to the influent, while in the suspended growth system, the relative levels of ARGs in the effluent were decreased by 96.5%

compared to the influent. The biofilm system exhibited higher diversity and persistence of ARGs during the secondary treatment process compared to the suspended growth system. Additionally, positive associations were observed between the presence of ARGs and antibiotic-producing genes (APGs) for specific classes of antibiotics and their corresponding resistance genes, suggesting that on-site antibiotic production could contribute to the selection for ARGs conferring resistance to those antibiotics [530]. WWTP bacteria serve as a reservoir for various resistance genes. The bacteria in the WWTP contain a diverse array of resistance genes, including some that were only recently identified in clinical settings. This indicates active genetic exchange between clinical and WWTP bacteria. The identification of around 64% of the 192 references in resistance genes on plasmids from bacteria found in the final effluents of the WWTP indicates a potential risk of these resistance determinants spreading to environments downstream of the plant, potentially aiding their spread among environmental bacteria. Additionally, the purification processes in the WWTP analyzed do not effectively reduce the variety of detectable resistance genes in the final effluents [303].

Treatment methods in WWTPs and spread of ARGs and AMR

Concerning secondary treatment, various studies with different plant configurations have reported decreases, no changes, or even increases in the prevalence of ARGs [253,302,419,420]. For instance, while some studies have noted no significant changes in the relative abundance of the sul1 gene following secondary treatment [302,531], others have reported an increase [253]. In addition, distinct ARGs exhibited varied responses during secondary treatment, particularly those associated with beta-lactamases (blaTEM, blaOXA-A, blaSHV, blaCTX-M) and quinolone resistance (qnrS) compared to those linked to sulfonamide resistance (sul1, sul2) and class 1 integrase (intI1). While the former group demonstrated a decrease, the latter group either remained unchanged or increased. The prevalence of sul genes remained unchanged (sul1), while it significantly increased after secondary treatment (sul2).

The elevation in most antibiotic resistance gene levels, presence of potentially pathogenic bacteria, and widespread presence of various resistance genes and cassettes in the tetracycline-degrading bacterial isolates indicate significant risks associated with the application of biodegradation methods in treating antibiotic-polluted wastewater. Indeed, in the biodegradation process for treating antibiotic-contaminated wastewater, prolonged acclimation of activated sludge under low antibiotic concentrations accelerated the emergence of antibiotic-resistant bacteria and the spread of resistance genes. Despite some resistance gene abundance decreasing at high tetracycline concentrations, the overall spread of most resistance genes and

MGEs was accelerated in the enriched cultures, suggesting that their abundance did not decrease but rather increased, even in the presence of high tetracycline concentrations as the sun carbon source [532]. Regarding biological treatment, it has altered the prevalence of various ARGs. The proportion of low erythromycin-resistant bacteria (0–4 mg/L erythromycin) remained notably high in both influent and treated wastewater samples, with little change observed during activated sludge treatment ($p = 0.873$), maintaining survival fractions at 70%. Conversely, the fraction of medium erythromycin-resistant bacteria (4–16 mg/L erythromycin) significantly decreased from 18% to 6% following biological treatment ($p = 0.007$). However, high erythromycin-resistant bacteria (>16 mg/L erythromycin) experienced substantial proliferation, particularly evident when erythromycin concentration increased to 64 mg/L, resulting in a dramatic rise in bacterial survival from 0.2% to 10% ($p = 0.011$). The erythromycin resistance spectrum of heterotrophic bacteria underwent a "shift" from medium to high levels in the bioreactor, indicating a clear amplification of bacterial tolerance to high erythromycin concentrations during activated sludge treatment. This notable increase in bacterial resistance to erythromycin within a short timeframe (8 hours) is likely attributed to the favorable environment for ARB proliferation during the biological process. In addition to the amplification of bacterial resistance, this study observed a significant increase in the diversity of erythromycin-resistant bacteria during biological treatment. This rise in diversity is believed to be linked to gene transfer within the activated sludge system [281]. Tertiary or Advanced Treatment Processes serve as an additional step to further enhance the quality of treated wastewater, particularly when specific constituents, substances, or contaminants persist after secondary treatment. In addition to conventional methods, advanced treatment technologies, such as membrane bioreactors (MBRs), may be employed for specific applications or to meet stringent water quality standards. However, it has been observed that the MBR environment facilitates the horizontal transfer of RP4 to cultivable bacteria in the sludge due to factors such as high bacterial density, microbial diversity, biofilms, and nutrient richness. Additionally, once the RP4 plasmid was transferred into bacteria, these transconjugants actes as secondary donors of RP4, establishing a dynamic equilibrium within the system. These findings imply that ARGs and ARB may be released into the natural environment from an MBR through the discharge and application of biosolids [533]. Dropa M et al. suggest that while disinfection methods are effective at reducing the number of live, culturable bacteria, they do not always succeed in eliminating bacterial DNA, including ARGs. These ARGs are often located on MGEs, which facilitate their spread even after the bacteria are no longer viable [497]. An overall analysis using qualitative PCR revealed only a slight

reduction in the frequency of ARG detection when comparing raw sewage to secondary effluent. For reuse water, the reduction varied: the blaCTX-M and intI1 genes saw a decrease of around 50%. The qnrB gene showed no reduction. For other ARGs, detection rates in reused water ranged from 0% to 16.7%. The quantitative data from WWTPs showed that each phase of wastewater treatment led to approximately a two-$\log^{10}$ reduction in the number of gene copies per liter. Although significant reductions in bacterial loads were achieved from the secondary to the tertiary treatment phase, the relative abundance of ARGs actually increased in the reuse water compared to the secondary effluent. This suggests that resistance genes can survive treatment processes aimed at reducing bacterial populations, becoming more concentrated as the treatment progresses because the genetic material of dead bacteria remains intact. These results highlight a critical gap in current wastewater treatment processes: while effective in diminishing the number of viable bacteria, these methods do not adequately address the persistence and potential spread of ARGs through surviving DNA. This underscores the need for advanced treatment strategies that can not only reduce bacterial counts but also effectively degrade resistant genetic material [497]. The conventional treatment process profoundly impacts resistance genes and their transcriptional activities across different stages of wastewater treatment. Compared to influent and effluent samples, the sludge from nitrifying and denitrifying bioreactors exhibited significantly higher levels of transcript copies of antibiotic, biocide, and metal resistance genes per liter. The strong correlations observed between all categories of resistance genes and biomass metrics (Spearman's $rs > 0.75$) support the notion that bacterial biomass primarily drives variations in the total concentration of both resistance genes and transcripts throughout WWTPs. Conversely, effluent and influent samples showed significantly higher transcript copies per gram of biomass and per gene copy of resistance genes compared to bioreactor sludge. This suggests that changes primarily occur within the mobilized resistome. In contrast, in the nitrifying and denitrifying bioreactors, smaller yet significant portions of the variations in resistome composition were attributed to three nitrogen metrics, three operational parameters, two metals (cadmium and nickel), and seven pharmaceuticals (including levofloxacin, trimethoprim, and sulfamethoxazole). They have further evidenced that (i) core resistome genes persist, are abundant, and undergo transcription across all compartments of the WWTP, and (ii) resistance genes and indicators of mobility exhibit higher transcriptional activity in the secondary effluent compared to activated sludge bioreactors. They highlight the importance of biotic factors, including mobility elements (such as intI1, conjugative transfer protein, and resolvase), and biomass in shaping the variations in influent and effluent resistomes. In the activated sludge, abiotic factors (such as inorganic nitrogen, pH,

dissolved oxygen, and various antimicrobials) also appear to influence resistomes. Within this compartment, nutrients and oxygen are significantly consumed by the activated sludge biomass, potentially acting as driving forces for both community and resistome composition. They also discovered notable positive correlations ($P < 0.05$) between the concentration (ng/L) of measured antibiotics (such as macrolides, sulfonamides, lincosamide, trimethoprim, and vancomycin) and the concentration of specific ARGs (170 instances) or ARG transcripts (43 instances). Most of these correlations were observed between an antibiotic class and ARGs or ARG transcripts that provide resistance to a different antibiotic class, suggesting potential associations arising from gene co-selection or co-expression. Their findings emphasize that the quantity of resistant bacteria and genes released into the environment by WWTPs is significantly influenced by the bacterial biomass present in the final effluent. Therefore, any intervention that effectively reduces bacterial biomass in the discharged effluent, such as enhancing sludge settleability in secondary clarifiers or implementing membrane filtration, would lead to a reduction in the discharge of resistance genes from WWTPs [534]. Xu H, et al examined the effects of ciprofloxacin (CIP) and dialkyldimethyl ammonium compound (DADMAC) on nitrifying systems using three sequencing batch reactors (R1, R2, R3) over 150 days. CIP had minimal impact on ammonia oxidation, while DADMAC, especially at higher concentrations, significantly inhibited it. Their combination had a stronger inhibitory effect and caused partial nitrification, with the highest nitrite accumulation in R3. Additionally, the mixture of CIP and DADMAC increased the size of sludge particles and boosted the abundance and transfer of ARGs, promoting their spread in the system. The findings suggest that while these chemicals can control bacterial activity, they also enhance the dissemination of ARGs[535]. Savin et al. revealed that wastewater, especially from clinical sources, is a significant source of ARGs linked to free-floating extracellular DNA (exDNA). In wastewaters treated by conventional biological processes, exDNA can constitute up to 85.8% of the total DNA. Traditional treatments were found to be ineffective in reducing exDNA and ARGs, unlike advanced methods such as ozonation and ultrafiltration. Ozonation, for instance, can reduce exDNA concentrations by up to 73.3% by breaking down bacterial cell walls and degrading DNA. Additionally, since bacteria in wastewater naturally lead to exDNA through cell death and lysis, removing them via ultrafiltration is crucial. To curb the release of exDNA and ARGs into municipal wastewater systems and aquatic environments, the study suggests using multi-barrier strategies that include pre-treatment at the source for clinical wastewaters and employing advanced technologies in municipal WWTPs [536]. Regarding anaerobic digestion coupled with pretreatments, it has been observed that this approach can reduce the abundance of ARB

in sludge by up to 90%. However, surprisingly, the pretreatments notably increased the abundance of specific ARB, such as tetracycline-resistant bacteria, in the supernatant, reaching levels of 2.3×10^2 CFU/mL compared to the relatively low value of 0.6×10^2 CFU/mL observed without pretreatment. The alkali-ultrasonication proved to be the most efficient pretreatment method in reducing the viable bacterial population within the sludge, achieving an overall removal rate exceeding 85%. Specifically, the maximum removal of heterotrophic plate count (HPC) in the supernatant treated with alkali-ultrasonication reached 91%, slightly surpassing that achieved with ultrasonication alone (84%) and alkali-hydrolysis (89%). Interestingly, a heightened conjugal transfer (0.015) of ARGs was observed when the digested supernatant was reintroduced into the biological treatment system. This suggests the potential for the spread of ARGs and subsequent ecological risks during anaerobic digestion aimed at reducing excess sludge, highlighting the need for further attention to the treatment of excess sludge, particularly the supernatant [537]. Machado EC et al. conducted a comprehensive study to examine the occurrence, removal, and characterization of ARB in WWTPs using different treatment technologies. The study compared three WWTPs: WWTP-A with conventional activated sludge (CAS), WWTP-B employing an upflow anaerobic sludge blanket (UASB) reactor followed by a biological trickling filter (BTF), and WWTP-C utilizing a modified activated sludge process followed by UV disinfection (MAS/UV). Samples were collected from both raw sewage (RI) and treated effluent (TE) to assess the efficacy of each treatment method. The findings showed that the MAS process was the most effective in reducing ARB counts, achieving a reduction of 2 to 3 log units. In contrast, CAS only achieved a 1 log unit reduction, and the UASB/BTF process showed the least reduction at just 0.5 log units. The composition of cultivable ARB differed between RI and TE samples. In the raw sewage, Escherichia was the predominant ARB, accounting for 56 out of 118 isolates. Post-treatment, Escherichia remained dominant in the treated effluent but was accompanied by Bacillus (22/118), Shigella (14/118), and Enterococcus (14/118). A vast majority of the isolates (370 out of 394) carried at least two ARGs. Notably, over 80% of these isolates contained four or more ARGs, including int1, blaTEM, TetA, sul1, and qnrB. The CAS and MAS processes effectively reduced the prevalence of ARB in the treated effluents. However, the UASB/BTF system, and particularly the UV disinfection stage, saw a slight increase in the prevalence of resistant bacteria. The study underscores the role of biological sewage treatment in significantly reducing ARB counts. Nonetheless, it also highlights the persistent risk of MDR bacteria and ARG dissemination into aquatic environments via treated effluents. This calls for the implementation of advanced or supplementary treatment processes to further mitigate the release of MDR bacteria into the

environment [538]. A pilot-scale study revealed that samples of civil wastewater from a traditional treatment plant, following the initial sedimentation/flocculation step, were rich in ARB and ARGs. However, when these samples were treated using innovative Constructed Wetlands (CWs) in different configurations, there was a significant reduction in both environmental contaminants and AMR, making the treated water suitable for reuse. The study demonstrated that various CW configurations are sustainable and low-cost solutions for wastewater treatment. They are effective not only in removing well-known contaminants, such as COD and nitrogen compounds, but also in reducing AMR. The CWs designed and implemented at the pilot scale highlighted the typical advantages of CW systems. Specifically, the use of diverse filling media, including plastic carriers with a high surface-to-volume ratio and sand of varying grain sizes, along with different plant species, provided a variety of habitats conducive to biofilm formation. Both CW configurations were able to produce high-quality reclaimed wastewater suitable for irrigation purposes in a short period. When comparing the two CW pilot plant configurations—Vertical Constructed Wetlands (VCW) and Horizontal Constructed Wetlands (HCW)—the HCW configuration showed superior efficiency in removing wastewater contaminants, particularly total nitrogen. The HCW was more effective than the VCW because it combines two different types of hydraulic flows in series (Vertical Flow, VF, and Horizontal Subsurface Flow, HSSF), harnessing the benefits of both systems. The use of various plant species likely contributed to enhancing the antimicrobial capabilities of both CW systems. Both CW configurations were effective in reducing ARB; in fact, CW treatments alone or in combination with UV irradiation were capable of removing up to 99% of ARGs [539]. Anaerobic treatment processes (anaerobic sequencing batch reactors, SBRs) lead to the presence and spread of ARGs when exposed to high, non-lethal concentrations of sulfamethoxazole-tetracycline-erythromycin (STE) and sulfamethoxazole-tetracycline (ST) combinations. Gene expression analysis indicated that microorganisms developed resistance to tetracycline and erythromycin through various mechanisms, including efflux pump proteins, modification of antibiotic targets, and modifying enzymes. Moreover, a significant correlation was found between the presence of class 1 integrons and sulfamethoxazole resistance genes[540]. ARG accumulation over time in the SBR system follows linear kinetics, with ARG quantities gradually attenuating without Sulfamethoxazole (SMX) selection pressure. Consequently, continuous antibiotic input in actual SBR system operation inevitably leads to ARG development and proliferation. Over a span of 55 days, sul I concentrations increased to 4.79 × 10^5 copies/mL, 2.10 × 10^5 copies/mL, and 1.05 × 10^5 copies/mL in groups exposed to 10 mg/L, 0.10 mg/L, and 0.001 mg/L of SMX, respectively. Additionally, higher SMX

concentrations corresponded with elevated ARG abundances at the same sampling intervals[541]. This observation aligns with the findings of Collado et al. (2013)[542]. Their research on a SBR system revealed that the abundance of ARGs increased progressively with the duration of treatment. Notably, the lowest ARG levels were observed in the group treated with 0.001 mg/L SMX. This pattern indicates that higher concentrations of antibiotics tend to promote the proliferation of ARGs. Remarkably, both sul I and sul II abundances linearly increased with SBR operation time in the presence of SMX, while slightly decreasing in control groups without SMX. The Chemical Oxygen Demand (COD) value may influence the impact of SMX on ARGs. Insufficient COD levels may inhibit bacterial growth, thereby limiting the proliferation of sul I and sul II genes. One plausible explanation is that compounds contributing to COD serve as nutrition for bacteria. Without an adequate nutrient source, organisms may struggle to grow normally, even under the selection pressure exerted by SMX. In contrast, regarding ultraviolet (UV) disinfection of secondary-treated wastewater, no significant alterations were observed in the bacterial community composition or the abundance of ARGs. However, UV treatment led to a significant decrease in viability, resulting in approximately a 2 log-unit reduction in culturable enterobacteria [201]. Furthermore, under laboratory conditions, it was observed that the descendants of surviving bacteria after UV disinfection exhibit a reduced ability to transfer genetic material compared to the initial bacteria without UV treatment. This indicates that although certain bacteria may "escape" UV exposure, their transfer ability is diminished overall. The results of the experiments suggest that even a low UV disinfection dose of 1 mJ/cm^2 can significantly reduce the frequency of conjugative transfer of surviving bacteria. However, the findings demonstrated that bacteria exposed to low UV doses can maintain their transfer ability after reactivation, whether through dark repair or photoreactivation. Conversely, after higher UV disinfection doses, the number of reactivated cells was lower, and their transfer ability was significantly reduced [543]. In contrast, another study on two municipal wastewater treatment plants where UV disinfection was implemented, the majority of resistance gene abundance decreased, although certain ARGs, such as sul1, tet (34), tetE, and tetS, showed slight increases that were not statistically significant. Under UV disinfection alone at a dose of 320 mJ/cm^2, the abundance of the 16S rRNA gene in Morganella morganii decreased by 0.40 log, while in Enterococcus faecalis, it decreased by 1.35 log. The reduction in the copy number of ARGs ranged from 0.58 to 1.60 log, indicating that UV disinfection's efficacy varied depending on the type of resistance. Chlorine disinfection exhibited a more potent effect in removing ARGs compared to UV treatment. Specifically, under chlorine disinfection alone at a dose of 320 mg × min/L, the abundance of the 16S rRNA

gene in Morganella morganii decreased by 4.19 log, and in Enterococcus faecalis, it decreased by 3.99 log. The reduction in the copy number of ARGs ranged from 4.30 to 5.18 log. The combination of UV treatment with a low dose of chlorine could significantly enhance the degradation of ARGs through a synergistic effect. Indeed, when UV disinfection was combined with 2 mg/L chlorine, the reduction in the copy number of five genes improved by 1–1.5 log with increasing UV dose, particularly at a high UV dose of 320 mJ/cm^2. Furthermore, it is important to highlight that the degradation of the tetB gene in Enterococcus faecalis was more pronounced than that observed in Morganella morganii when subjected to the same disinfectant and dose ($P < 0.05$). Therefore, the efficacy of disinfection was influenced by the specific type of resistance and the bacterial characteristics. Compared to UV treatment alone, the inactivation of ARB was enhanced by 1.4 log, and photoreactivation was effectively suppressed by the UV/chlorine combination (UV dose: 8 mJ/cm2, chlorine concentration: 2 mg/L). The synergistic effect of adding low-dose chlorine (1–2 mg/L) during UV irradiation effectively improved the removal of both ARB and ARGs simultaneously. This synergistic effect was also observed in HGT. Non-lethal doses of chlorine (0.5 mg/L) increased the frequency of conjugation transfer, as evidenced by the significantly enhanced mRNA expression levels of type IV secretion system (T4SS) proteins vir4D, vir5B, and vir10B. The risk of RP4 plasmid conjugation transfer was significantly reduced with UV/chlorine treatment (UV dose ≥ 4 mJ/cm2, chlorine concentration ≥ 1 mg/L) [544]. However, in a study examining the efficacy of chlorination in an on-site hospital wastewater treatment system, Escherichia coli and Enterococcus were evaluated for their viability and ability to enter a viable but non-culturable (VBNC) state. After chlorination, reductions were observed, with viable/VBNC E. coli decreasing by 0.44 to 1.88 log and Enterococcus by 0.29 to 1.29 log. The data indicated that gram-negative E. coli exhibited a lower tolerance to chlorination compared to gram-positive Enterococcus. However, while chlorination effectively reduced the bacterial load, it did not completely eliminate ARGs and MGEs. Chlorination appeared to cause these genetic elements to be released from cells, potentially enhancing the antibiotic resistance of the remaining viable bacteria. Interestingly, the presence of ARB in the hospital wastewater did not directly correlate with the levels of detectable antibiotics, suggesting that ARB can persist even in the absence of significant antibiotic concentrations [545]. Additionally, it has been observed that chlorination promotes the selection of chlorine-resistant pathogenic bacteria, and the regrowth of such bacteria in reclaimed water with long retention times. Indeed, the inactivation of total viable cells, measured through metabolic activity, typically exhibited a linear correlation at lower chlorine doses (0-22 mg.min/L), followed by a trailing region as the chlorine dose increased

from 22 to 69 mg.min/L. Reactivation and regrowth of bacteria were most likely to occur after exposure to lower chlorine doses, with reactivation decreasing gradually as the chlorine dose increased. Unlike total coliforms and Enterococcus, Salmonella exhibited significant regrowth and reactivation, maintaining 2% regrowth even after chlorination at 69 mg.min/L and 24-hour storage [483]. Furthermore, exposure to sub-inhibitory concentrations of chlorine during disinfection resulted in an increased number of ARB in the disinfection effluent, potentially due to enhanced horizontal transfer of ARGs during chlorination. In particular, for most ARB, except tetracycline-resistant bacteria, a decrease in concentration was observed only at higher chlorine doses (> 8 mg/L) (*P* value=3.5×10^{-3}, 6.7×10^{-17}, 4.32×10^{-17} and 7.2×10^{-17} for ampicillin-, ciprofloxacin-, erythromycin- and sulfamethoxazole-resistant bacteria). The effectiveness of chlorination depended on the dosage, with concentrations of NaClO at 2 mg/L and 8 mg/L resulting in higher concentrations of ampicillin-, ciprofloxacin-, tetracycline-, and sulfamethoxazole-resistant bacteria compared to those in the secondary effluent. However, Surprisingly, the concentration of tetracycline-resistant bacteria increased with higher NaClO doses, reaching removal only at a chlorine dose of 32 mg/L. In contrast, the number of erythromycin-resistant bacteria decreased with increasing NaClO concentration, indicating their higher sensitivity to chlorination [546]. This finding contrasts with a previous study showing that chlorine concentrations exceeding 1.0 mg Cl_2/L and a contact time of 10 minutes also increased tetracycline resistance in E. coli [547] . Furthermore, the selective pressure induced by chlorine may facilitate the proliferation of resistant bacteria, potentially establishing this stage of the treatment process as a reservoir for both resistant bacteria and resistance determinants. For instance, P. aeruginosa isolates collected during the chlorination phase displayed resistance to multiple antibiotics[548]. Similarly, Salmonella isolated from effluents after chlorination showed higher rates of antibiotic resistance compared to those found in influent water. The resistance rates to TET, sulfamethoxazole (SMZ), chloramphenicol (CHL), streptomycin (STP), ampicillin (AMP), and gentamicin (GEN) of Salmonella isolated from effluent after chlorination exhibited a significant increase compared to those before chlorination in WWTP[317]. Moreover, chlorine disinfection may elevate the abundances of both intracellular antibiotic resistance genes (iARGs) and extracellular antibiotic resistance genes (eARGs), thus increasing the risk of antibiotic resistance dissemination in the environment. ClO_2 treatment led to an increase in the presence of eARGs in the final wastewater, significantly elevating emissions of eARGs associated with tetracycline, sulfonamide, β-lactam, aminoglycosides, and vancomycin. Among the 22 eARGs analyzed, concentrations of tetA, tetB, sul3, ampC, aph(2')-Id, and vanA in all final sewage samples exhibited significant

increases ($p < 0.05$, $n = 33$) compared to their counterparts in other wastewater samples, suggesting that these genes may display greater resilience to ClO_2 [549].

The interaction between water quality parameters and the spread of ARGs in treatment plants

Certain water parameters may serve as drivers and play a crucial role in the persistence and proliferation of ARGs in the wastewater treatment plant. Factors such as conductivity, pH, heterotrophic plate count (HPC), total nitrogen (TN), ammonium, and chemical oxygen demand (CODMn) showed significant correlations with certain eARGs (e.g., ampC, catA1, catG) during the disinfection process. Water temperature did not show any correlations with variations in the concentration of total observed eARGs ($p > 0.05$, $n = 3$), but a significant increase in eARG abundance was noted when the water temperature ranged between 25 °C and 27 °C ($p < 0.01$, $n = 3$). Moreover, a much greater increase in the median concentration of total iARGs was observed when the water temperature in the secondary effluent decreased, indicating that lower environmental temperatures (10–20 °C) may promote an elevation in iARGs concentration under chlorine exposure. Additionally, NH4+-N concentration in the range from 1.8 to 2.5 mg/L also exhibited a significant impact on iARGs concentration, leading to an increase in iARGs concentration [549]. Huang H et al., 2016 found that different pH levels, during sludge anaerobic treatment, not only influenced the community compositions of tetracycline-resistant bacteria (TRB) but also impacted their relative abundances, favoring the proliferation (under acidic pH conditions) or suppression (under alkaline pH conditions) of TRB [550]. Further analysis revealed that acidic pH conditions significantly enhanced both the quantity and genetic capabilities of key genetic elements responsible for the horizontal transfer of tetracycline resistance genes (TRGs), whereas alkaline pH conditions restricted their presence and activity. In addition, Li L et al. 2018 conducted redundancy analysis (RDA) to investigate relationships among ARGs, mobile elements (int1 and int2), and external factors, such as tetracyclines (TC), sulfonamides (SA), quinolones (QN), macrolides (MC), pH, dissolved oxygen (DO), electrical conductivity (EC), total organic carbon (TOC), and total nitrogen (TN) [551].The findings revealed no significant correlations between antibiotics and ARGs, except for the sul genes, which exhibited a negative correlation with sulfonamides (SA), indicating potential co-selection with other ARGs or factors. The tetB, sul1, sul2, gyrA genes, and 16S rRNA gene copies showed positive correlations with pH and EC ($p < 0.05$). Additionally, the abundance of tetW, sul1, and sul2 genes correlated with the 16S rRNA gene, suggesting that bacteria in wastewater played a crucial role in the proliferation of tetW and sul

genes. Furthermore, the sul1 and sul2 genes exhibited significant correlations with TOC ($p < 0.05$), indicating that high TOC content likely contributed to bacterial reproduction. TetW was also positively correlated with TN and DO ($p < 0.05$). Wu F et al. explored how polyaluminum chloride (PAC), and ferric chloride ($FeCl_3$) affect the reduction of ARB in secondary wastewater. Coagulation effectively reduced four types of ARB (clindamycin, sulfamethoxazole, tetracycline, and ciprofloxacin-resistant bacteria) by 37% to 95%. Despite this initial reduction, the abundance of ARB significantly increased in the reclaimed effluent after 4 days. The use of PAC and $FeCl_3$ notably boosted the frequency of bacterial conjugation, the process where bacteria exchange genetic material, including ARGs. Factors like proper mixing, extended incubation, and optimal temperature during coagulation enhanced this gene transfer. Larger bacterial aggregates formed during coagulation promoted closer cell interactions, facilitating gene exchange. Additionally, PAC and $FeCl_3$ regulated the expression of genes related to conjugation, increasing the efficiency of genetic material transfer. The findings indicate that while coagulation with PAC and $FeCl_3$ reduces ARB initially, it could potentially accelerate ARG spread during tertiary wastewater treatment, necessitating careful management and follow-up processes [552]. Furthermore, Johansson MHK et al., in their research, indicate that multiple factors play a role in the network responsible for disseminating ARGs. They observed that climate conditions, or factors similar to climate, influence the types of MGEs present in specific geographic regions. This, in turn, restricts the availability of MGEs accessible to bacteria and the potential interactions between different MGEs. Additionally, they discovered that certain MGEs have a wider range of host compatibility than others, which may enhance their ability to transfer genes across diverse bacterial species. These collective factors act as constraints on the potential pathways for gene transposition [553].

The mechanisms through which genetic material is exchanged between clinical and environmental bacteria in WWTPs

Comprehending the mechanisms through which genetic material is exchanged between clinical and environmental bacteria is essential for managing the dissemination of AMR. Many resistance genes are located on MGEs, such as plasmids, transposons, and integron-specific gene cassettes, acting as vehicles for these determinants and enabling their dissemination**.** HGT facilitates the transfer of genetic material between distinct species or even across different domains of life. It serves as a significant catalyst for microbial evolution, enabling the rapid acquisition of novel traits such as antibiotic resistance. This exchange of genetic material empowers microorganisms to effectively adapt to varying environmental conditions and

evolutionary pressures. This adaptability becomes especially pertinent when confronted with environmental stressors and shifting circumstances. There are three primary mechanisms of HGT: transformation (direct DNA uptake), transduction (virally mediated transduction), and pili-mediated conjugation. Transformation entails the absorption of free DNA from the surrounding environment, transduction is facilitated by bacteriophages, and conjugation involves the direct transfer of genetic material through direct cell-to-cell contact. Recent findings indicate that bacteriophages have a significant impact on horizontal gene transfer and recombination. Significant proportions of clinically relevant carbapenemase genes (blaCTX-M, blaKPC, blaOXA-48-like, and blaNDM-1) were detected in phage fractions isolated from environmental water. This indicates that bacteriophage transduction could potentially play a role in the horizontal transfer of resistance genes. The study revealed a notable disparity in the prevalence of resistance genes between the bacteriophage and bacterial fractions of water samples. Resistance genes were detected in 7.3% to 64.9% of the phage samples, whereas only 5.4% to 36.8% of the bacterial samples tested positive. Among the resistance genes identified, blaOXA-48-like and blaTEM were the most widespread, occurring in 57.3% and 64.0% of the samples, respectively, across all sampling locations. These findings underscore the significant role of bacteriophages as reservoirs for ARGs and imply that phage-mediated transduction could be a crucial mechanism in the horizontal gene transfer of resistance traits among microbial communities [554]. Phage-mediated transduction is a significant mechanism facilitating horizontal gene transfer, as evidenced by metagenomic studies detecting a substantial proportion of bacterial genes within viral DNA fractions [555]. Using a network-based approach, it demonstrated that staphylococcal hosts from different species, ecosystems, or antibiotic resistance phenotypes are closely interconnected through numerous phages. Additionally, it was revealed that phages facilitating these connections have the ability to incorporate foreign genetic material, as indicated by the presence of a chloramphenicol resistance gene. These findings challenge the commonly reported host specialization of phages and highlight phages as potent vehicles for bacterial genetic exchange[556]. Phages, especially those with broad host ranges, can serve as reservoirs and carriers for antibiotic resistance, potentially aiding its spread in the environment. In a study, the majority of phages exhibited the ability to infect a wide variety of STEC strains, highlighting their broad host specificity. Notably, twelve E. coli phages were capable of infecting all four tested E. coli O157 strains. Among these, the blaTEM gene was detected in 15 phages, and six of them were found to transfer blaTEM into E. coli ATCC 13706 via transduction. This widespread presence of antibiotic resistance genes has significant ecological implications, as it could promote extensive genetic

exchange among bacterial hosts. Furthermore, the observation that three out of six transducing phages successfully transferred blaTEM genes across different serotypes provides further evidence of antibiotic resistance transfer facilitated by phages originating from the environment [557]. Although rare, certain transducing phages containing ARGs likely arise due to errors in packaging during the infection process of ARB. Some undegraded ARGs are released from antibiotic-resistant bacteria along with progeny phages, which may then be assimilated by other bacteria. This raises concerns about the potential for horizontal transfer of ARGs facilitated by such phages. The E. coli strain XX13, resistant to multiple antibiotics (at least eight antibiotics), along with its associated phage (YZ1), was isolated from a municipal wastewater treatment facility. It's worth noting that although transducing phage particles carried some ARGs, the actual transduction process might be influenced by various environmental factors, such as concentrations of host bacteria and phages, temperature fluctuations, the presence of other substances in the medium, or other unknown factors [532].

Wastewater environments are hotspots for the selection of conserved chromosomal structures and diverse, adaptable plasmids. These conditions promote extensive gene exchange between plasmids, leading to the widespread dissemination of resistance genes. A significant factor in this process is the high diversity of plasmids found in WWTP isolates, which correlates with an increased variety of ARG. This diversity facilitates the proliferation of conserved E. coli sequence types (STs) and their specific associations with plasmids, driven by the continuous introduction of new resistance plasmids and the varying environmental conditions in WWTPs. These dynamic interactions enhance the exchange of genetic material, perpetuating the persistence and spread of resistance genes within microbial communities in wastewater[558]. Bacteria from the Aeromonadaceae, Moraxellaceae, and Bacteroidetes families act as crucial reservoirs of antibiotic resistance in WWTPs. Specifically, Aeromonadaceae bacteria may play a significant role in the spread of antibiotic resistance within these systems. Additionally, IncQ plasmids and class 1 integrons, noted for their broad host range, are highlighted as key vectors for horizontal gene transfer [559]. It has been observed that specific ERY-ARGs can be efficiently transmitted between bacteria via mobile elements like plasmids and transposons, resulting in the extensive spread of these resistance traits within the environment [144]. The discovery of plasmid-mediated genes responsible for beta-lactam resistance in nearly 10% of bacteria present in the final effluents of WWTPs and in over 32% of bacteria in the vicinity of WWTP areas confirms the dissemination of these genes into the environment, facilitating their further transmission among environmental bacteria. Moreover, the ability of these genes to transfer to an E. coli recipient strain indicates a significant potential for horizontal gene transfer

among strains of different genera within sewage and environmental samples. All examined bla genes were transferable to E. coli J53 (RifR) through conjugation assays, indicating their location on transferable elements irrespective of their class. The frequency of conjugation ranged from 1.0×10^{-5} to 3.5×10^{-5} per donor strain, suggesting a substantial potential for horizontal gene transfer among strains of different genera within both sewage and environmental samples [299]. Continuous discharges of treated wastewater exert a significant influence on the river's resistome, impacting not only the quantity and diversity of ARGs but also facilitating their potential spread by enriching the river's mobilome with a diverse range of MGEs. In all cases examined, downstream metagenomes exhibited notably elevated levels of sequences associated with both the phage integrase family (PFAM no. PF00589) and transposases, with more pronounced variations observed among sequences linked to the transposase 7 family (PFAM no. PF01526), which includes transposases from Tn3, Tn21, Tn1721, Tn2501, and Tn3926. The substantial abundance and diversity of various integrase and transposase genes in downstream metagenomes strongly suggest that HGT likely plays a significant role in the acquisition and dissemination of ARGs in both the source UWWTP and the surrounding environment [560]. Simultaneously, Munke et al. have highlighted that the overall dissemination of resistance genes from WWTPs is comparable to that observed in soil environments. Their analysis reveals a significant correlation ($P < 0.001$, based on 999 permutations) between the resistome of WWTPs and their microbial phylogeny, indicating a close association between the core resistome of WWTPs and the microbial community. Through sequencing the 16S rRNA of longitudinal samples collected over a 7-year period from the same WWTP, they observed remarkable stability in the microbial community within the WWTP, suggesting that the exchange of the WWTP resistome occurs to a limited extent within the WWTP environment [561]. Effluents from WWTP have the potential to facilitate the horizontal transfer of MGEs and multidrug resistance phenotypes. Notably, a statistically significant correlation was found between the presence of integrons in E. coli isolates and resistance to fluoroquinolones, trimethoprim/sulfamethoxazole, amoxicillin/clavulanate, and piperacillin/tazobactam [562]. Despite the wastewater treatment process effectively reducing the concentration of integrons, their levels remained substantial (ranging from 10^5 to 10^6 copies/ml) with significant diversity in the effluents. This highlights the potential risks associated with the integron-mediated dissemination of ARGs into downstream environments. Among the three classes of integrons studied, class 1 integrons were found to be the most prevalent, consistent with previous research [563,564]. In contrast, the concentrations of class 2 and 3 integrons were relatively low compared to class 1 integrons. Additionally, class 1

integron gene cassettes were observed to carry more antibiotic resistance genes than class 3 integron gene cassettes, indicating that class 1 integrons may play a primary role in the spread of integron-mediated antibiotic resistance. In activated sludge, where most ARG cassettes were located, class 3 integron gene cassettes exhibited greater diversity. This suggests that activated sludge may offer favorable conditions for the enhanced exchange and recombination of ARG cassettes within class 3 integrons [565]. A positive correlation was found between the increasing number of MDR strains and the presence of class 1 integrons, either individually or in conjunction with class 2 integrons, suggesting the involvement of integrons in facilitating multiple antibiotic resistance. Additionally, the prevalence and diversity of sul and dfr genes indicate redundancy among plasmid-mediated sulfamethoxazole and trimethoprim-resistant genes. These findings collectively underscore the significance of transmissible plasmid-mediated and integron-associated ARGs, specifically those conferring resistance to sulfamethoxazole and/or trimethoprim, among bacteria. This diversity potentially contributes to the persistence and dissemination of antibiotic resistance [566]. Promiscuous, broad-host-range plasmids from the P incompatibility (IncP) group have been frequently implicated in the emergence and dissemination of antibiotic resistance genes among gram-negative bacteria. These versatile plasmids facilitate the transfer of resistance determinants across diverse bacterial species, significantly contributing to the spread of antimicrobial resistance. The combined action of an integron and the conjugative DNA-transfer modules of an IncP-1 broad-host-range plasmid is crucial for the rapid emergence and spread of enhanced resistance traits. It has been shown that an integron on the IncP-1β multiresistance plasmid, specifically pB10, can function by integrating an additional resistance gene cassette into the central segment of the integron. Moreover, the insertion of an IS10 element can lead to increased levels of β-lactam resistance [567]. The presence of genes for ESBL (Extended-Spectrum Beta-Lactamase), integron-integrase, antibiotic resistance, and MDR efflux in bacteria isolated from the Municipal Wastewater Treatment Plant (MWWTP) suggests that this facility's wastewater may be a critical area for the development and spread of MDR bacteria. This situation could pose significant risks to both human health and the environment [568]. In addition to horizontal gene transfer and the selection of resistant organisms/genetic determinants, the preferential elimination of susceptible organisms compared to their resistant counterparts can also play a significant role in the increase of antimicrobial resistance. Notably, certain subsets of E. coli, such as type A0, which were selectively removed during wastewater treatment, were found to contribute to the observed increase in antimicrobial resistance prevalence in the final effluent [569]. Abdulkadir N et al.'s analyses indicated that only 10.26% of the annotated ARGs were

located on plasmids, suggesting that the majority of ARGs are not transmitted through plasmids. Most of the annotated ARGs were found on chromosomes, indicating that these genes are primarily passed down through vertical gene transfer (VGT), from parent to offspring. While ARGs on plasmids are prone to be transferred between different bacterial species, chromosome-located ARGs are the main contributors to the spread of ARGs within bacterial communities [570] . In addition, Meng L et al. (2017) evaluated five ARGs and found that none exhibited a significant positive correlation with intI1 ($p < 0.05$) over the operational period. This indicates that horizontal transfer pathways may not be the primary mechanism for the spread of ARGs in the system. Instead, it suggests that vertical gene transfer played a major role in shaping the ARG profiles observed [571]. Amirsoleimani et al. (2021) emphasized that out of the ten plasmids examined in their study, five originated and were exclusively carried within a single species or clonal complex of a species. The exclusive presence of these plasmids within a clonal complex of S. aureus or a staphylococcus species suggests their capacity for conservation within a specific group, with higher coverage levels indicating more recent acquisition and greater preservation. This pattern also suggests vertical gene transmission [572].

7.2. Results of the Scoping Review on "Updates on developing and applying biosensors for the detection of microorganisms, antimicrobial resistance genes and antibiotics: a scoping review."

Following the removal of duplicate articles, a comprehensive search of literature databases yielded a total of 786 unique articles. Through the screening of titles and abstracts, 594 articles were excluded. Subsequently, 192 full-text articles were retrieved and assessed for eligibility. Out of these, 144 articles were excluded for various reasons: 91 were not relevant for the objective of the current scoping review, 11 lacked full-text availability, and 42 were identified as reviews. Finally, a total of 48 studies met the eligibility criteria and were included in this scoping review (Figure 3).

Development and validation of biosensors for the detection of microorganisms and antimicrobial resistance

Characteristics of the studies

In this scoping review, 25 studies were included, all focused on the development and/or validation of biosensors for detecting ARGs and/or antimicrobial-resistant (AMR) microorganisms in clinical or bacterial samples. The studies spanned a time frame from 1999

to 2022. Among them, 6 studies were conducted in the United States of America [573-578], 8 studies in China [579-586], while 2 studies were conducted in Canada [587,588] and UK [589,590], respectively. Only 1 study was conducted in Australia [591], Italy [592], Spain [593], France [594], Switzerland [595], Thailand [596] and Turkey [597], respectively. In these studies, the authors described a wide range of biosensors, and different samples used for their validation. In particular, the authors of included studies defined their biosensors as: silicon-based biosensors [573,574,576]; based on nanoscale film on optical fiber [577]; surface plasmon resonance biosensor [588]; sensitive surface-enhanced Raman scattering biosensor [586]; biosensors based on colorimetric assays [581,583] or on new nanocomposite materials [594]; binary deoxyribozyme biosensor [578]; electric and electrochemical biosensors [575,589-592,595-597]; electrochemical aptasensor [585]; conventional multiple cross displacement amplification technique combined with nanoparticle-based lateral flow biosensor [580,582,584], ultrasensitive nanophotonic bimodal waveguide interferometer [593], and biochip systems [579,587]. With respect to the samples used for the development and/or validation of biosensors, 9 studies developed and applied biosensors for clinical samples (e.g., human serum and urine samples) [573,576,577,579,586,587,589,595,597], while 13 for bacterial samples [574,575,580,581,583,584,588,590-594,596]. Three studies utilized both clinical and bacterial samples for their analyses [578,582,585].

Biosensors in clinical samples

In total, 9 studies focused on the development and/or validation of biosensors for detecting microorganisms and/or ARGs in clinical samples [573,576,577,579,586,587,589,595,597]. Nucleic acid hybridization was commonly analyzed using detection methods such as colorimetric, chemiluminescent, radioactive, or fluorescent labeling. However, it is well-established that biosensors can also detect nucleic acid hybridization without the need for labeled reporter molecules. For instance, Ostroff and colleagues developed a silicon-based biosensor capable of generating a visual signal in response to nucleic acid targets by directly interacting with light and thin films on a modified silicon surface. The biosensor's surface was functionalized with capture oligonucleotide probes, which, through hybridization with complementary targets and biotinylated detector oligonucleotides, facilitated the formation of an organic thin film. This process was further amplified using peroxidase, leading to the deposition of an insoluble product on the silicon surface. Changes in reflected light interference patterns caused a visible color shift from gold to purple, corresponding to variations in surface thickness due to target hybridization. Notably, this biosensor demonstrated the ability to detect

a wide range of targets, including single-nucleotide polymorphisms, in human serum and urine samples. To evaluate its discriminatory power, six synthetic mecA target molecules were generated, and the biosensor's performance was validated. Results can be obtained within just 25 minutes through direct visual inspection, or quantified using ellipsometry. The biosensor also exhibited an impressive detection limit of 5 pmol/L [573]. Similarly, Lindsey and colleagues utilized a silicon-based biosensor to detect the *mecA* gene, enabling the identification of methicillin resistance. In addition, they targeted the *tuf* and *femB* genes to distinguish the *Staphylococcus* genus and species, respectively. The assay was tested on 107 consecutive positive blood cultures from clinical patients and demonstrated 100% concordance with standard microbiological methods. The detection process involved target immobilization, which triggered a series of reactions that converted hybridization events into a molecular thin film, causing the surface color to shift from gold to blue. Remarkably, this biosensor delivered results within just 90 minutes from signal-positive samples, all without the need for instrumentation [576]. With the rising prevalence of methicillin resistance among *Staphylococcus* species in hospitals, communities, and animals, there is an increasing need for culture-free diagnostic assays. In this context, optical fiber biosensors offer several key advantages, including their lightweight nature, cost-effectiveness, and ability to capture light emitted from targets after excitation. Bandara and collaborators introduced a nanoscale-film optical fiber sensor designed for the detection of methicillin-resistant staphylococci. The team developed an optical fiber coated with a self-assembled nanoscale film, which was functionalized with monoclonal antibodies specifically targeting penicillin-binding protein 2a (PBP2A), a key marker for methicillin resistance. Samples were collected from healthy volunteers using swabs from the ear canal, nostrils, palms, or arm skin. When the target antigen (PBP2A) bound to the monoclonal antibodies, it induced changes in the thickness and refractive index of the optical fiber's thin film coating. These changes generated a detectable signal, clearly indicating the presence of the target antigen. The system demonstrated promising potential for the rapid and specific detection of MRSA (methicillin-resistant *Staphylococcus aureus*) [577]. Suea-Ngam and colleagues introduced an innovative electrochemical CRISPR/Cas biosensor, known as E-Si-CRISPR, which leveraged silver metallization for the detection of MRSA. This cutting-edge biosensor eliminated the need for nucleic acid amplification, streamlining the detection process while maintaining exceptional analytical performance. Remarkably, E-Si-CRISPR demonstrated high selectivity for MRSA in human serum, effectively distinguishing it even in the presence of other commonly encountered bacterial species [595]. Beyond *S. aureus*, the growing prevalence of drug-resistant

Mycobacterium tuberculosis strains is a significant concern. In response, Kara and collaborators developed an innovative electrochemical genosensor designed to detect multiple point mutations in the *rpoB* gene, a key marker for rifampicin resistance. This technology utilized bacterial DNA from clinical isolates and featured graphite electrode arrays modified with five distinct guanine-free aminohexyl-tethered oligonucleotide probes. These probes targeted specific segments within the hotspot region of the *rpoB* gene. The hybridization process between PCR amplicons and inosine-modified capture probes on the graphite surface generated an electrochemical signal, which was monitored through changes in peak voltage related to guanine oxidation. This signal provided a reliable method for detecting mutations associated with rifampicin resistance [597]. Guo and colleagues introduced a rapid biochip system for the simultaneous identification of the most common mutations in the *rpoB*, *katG*, and *inhA* genes of *M. tuberculosis* isolates and clinical sputum samples. The biochip, which featured covalently immobilized oligonucleotide probes, consisted of four distinct arrays. After a multiplex asymmetric PCR and subsequent hybridization on the biochip, software was used to quantify fluorescent intensities. When compared to conventional susceptibility testing and DNA sequencing, the biochip showed excellent agreement with both methods, offering a simple and accurate clinical assay that could be completed in just six hours [579]. To overcome the need for an active multiplexing strategy in highly multiplexed arrays, Lam and collaborators introduced a novel approach using a two-dimensional array of electrodes. This method allowed for the programming of transient solution-based circuits, enabling individual sensor analysis using shared contacts. Compared to integrating costly active electronics on silicon surfaces, this solution offered significant advantages. The system included 100 working electrodes, 30 off-chip contacts, and 5 liquid channels, each containing 20 sensors. The authors adapted the chip's multiplexing capabilities to detect various pathogens and analyze antibiotic resistance. To develop and validate the system, the team designed probes based on the *rpoB* gene sequences of the target bacteria. The set of probes, aimed at mRNA targets to confer pathogen specificity, covered 90% of common urinary tract pathogens and was capable of detecting clinically relevant levels of *Escherichia coli*. Additionally, the system was compatible with unpurified bacterial lysates and samples ranging from microliters to milliliters, demonstrating its versatility. This technology represents a major advancement in biomolecular sensing, with broad potential applications in pathogen detection and antibiotic resistance analysis [587]. In the field of optical biosensors, Surface-Enhanced Raman Scattering (SERS) has emerged as a powerful technique for bacterial detection, prized for its exceptional sensitivity and ease of use. Leveraging this approach, Wang and collaborators developed a SERS-based biosensor that demonstrated

impressive bacterial capture efficiency, achieving 65% efficacy across various pathogens, including *E. coli*, *S. aureus*, and MRSA, in complex solutions. Notably, the biosensor required only a short assay time of 30 minutes, and its effectiveness was validated in both milk and blood samples [586]. In response to the increasing prevalence of MDR Gram-negative pathogens, researchers have focused on developing biosensors capable of detecting key resistance genes. Huang and colleagues, for instance, created an electrochemical biosensor specifically designed to detect *blaNDM*, the gene responsible for producing New Delhi metallo-beta-lactamase (NDM), a major contributor to antibiotic resistance. Their study utilized a clinical isolate of *Citrobacter freundii* producing NDM-1. Using in silico techniques, the team designed nucleic acid probes targeting *blaNDM*, which were immobilized on gold screen-printed electrodes. The biosensor employed label-free electrochemical impedance spectroscopy to detect *blaNDM* PCR products and enabled direct, label- and amplification-free detection of *blaNDM* plasmid DNA. The assay's effectiveness was demonstrated through the detection of synthetic targets, PCR products, and direct detection of plasmid DNA without amplification, offering a robust method for identifying NDM-related resistance genes [589].

Biosensors in bacterial samples

A total of 13 studies focused on the development and/or validation of sensors for detecting microorganisms and/or ARGs in bacterial samples. Additionally, three studies extended this work by validating such sensors in both clinical and bacterial samples [578,582,585]. Among the studies investigating sensors for microorganism or resistance gene detection, some utilized bacterial DNA [574,575,580,584,588,592-594], while others employed bacterial cultures [581,583,590,591,596]. Building on the work of Ostroff and Lindsey, Jenison and colleagues designed a silicon-based biosensor specifically engineered to detect the *mecA* gene. Using DNA extracted from a MRSA strain, they demonstrated how enzymatic transduction promoted hybridization, forming a molecular thin film that was visually detectable under white light. Initially, the unreacted surface had a thickness of 0 Å, giving it a gold appearance. As the surface thickness increased to 100 Å, the color transitioned from gold to red, then to purple, and finally white, before returning to its original gold hue. The sensor successfully detected a color signal when DNA from an MRSA strain was used, while no such signal was observed with a *Staphylococcus aureus* strain lacking the *mecA* gene [574]. Tombelli and colleagues advanced the development of a biosensor for detecting the *mecA* gene in MRSA by proposing a mechanical DNA-based piezoelectric biosensor. This sensor detects hybridization by measuring frequency changes caused by the interaction between a specific DNA probe and its

complementary strand. The probe was immobilized on a gold electrode positioned on a quartz crystal. The authors compared two methods of probe immobilization: one utilizing streptavidin-biotin interaction, and the other using direct immobilization of thiolated probes. The goal was to identify the most efficient and effective immobilization technique for probes targeting the *mecA* gene. In addition to testing immobilization methods, the researchers evaluated two different bacterial DNA denaturation procedures as part of the biosensor's preparation. The optimal results were achieved using the biotinylated probe immobilization method and PCR-amplified DNA samples treated with thermal denaturation combined with blocking oligonucleotides. With further optimization of PCR conditions, the proposed biosensor shows promise for broader application in detecting PCR-amplified DNA from clinical samples [592]. In contrast to earlier approaches, Wei and colleagues introduced a biosensor based on aptamers—short oligonucleotide molecules that offer enhanced selectivity and binding properties compared to natural DNA and RNA. Their aptamer-based colorimetric biosensor leveraged the CRISPR/Cas12a system and recombinase polymerase amplification to detect MRSA. Testing on clinical samples confirmed the biosensor's strong potential as a robust platform for detecting antibiotic-resistant bacteria [585]. In a similar context, Wang and collaborators developed a biosensor for detecting *Staphylococcus aureus* while also identifying methicillin resistance. Their approach utilized the conventional multiple cross displacement amplification (MCDA) technique, which is known for its high selectivity in target sequence detection through a specific set of 10 primers. This MCDA assay was combined with a nanoparticle-based lateral flow biosensor (MCDA-LFB). The biosensor was rigorously evaluated using 58 bacterial strains, including a range of Gram-positive and Gram-negative species. The results demonstrated exceptional analytical specificity, achieving 100%, with no cross-reactivity to non-*S. aureus* strains during testing. These findings underscore the biosensor's potential as a simple, rapid, and reliable tool for detecting *S. aureus* strains and identifying MRSA infections, thereby aiding in timely and accurate antibiotic therapy decisions [580]. Similarly, Chen and colleagues developed a multiplex loop-mediated isothermal amplification biosensor (m-LAMP-LFB), also paired with a nanoparticle-based lateral flow system, to detect all *S. aureus* species and MRSA. The primers for this biosensor targeted the *femA* gene, specific to *S. aureus*, and the *mecA* gene, responsible for methicillin resistance. The multiple-LAMP products were analyzed using a colorimetric indicator and a lateral flow biosensor, where the amplification process triggered a color change from colorless to light green, enabling straightforward visual detection. The assay's optimization demonstrated a specificity of 100%. Given its speed, accuracy, and ease of use, this biosensor shows great promise as a

reliable method for rapid detection of *S. aureus* and MRSA, even in clinical samples [584]. While previous studies focused on the MCDA-LFB for detecting *Staphylococcus aureus*, Hu and colleagues adapted this biosensor for the detection of *Acinetobacter baumannii* strains, specifically targeting the carbapenem-resistance gene *blaOXA-23-like*. Using bacterial strains from the American Type Culture Collection and Shougang Hospital, they validated the optimal reaction conditions and demonstrated 100% specificity for the assay. Notably, the MCDA-LFB assay was also evaluated with clinical samples, specifically DNA templates extracted from 110 sputum samples, where it showed consistent results compared to conventional culture-based techniques [582]. There has also been increasing interest in developing biosensors for detecting *M. tuberculosis* and its antibiotic-resistant strains, as indicated by two studies [575,594]. Mac Sweeney and colleagues introduced an optical DNA hybridization biosensor using ruthenium electrochemiluminescence for detecting the *rpoB* gene of *M. tuberculosis*. The target DNA strand was labeled with a ruthenium ester, which, upon hybridization, emitted light at a wavelength of 620 nm. The biosensor utilized a solid-state silicon PIN photodiode as the transduction element. The researchers focused on characterizing and optimizing each component of the biosensor to enhance its sensitivity in detecting the electrochemiluminescent optical signal generated during the DNA hybridization event [575]. Bizid and colleagues developed a direct DNA sensor using novel nanocomposite materials (Fc-ac-OMPA/MWCNTs). The nanocomposite membrane significantly enhanced the anchoring of DNA strands, resulting in an improved electrochemical response compared to sensors based solely on redox oligomers. By integrating PCR amplification with electrochemical devices, this biosensor offered a highly sensitive method for detecting DNA from the *rpoB* gene of *M. tuberculosis*. The biosensor was notable for its ease of construction, excellent reproducibility, and minimal time requirements, making it a competitive option for future applications in pathogen diagnostics and therapeutics [594]. In a similar context, Bengtson and colleagues introduced binary deoxyribozyme sensors for multiplex detection of extensively drug-resistant *M. tuberculosis* in both clinical and bacterial samples [578].Their sensor integrated multiplex PCR with the detection of single nucleotide polymorphisms (SNPs), specifically targeting genes associated with drug resistance: *rpoB*, *katG*, *inhA*, and *gyrA*. These genes are linked to resistance against rifampicin, isoniazid, and fluoroquinolones. The sensor employed highly selective binary deoxyribozymes, allowing for precise detection of five key mutations within these genetic loci. The assay demonstrated the ability to accurately identify *M. tuberculosis* strains, as well as mutations conferring resistance to the three antibiotics, even in clinical isolates. Furthermore, the sensor proved capable of detecting minor populations of drug-resistant strains and previously unknown

mutations at the targeted loci, showcasing its potential for high-precision diagnostics in tuberculosis treatment [578]. Rachkov and colleagues proposed a surface plasmon resonance (SPR) biosensor system capable of detecting single-base mismatched oligonucleotides associated with the *rpoB* gene of *M. tuberculosis*. Their work detailed the design of the SPR instrument, the immobilization of DNA probes, and the subsequent hybridization process with target sequences. The study focused on understanding the hybridization dynamics between the immobilized probe and two types of targets: perfectly matched oligonucleotides and single-base mismatched variants. This analysis was conducted in buffer solutions with varying stringency levels. The results revealed distinct hybridization patterns between the two target types and the probe, allowing for effective discrimination between perfectly matched and single-base mismatched sequences. The ability to differentiate these targets with precision and a rapid response time underscores the biosensor's potential for identifying highly virulent microbial organisms through DNA hybridization. Additionally, the SPR technology could be further developed to create a multiplexed array, significantly enhancing its utility for antimicrobial resistance profiling of *M. tuberculosis*, providing valuable insights for treatment and diagnostics [588]. Researchers have increasingly focused on the urgent need for rapid and direct detection of key antimicrobial resistance genes in Gram-negative bacteria. Maldonado and colleagues introduced an innovative biosensor that employs an ultrasensitive nanophotonic bimodal waveguide interferometer for the direct detection of the *blaCTX-M-15* and *blaNDM-5* genes, eliminating the need for amplification. This approach leverages the evanescent field principle, which detects local changes in the refractive index at the sensor's surface, enabling real-time monitoring of molecular interactions. To enhance detection, the researchers incorporated pre-analytical steps such as DNA extraction, fragmentation, and denaturation, optimizing the biosensor's ability to detect resistance gene sequences. The results showed that this biosensor achieved outstanding sensitivity and specificity, successfully identifying antimicrobial resistance within 30 minutes. These features make the biosensor a valuable tool for the rapid diagnosis of drug resistance, particularly in healthcare-associated infections caused by multidrug-resistant bacteria. Its speed and accuracy hold significant potential for improving infection control and antimicrobial stewardship in clinical settings [593]. Additionally, five studies reported the development of sensors for detecting microorganisms and/or ARGs using bacterial cultures [581,583,590,591,596]. Zhou et al. reported the development of a colorimetric biosensor utilizing engineered polydiacetylene liposomes for the detection of various pathogenic bacteria. The study targeted several key bacterial species, including *P. aeruginosa*, *S. aureus*, *E. coli*, *S. pneumoniae*, *K. pneumoniae*, and *E. faecalis*, which were

sourced from reputable repositories like the ATCC and the National Center for Medical Culture Collections (CMCC). The striking chromatic changes observed in the biosensor were attributed to the unique interactions between bacterial toxins and the biomimetic lipid bilayers of the liposomes. Zhou and colleagues developed a colorimetric fingerprint array capable of differentiating between microorganisms by analyzing how bacterial toxins interacted with the liposome bilayers. This label-free, liposome-based system demonstrated faster response times and greater sensitivity compared to other chromatic bacterial detection assays. Notably, the biosensor could accurately distinguish between pore-forming hemolysis and phospholipase-induced hemolysis, even without ligand conjugation, underscoring its high sensitivity and effectiveness in identifying bacterial species based on their secreted toxins [583].

In this context, Sun and colleagues developed a colorimetric and electrochemical bioassay for detecting *E. coli* and assessing its antibiotic resistance, utilizing p-benzoquinone as a redox mediator. The assay produced a visible color change that could be captured using a smartphone within a “light box,” with the intensity of the color correlating to the bacterial concentration. To demonstrate its practical application, the bioassay was tested on two artificially induced antibiotic-resistant strains of *E. coli*, successfully detecting their resistance levels. Unlike existing electrochemical bacterial biosensors, this assay required no additional sample processing, enzymes, or complex instrumentation for result visualization. These features make it a rapid, cost-effective solution for identifying *E. coli* and its antibiotic resistance, offering significant advantages in both simplicity and speed compared to more traditional detection methods [581]. Abeyrathne and colleagues developed a chip-based sensor for the rapid detection of *S. aureus* and its sensitivity to flucloxacillin. The biosensor utilized interdigitated electrodes to measure impedance changes resulting from the binding of *S. aureus* to immobilized antibodies specific to the bacterium on the sensor surface. In addition, the sensor monitored changes in the medium's conductivity caused by bacterial growth. The biosensor demonstrated the ability to differentiate between positive and negative control samples within just one hour and accurately assess flucloxacillin sensitivity within two hours. These promising results underscore the potential of the sensor for further refinement and broader application, including use in whole blood samples and detection of other bacterial subtypes, positioning it as a valuable tool for rapid diagnostics [591]. Pintavirooj and colleagues developed an innovative electrochemical biosensor utilizing molecularly imprinted polymers for the detection of *K. pneumoniae*. Their approach employed cyclic voltammetry to monitor current variations at the sensor surface, reflecting biochemical interactions occurring on the electrode. This biosensor facilitated quantitative detection by measuring changes in electrical signals

across a range of *K. pneumoniae* concentrations. The sensitivity tests revealed that the biosensor featuring a gold electrode demonstrated superior sensitivity compared to those designed for other microorganisms, such as *P. aeruginosa* and *E. faecalis*. The sensor's ease of use, combined with its high accuracy and specificity for detecting *K. pneumoniae*, positions it as a promising tool for clinical diagnostics and environmental monitoring [596]. The detection of Gram-positive microorganisms has seen advancements through the development of innovative biosensors. For example, Schulze and colleagues introduced a novel electrochemical impedance spectroscopy-based sensor that utilizes a new class of non-biological binder molecules for the direct, label-free detection of *Staphylococcus carnosus*. This approach leverages specific conformational changes in the polymers immobilized on the surface of gold screen-printed electrodes in response to the presence of the bacteria. Remarkably, *S. carnosus* was detected within just 20 minutes of incubation when the sample solution was applied to the polymer-functionalized electrodes. The results showcased a clear differentiation between Gram-positive and Gram-negative bacteria, marking a significant advancement over existing bacterial detection assays. This enhanced specificity and rapid detection capability highlight the sensor's potential for improving diagnostic accuracy in clinical and research settings [590].

Development and validation of biosensors for the detection of antibiotics

Characteristics of studies included

The scoping review encompassed 23 studies focused on the development and validation of biosensors for antibiotic detection, with research conducted between 2002 and 2021. Among these studies, 3 studies were conducted in the United States of America [598-600], 3 in Germany [601-603], 5 in China [604-608], 2 in Switzerland [609,610] and India [611,612], respectively. Only 1 study was conducted in UK [613], Denmark [614], New Zeeland [615], Japan [616], Taiwan [617], Iran [618], Australia [619], and Saudi Arabia [620], respectively. The studies reviewed showcased a diverse array of biosensors and the various samples used for their validation. In particular, the authors of included studies defined their sensors as: *E.coli* whole-cell biosensors [614,615], optical biosensors [612,613,617], surface plasmon resonance sensor [602], generic dipstick-based sensor [610], electrochemical biosensors [598,600,601,606,619,621], biosensor based on aptamers [607,608,618], amperometric immunosensor [611], bioluminescent assays [603-605], colorimetric paper-based biosensor [616], biosensor based on proteins of prokaryotic origin [609], and highly sensitive square wave voltammetry [620]. With respect to samples used for the development and/or validation of these biosensors, 5 studies regarded the development and application of biosensors in clinical (e.g.,

human blood and serum and urine samples) or bacterial samples [598,601,605,611,615], while 18 in environmental samples (e.g., milk and meat samples, animal feces and serum, water samples) [599,600,602-604,606-610,612-614,616-620].

Biosensors in clinical or bacterial samples

In their work on developing biosensors for antibiotic detection in clinical samples, Kling and colleagues introduced a cutting-edge microfluidic platform capable of performing up to eight simultaneous enzyme-linked assays with electrochemical readouts. This innovation offers a highly adaptable solution for the rapid and efficient detection of multiple analytes in clinical environments. The system features microfluidic channel networks with discrete immobilization zones, each of which can be independently activated for specific assay protocols. The platform was successfully employed to detect two major antibiotic classes—tetracycline and streptogramin—in human plasma samples, achieving results in under 15 minutes [601]. Yadav and colleagues developed an innovative amperometric biosensor for detecting norfloxacin using urine samples. This biosensor utilized nanostructured yttrium oxide modified with chitosan, and the electrode was functionalized with fluoroquinolone antibodies, specifically targeting norfloxacin through covalent interactions. The result was a highly sensitive biosensor with a rapid response time of just 10 minutes. Its effectiveness was demonstrated through successful recovery of norfloxacin from spiked samples, achieving recovery rates between 90.5% and 101.1%. To further validate its performance, the biosensor was compared with the enzyme-linked immunosorbent assay (ELISA), showing greater sensitivity and a lower detection limit than existing ELISA methods. These findings highlight the potential of this approach for developing highly sensitive, fast-responding biosensors and biochip devices [611]. In the field of medical diagnostics, RNA-aptamer-based biosensors have gained attention for their potential use in point-of-care testing. However, one significant limitation has been their susceptibility to nuclease degradation. To overcome this challenge, Rowe and colleagues engineered an electrochemical sensor utilizing aptamers designed to target aminoglycosidic antibiotics in human serum. This platform offered a robust and reliable solution, allowing for the rapid detection of aminoglycoside concentrations within a clinically relevant range, with measurements completed in under 10 seconds and the entire process taking less than 30 minutes. To mitigate the vulnerability to nuclease degradation, ultrafiltration was employed, enabling accurate measurements in filtered serum calibrators. This suggests a promising general strategy for using RNA-based probes in biosensing applications [598]. To enhance the performance and stability of their previously developed biosensor for detecting cephalosporins and class C β-

lactamase inhibitors, Tsang and colleagues introduced a strategic mutation. By incorporating a Y150S mutation, they aimed to suppress the hydrolytic activity of the fluorescein-labeled class C β-lactamase mutant (V211Cf). This modification resulted in the creation of the Y150S/V211Cf biosensor, which demonstrated improved sustainability of the fluorescence signal and a rapid, targeted response to cephalothin, a first-generation cephalosporin. These results suggest that the Y150S/V211Cf biosensor has significant potential as a reliable tool for the quick and specific detection of cephalosporins [605]. Additionally, Song and colleagues developed a genetically engineered *E. coli* biosensor to detect tetracycline, using the bacterial respiratory gene *nuoA* as a reporter. To optimize sensor performance, they incubated the biosensor cells at a lower temperature (15°C), which reduced the risk of over-expressing the reporter gene. Additionally, they utilized a low-copy plasmid (pBR322) to carry the transgene, further minimizing gene over-expression and background noise. These modifications significantly improved the biosensor's sensitivity and response, demonstrating a more efficient and reliable approach to tetracycline detection [615].

Biosensors in environmental samples

The overuse and misuse of antibiotics in non-medical settings, particularly in livestock farming to promote growth, have significantly contributed to the rise of multidrug-resistant human pathogenic bacteria. To address this issue, Hansen and colleagues developed an E. coli whole-cell biosensor for detecting chlortetracycline in pig feces, investigating its correlation with the presence of tetracycline-resistant bacteria. Their findings showed a strong link between tetracycline concentrations and the emergence of tetracycline-resistant fecal coliform bacteria. Even as tetracycline levels declined, a high degree of resistance persisted among the bacterial population, highlighting the lasting impact of antibiotic exposure [614]. In response to growing concerns about antibiotic resistance, particularly in agriculture, European lawmakers have introduced restrictions on the use of antibiotics in veterinary medicine and banned their use in livestock breeding. To help enforce these regulations, Link and colleagues developed a dipstick-based assay to monitor antibiotic levels in serum, meat, and milk. This user-friendly test demonstrated detection limits up to 40 times lower than the legally allowed thresholds for antibiotics like tetracycline, streptogramins, and macrolides. The assay utilized membrane strips coated with streptavidin and immobilized biotinylated operator DNA, which served as a capture element for a bacterial biosensor tagged with a hexa-histidine (His6) marker. Upon exposure to samples containing antibiotics, the biosensor released the capture DNA in a dose-dependent manner, disrupting its interaction with the biosensor. The dipstick enabled visual

detection of antibiotic concentrations via a simple colorimetric change. After dipping the strip into two solutions, a chromogenic substrate was activated by a His6-targeted enzyme complex, producing a color shift that indicated the presence and concentration of antibiotics. This rapid, sensitive, and easy-to-use assay offers a powerful tool for monitoring antibiotic use in food production, helping to ensure regulatory compliance and promoting responsible antibiotic stewardship [610]. Weber and colleagues developed a protein-based biosensor of prokaryotic origin for the detection of tetracycline, streptogramin, and macrolide antibiotics in milk and cow serum samples. The biosensor achieved detection levels up to two orders of magnitude lower than the current European Commission threshold values. To optimize the system for high-throughput use in an ELISA-like format, the authors designed the biosensor to convert the interaction between the biosensor protein and DNA, which was chemically linked to a solid surface, into a colorimetric immuno-based readout. This color change directly correlated with specific antibiotic concentrations, allowing for efficient and sensitive detection of contaminants in food samples [609]. Furthermore, Kao and colleagues developed a method for detecting ciprofloxacin in food samples—such as milk, egg white, chicken essence, and egg yolk—at concentrations below the minimal allowed values. Their approach utilized luminescence induction by live bacterial sensor strains integrated into a windowless linear charge-coupled device (CCD)-based lens-free optical analyzer, known as the LumiSense System. The biosensor system incorporated microfluidic sample chips, a bacterial incubation chip (BacChip), and a linear CCD for photodetection. Luminescence emitted by bacteria within the BacChip was captured and recorded by PC-based software, offering a precise, real-time measurement. With response times ranging from 20 to 80 minutes and requiring only simple procedures such as mixing, dilution, and homogenization, this streamlined, high-throughput approach provided an efficient and effective means for screening food samples for antibiotic contamination [617]. Lai and colleagues introduced an innovative biochemiresistor for the detection of enrofloxacin, utilizing gold-coated magnetic nanoparticles modified with antibodies. These nanoparticles were magnetically assembled into films over interdigitated electrodes, forming the core of the biosensor. The gold-coated magnetic nanoparticles were functionalized with anti-enrofloxacin IgM antibodies (Ab-Au@MNPs), which served as the biosensing elements. When the biochemiresistor was exposed to a sample containing enrofloxacin, specific binding sites on the surface of the nanoparticles allowed enrofloxacin molecules to interact with the anti-enrofloxacin antibodies. This interaction caused some antibodies to dissociate from the Ab-Au@MNPs and form complexes with enrofloxacin in the solution. As a result, the nanoparticles within the film moved closer together, decreasing the film's resistance. The sensor exhibited a

direct relationship between the concentration of enrofloxacin and the reduction in resistance: the higher the analyte concentration, the more antibodies dissociated, leading to lower resistance. To assess its practical utility, the biochemiresistor was tested with a complex milk matrix, achieving an impressive detection limit of 2.8 pM for enrofloxacin. These results highlight the biochemiresistor's versatility, as it can be easily adapted to detect various analytes by modifying the antibodies on the Au@MNPs. Its high sensitivity and adaptability make it a powerful tool for a wide range of applications in biosensing and diagnostics [619]. Given the widespread use of fluoroquinolones (FQNs) as broad-spectrum antibacterial agents in animal husbandry and aquaculture, Cheng and colleagues developed a bioluminescent bacteria-based assay to detect FQNs in animal-derived food products. This innovative method is designed to enhance food safety and quality control. The assay leverages the *recA* promoter-fused luciferase reporter gene in *E. coli* pK12, containing the plasmid pRecAlux3, which triggers an SOS response in the presence of FQNs. This system successfully detected 11 FQNs, including enrofloxacin, across 11 different tissue types such as milk, fish muscle, and the muscles, livers, and kidneys of cattle, chickens, and pigs. With detection limits ranging from 12.5 to 100 μg kg^{-1}, —well below maximum residual limits—this assay is not only sensitive and accurate but also straightforward, requiring only simple extraction procedures, making it an effective tool for detecting FQNs in animal-derived foods [604].In the field of food safety and antibiotic residue detection, Wang and colleagues developed a homogeneous electrochemical aptasensor based on a target-induced, T7 exonuclease-assisted dual recycling signal amplification strategy. This aptasensor was applied to detect ampicillin in milk, demonstrating strong anti-interference capability in complex matrices, thus showing great potential for practical applications. The detection limit was as low as 4.0 pM, surpassing previously reported methods [606]. In addition, other studies have explored biosensors for detecting antibiotic residues in milk. Altintas and colleagues, for example, developed a surface plasmon resonance (SPR) sensor for detecting glycopeptide antibiotics in milk. They created molecularly imprinted nanostructures with high-affinity synthetic receptors that were integrated with the SPR sensor. These synthetic receptors exhibited superior affinity for target molecules compared to natural and other synthetic receptors. The sensor demonstrated impressive sensitivity and selectivity, detecting vancomycin in concentrations ranging from 10–1,000 ng mL^{-1}. This highlights the potential of such biosensors for accurate and sensitive detection of antibiotic residues in food safety applications [602]. In this context, Mohammad-Razdari and colleagues introduced an impedimetric aptasensor for the detection of Penicillin G in cow, sheep, goat, and water buffalo milk, as well as water samples. The aptasensor utilized a pencil graphite electrode modified

with reduced graphene oxide and gold nanoparticles to enhance sensitivity. By measuring charge transfer resistance, the aptasensor achieved an impressive detection limit as low as 0.8 fM. This demonstrates the aptasensor's potential as a highly sensitive and cost-effective tool for early screening of food samples, making it ideal for detecting Penicillin G in various matrices [618]. Similarly, Yazgan-Karacaglar developed a novel bioassay utilizing whole-cell organisms to detect several antibiotics, including ampicillin, benzylpenicillin, gentamicin, neomycin, and tetracycline, in milk samples. This bioassay employed *E. coli* cells expressing Green Fluorescent Protein (GFP) as a recognition agent. When viable cells were inhibited by antibiotics, a decrease in fluorescence intensity was observed, correlating with the rate of inhibition. The bioassay showed detection limits of 3.33, 0.29, 28.00, 618.36, and 33.17 μg/L for ampicillin, benzylpenicillin, gentamicin, neomycin, and tetracycline, respectively. These promising results suggest that this bioassay could be effectively used in the dairy industry for the detection of antimicrobial residues [603]. Among aminoglycoside antibiotics, tobramycin, which is derived from the fermentation of *Streptomyces tenebrarius*, has broad-spectrum antibacterial activity and can also be found in milk. Liu and colleagues developed a SnO_2/Bi_2S_3-based photoelectrochemical aptasensor specifically designed to detect tobramycin in milk samples. Under optimal conditions, the sensor achieved a detection limit of 4.28 nmol/L. The aptamer selectively captured tobramycin molecules, which increased electron transfer resistance and caused a subsequent decrease in photocurrent. The high selectivity and sensitivity of this aptasensor make it a promising tool for on-field food safety testing, particularly for controlling antibiotic residues in dairy products [607]. Beyond milk, Li and Stevenson explored the detection of antibiotics in meat samples, presenting promising advancements in this area. Li and colleagues developed an innovative electrochemical biosensor that integrates a hybrid nanowire/nanoparticle array with biomolecular receptors for the simultaneous detection of penicillin and tetracycline. The biosensor's electrode was composed of a multisegmented Au (L-cysteine)–Pt (penicillinase) nanowire array, which demonstrated exceptional sensitivity in detecting both antibiotics. The biosensor achieved detection sensitivities of 41.2 μA μM^{-1} cm^{-2} for penicillin and 26.4 μA μM^{-1} cm^{-2} for tetracycline, offering high precision and reliability for simultaneous antibiotic detection. Real-world application tests were performed using chicken and beef extracts, where the biosensor showed excellent recovery performance, highlighting its practical utility in food safety testing. The unique multisegmented and vertically aligned 3D nanowire array structure not only contributed to the high sensitivity but also provided a robust platform for detecting multiple bioanalytes at once. This cutting-edge biosensor holds significant potential for rapid, accurate

detection of antibiotics in a variety of applications, particularly in the food industry, where simultaneous monitoring of contaminants is essential for ensuring product safety and quality [599]. In this context, Stevenson and colleagues developed an affinity-based electrochemical biosensor for the detection of ceftiofur residues in meat samples. This biosensor employed electrochemical impedance spectroscopy (EIS) to measure the binding interactions between ceftiofur and the sensor surface, which induced detectable changes in interfacial capacitance. The results demonstrated the biosensor's ability to detect ceftiofur within just 15 minutes, even at low concentrations of 0.01 ng/mL in 1× phosphate-buffered saline (PBS). Additionally, it exhibited a detection limit of 10 ng/mL when tested with 220 mg of ground turkey meat samples. These findings underscore the biosensor's accuracy and speed, making it a valuable tool for sensitive ceftiofur detection across various meat products, with significant implications for food safety and veterinary medicine [600]. Similarly, there has been a rising use of tylosin in apiculture due to bacterial brood diseases developing resistance to oxytetracycline. To address this, Caldow and colleagues developed and validated an optical biosensor for detecting tylosin residues in honey. This biosensor demonstrated a detection capability of 2.5 $\mu g\,kg^{-1}$, complementing existing confirmatory methods. With its fully automated system, the biosensor can efficiently process and analyze up to 25 samples within a standard working day. The high throughput, combined with its precise detection, suggests that this optical biosensor offers a reliable and efficient solution for monitoring tylosin in honey, contributing to improved quality control in apiculture and honey production [613]. Nag and colleagues introduced an optical enzymatic biosensor capable of rapid, point-of-use detection of β-lactam antibiotics, such as penicillins and cephalosporins, in both food and water samples. The biosensor leveraged the enzymatic hydrolysis of β-lactams, which induced structural changes in nanofibers, resulting in increased evanescent wave absorbance. A key advantage of this sensor was its minimal sample preparation, requiring only the removal of fat. To calibrate the system, ceftazidime was spiked into antibiotic-free milk, meat, and tap water, demonstrating the biosensor's ability to detect a range of β-lactam antibiotics, including ampicillin, amoxicillin, and cefotaxime, across multiple matrices. The biosensor exhibited impressive detection limits: 0.18 nM in milk, 9 nM in chicken, and 0.18 nM in water. These low detection limits highlight the sensor's potential for screening β-lactam antibiotics not only in food products but also in wastewater sources. The biosensor's performance was further validated using real wastewater samples, with qualitative analysis confirmed through high-resolution liquid chromatography-mass spectrometry (LC-MS). Given its broad detection capability and high sensitivity, the biosensor presents a valuable tool for quality control in food processing industries and for monitoring β-lactam antibiotics in

wastewater treatment plants. Its ability to monitor antibiotic contamination in influent and track removal efficiency in treatment processes underscores its potential utility for environmental safety and food industry applications [612]. Several studies have explored the potential use of biosensors for detecting antibiotics in various environmental matrices, particularly water samples. In one such study, Chinnipan et al. developed a highly selective and sensitive voltammetric aptasensor designed to detect azlocillin, a broad-spectrum β-lactam antibiotic. This aptasensor exhibited strong performance in accurately identifying and quantifying azlocillin in water samples, underscoring its potential for environmental monitoring. The aptamer used, Az9, was selected through the systematic evolution of ligands by exponential enrichment (SELEX) process. The resulting sensor demonstrated an impressive detection limit of 1.2 pg/mL and required 30 to 50 minutes to quantify the target antibiotic. Notably, it performed well in complex settings such as tap water and wastewater, achieving high recovery rates [620]. Similarly, Du and colleagues developed a lateral flow aptasensor, combined with recombinase polymerase amplification (RPA), for detecting erythromycin, an antibiotic that has polluted aquatic environments for decades. The DNA aptamers designed for this sensor allowed for a remarkable detection limit of 3 pM. When tested with real water samples, the sensor effectively detected erythromycin at trace levels [608]. Additionally, Duyen and colleagues proposed a colorimetric, paper-based biosensor to detect antibiotics that inhibit bacterial protein synthesis, such as aminoglycosides, tetracyclines, chloramphenicol, and macrolides. The paper-based sensor relied on b-galactosidase synthesis, which caused a color change from yellow to purple on freeze-dried paper discs. In the presence of antibiotics, this color change was inhibited due to the suppression of b-galactosidase synthesis. The sensor functioned within a broad range of temperatures (15–37°C) and pH levels (6–10), and it demonstrated the ability to detect paromomycin at concentrations as low as 0.5 mg/mL in environmental water samples, with results visible to the naked eye. These studies highlight the versatility and potential of biosensors in environmental antibiotic detection, offering sensitive, rapid, and cost-effective solutions for water quality monitoring [616].

7.3. Results of Laboratory Activities

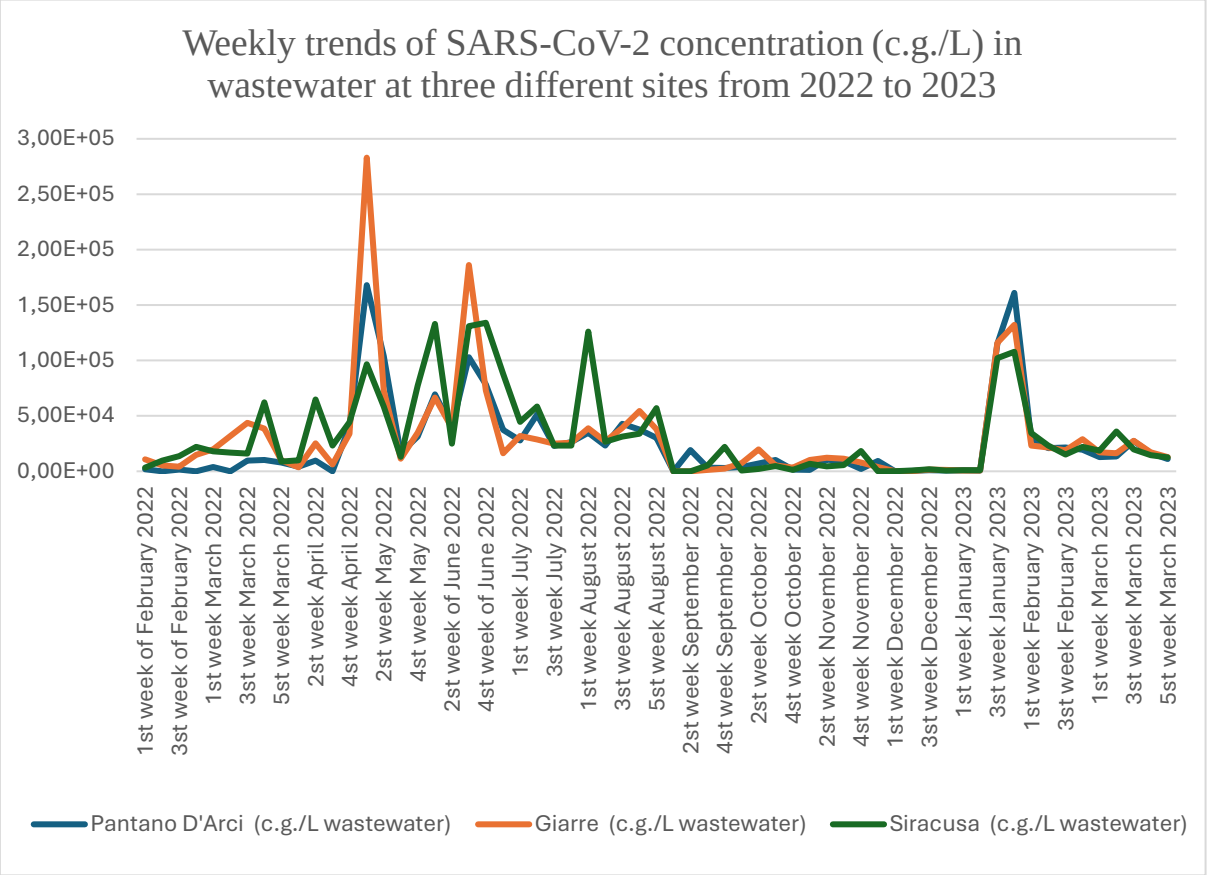
7.3. 1. SARS-CoV-2 Detection in Wastewater Samples and Temporal Trend Analysis

The presence of SARS-CoV-2 in the collected samples was detected using one-step real-time RT-qPCR (i.e., reverse transcription of viral RNA followed by gene amplification in a single

reaction). Specifically, SARS-CoV-2 was detected in 293 out of the 316 samples analyzed. The weekly sampling results from February 2022 to March 2023 revealed that the trend in SARS-CoV-2 concentration in wastewater from the three sites (Pantano D'Arci, Giarre, and Siracusa) showed significant fluctuations, as illustrated in the graph (Graph 1.). In particular, the graph presents the weekly trend of SARS-CoV-2 concentration (measured in c.g./L) in wastewater, over the period from February 2022 to March 2023. The data is visualized through line plots for each site. The graph shows that the SARS-CoV-2 concentration in Pantano D'Arci (blue line) exhibited various peaks throughout the observed period. Significant peak occurred in the first weeks of May 2022, in the last weeks of June 2022, in the last week of January and the first week of February 2023. These peaks are followed by sharp declines, with the trend indicating a slight decrease in concentration over the year.

Similarly, Giarre (orange line) showed several peaks in concentration, with the highest values recorded in the first week of May, the penultimate week of June 2022 and the last week of January 2023. The data indicate fluctuations with periods of low concentration interspersed with sudden increases. The trend for Giarre also shows a slight downward trend, although the high peaks suggest significant variability in viral load at this site. Siracusa (green line) showed frequent fluctuations, with multiple peaks particularly evident in late May, mid-June and the first week of August 2022. This fluctuation is characterized by a drastic decrease in the second week of June 2022 and then a rapid increase. In addition, a significant increase was observed in early January 2023, similar to the other sites. After January 2023, the concentration decreased dramatically and remained low with small fluctuations. The overall concentration remains relatively lower compared to Giarre. The trend for Siracusa indicates a slight decrease in SARS-CoV-2 concentration over time, similar to the other two sites. Despite the fluctuations, the trends for all sites are slightly decreasing, suggesting a general decline in the SARS-CoV-2 concentration in wastewater over the observed period. All three sites experienced significant spikes in May 2022 and January 2023. The data indicates variability in the concentration of SARS-CoV-2 across different sites and times, highlighting the importance of continuous monitoring to understand the spread and prevalence of the virus. This graph underscores the importance of consistent and frequent wastewater monitoring to detect changes in viral load, as it highlights both short-term spikes and long-term trends in SARS-CoV-2 concentration across different locations. This data is crucial for understanding and managing public health risks associated with the virus.

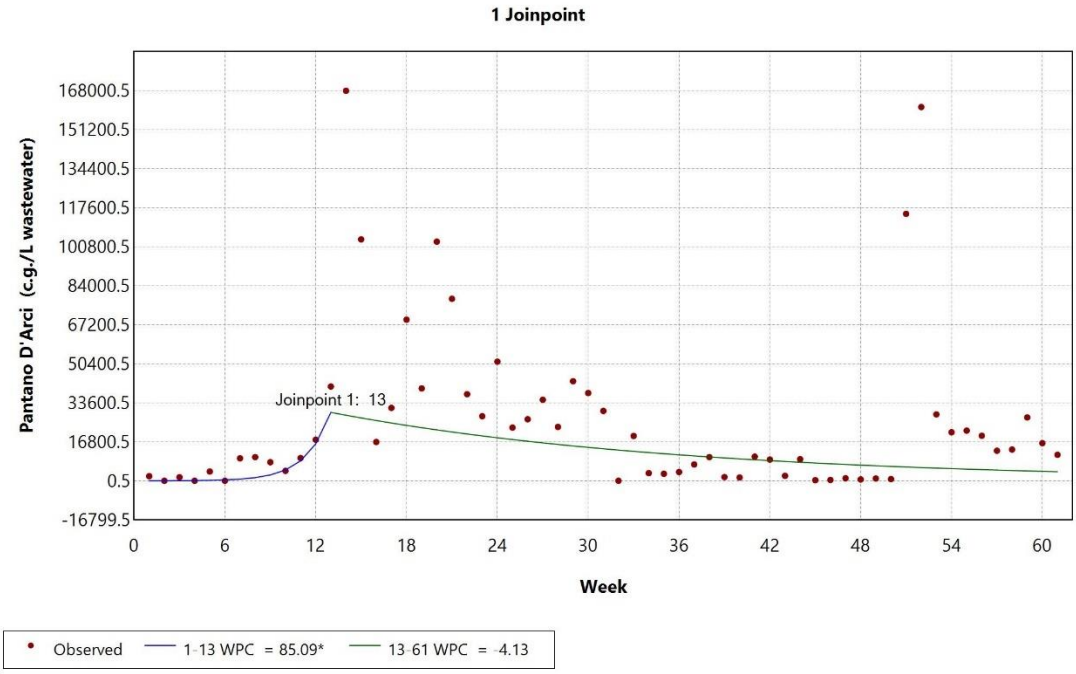
Graph 1. Weekly trends of SARS-CoV-2 concentration (c.g./L) in wastewater at three different sites from 2022 to 2023



Specifically, the observed peaks in CoV-2 concentrations in wastewater from Siracusa, Giarre, and Pantano D'Arci coincided with reported case surges in the same periods. This association highlights the reliability of wastewater data in reflecting community infection levels. Notably, the trend reveals significant spikes in June 2022, coinciding with a period of increased COVID-19 cases in the region, as documented by the Sicilian region website (www.regione.sicilia.it). This alignment between wastewater data and reported cases reinforces the value of wastewater monitoring in tracking and responding to pandemic dynamics. In addition, in the "Weekly Bulletin No. 37 of 06/15/2022: Epidemiological and Vaccination Data," it was noted that for the first time in two months, there was an increase in new infections during the week of June 6 to June 12, 2022. The incidence of new cases rose to 17,316 (+21.9%), with a cumulative rate of 360.64 per 100,000 inhabitants. Notably, the provinces of Catania (424/100,000) and Siracusa (415/100,000) reported higher rates than the regional average (www.regione.sicilia.it). This trend is particularly significant because, as shown in graph 1, a peak in viral concentrations in wastewater samples was already observed in the early weeks of May. This indicates that wastewater data can serve as an early warning system, detecting the presence of the virus before

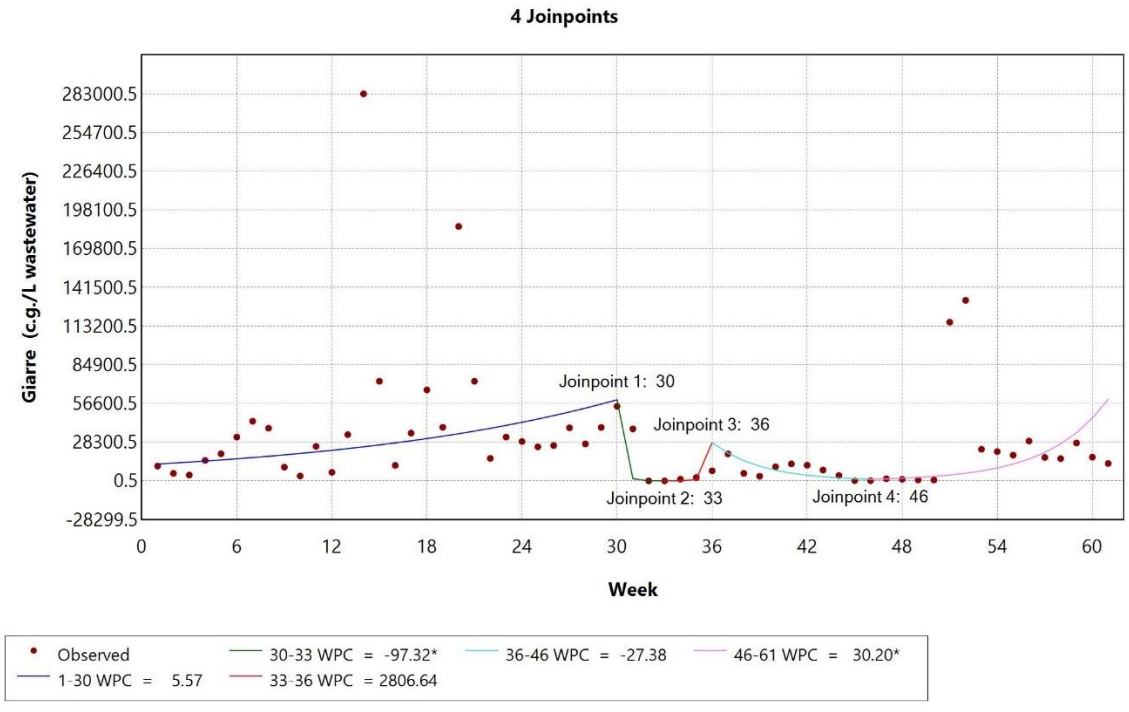
clinical cases are officially reported. The reason is that infected individuals can shed the virus in their stool even before they develop symptoms or undergo testing. Moreover, wastewater analysis captures data from the entire population served by a treatment plant, including asymptomatic individuals who do not get tested, potentially providing a more comprehensive picture of the virus's spread than clinical case reports alone. Additionally, we examined the temporal trends in SARS-CoV-2 concentrations on both a weekly and monthly basis, conducting the analysis separately for each of the three sites. For Pantano D'Arci, the analysis revealed a single joinpoint occurring at week 13 (Figure 4). Specifically, SARS-CoV-2 concentrations showed a steady increase from week 1 to week 13 (4st week April 2022) (WPC = 85,01%), followed by a gradual decline extending through week 61 (5st week March 2023) (WPC = -4,13%). For Giarre, the analysis revealed four joinpoints that occurred at week 30, 33, 36, and 46 (4st week August 2022, 2st week September 2022, 1st week October 2022, 2st week December 2022) (Figure 5). Specifically, SARS-CoV-2 concentrations showed an increase from week 1 to week 30 (WPC = 5.57%), followed by a rapid decline from week 30 to week 33 (WPC = 97.32%), a sudden increase from week 33 to week 36 (WPC = 2806.64%), followed by a gradual decline that extended until week 46 (WPC = -27.38%). Finally, for Siracusa, the analysis revealed two joinpoint occurring at week 29 and 32 (Figure 6). Specifically, SARS-CoV-2 concentrations showed a steady increase from week 1 to week 29 (3st week August 2022) (WPC = 7,78%), followed by a rapid decline extending through week 32 (1st week September 2022) (WPC =-87,49%).

Figure 4: Joinpoint regression of temporal trends of SARS-CoV-2 for Pantano D'Arci



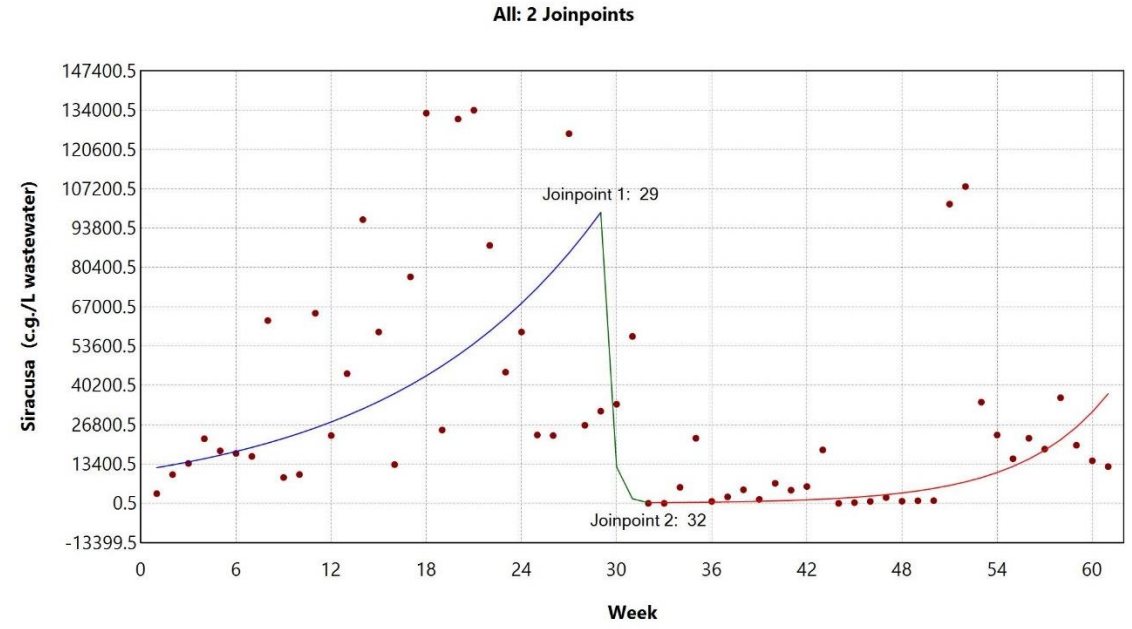
* Indicates that the Week Percent Change (WPC) is significantly different from zero at the alpha = 0.05 level.
-- Test Statistic and P-Value not available for the Empirical Quantile method.
Final Selected Model: 1 Joinpoint.

Figure 5: Joinpoint regression of temporal trends of SARS-CoV-2 for Giarre



* Indicates that the Week Percent Change (WPC) is significantly different from zero at the alpha = 0.05 level.
-- Test Statistic and P-Value not available for the Empirical Quantile method.
Final Selected Model: 4 Joinpoints.

Figure 6: Joinpoint regression of temporal trends of SARS-CoV-2 for Siracusa

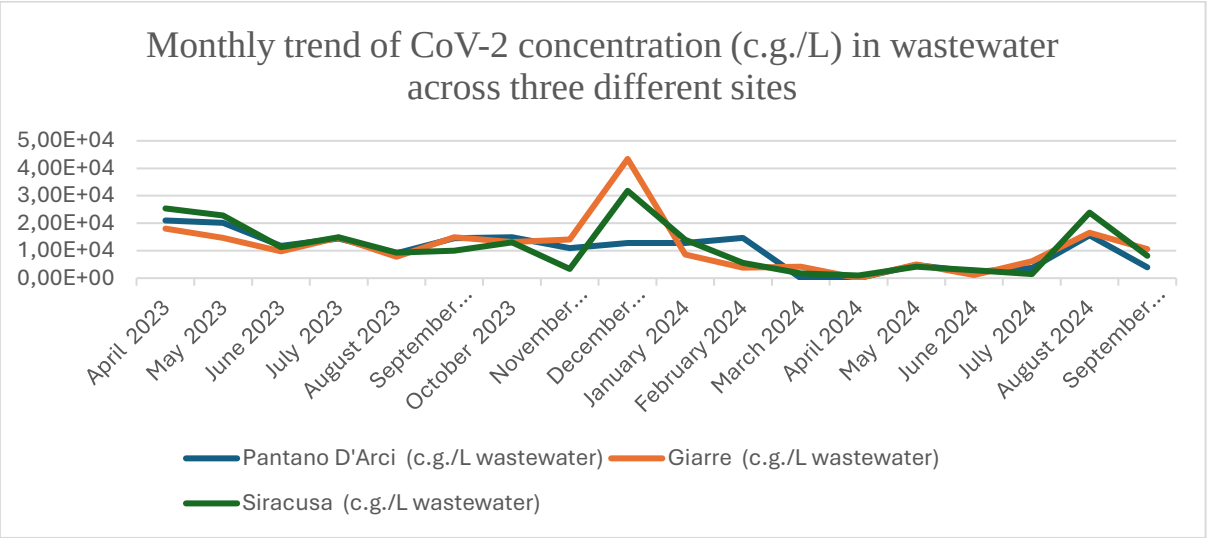


* Indicates that the Week Percent Change (WPC) is significantly different from zero at the alpha = 0.05 level.
-- Test Statistic and P-Value not available for the Empirical Quantile method.
Final Selected Model: 2 Joinpoints.

Over time, with the implementation of containment measures and widespread vaccination efforts, the virus began to stabilize in many regions. As a result, from April 2023 to September 2024, epidemiological surveillance of wastewater was adjusted, shifting from weekly to monthly sample collection. This change reflected the need to monitor the virus's presence in a more sustainable way while still capturing essential data. Graph 2. illustrates the monthly trend of SARS-CoV-2 concentration (measured in c.g./L) in wastewater during this period. At the beginning of the observed period (April to September 2023), the concentrations across the three sites were relatively stable, fluctuating between 1,500 and 2,500 c.g./L. There is a slight decrease from May to June, followed by a steadying trend. This suggests that viral concentrations in the wastewater were not significantly impacted during this time frame, possibly indicating stable infection rates within the population. A sharp increase in viral concentrations is seen from December 2023 to January 2024, with Giarre experiencing the most significant spike, reaching nearly 50,000 c.g./L. Pantano D'Arci and Siracusa also show noticeable increases, although less pronounced than Giarre. This spike could be attributed to a surge in COVID-19 cases during the winter months, which is common for respiratory viruses due to factors such as indoor gatherings and colder weather. Following the peak, the

concentration levels drop significantly across all three sites, stabilizing by April 2024. The concentrations remain at lower levels for the next few months, suggesting a reduction in virus transmission. From April to July 2024, the viral load stays below 1,000 c.g./L in all locations, indicating a possible control of the infection in these cities. A smaller, more gradual increase is observed again towards the end of the period, from August to September 2024.This could indicate the start of another wave of infections, possibly influenced by environmental factors or changes in public behavior as summer ends and the new season begins. In detail, Pantano D'Arci (blue line) exhibits a relatively stable trend in SARS-CoV-2 concentration throughout the period, with a gradual decrease in SARS-CoV-2 concentration over time. In 2023, the concentration gradually decreases until reaching its lowest point around August, followed by a slight increase around September and October. In 2024, a peak is observed in February, followed by a sharp decrease in March and April and a subsequent increase in the last months until August 2024. Giarre (orange line) shows notable variability in SARS-CoV-2 concentration, with a pronounced peak in December 2023, where the concentration temporarily surged before dropping sharply. Afterward, the concentration remains relatively low, with a slight increase observed in August 2024. Despite these fluctuations, the overall trend for Giarre also shows a slight decrease over time. Siracusa (green line) also demonstrates fluctuations in SARS-CoV-2 concentration. The concentration decreases steadily from April to November 2023, after which it spikes in December 2023. Following this spike, the levels drop significantly before rising again towards August 2024. The trend for Siracusa shows a slight overall decline, though the trend is less pronounced compared to the other sites. Overall, the data highlights distinct periods of viral concentration changes in wastewater, with notable peaks during the winter months. This graph underscores the critical role of continuous wastewater monitoring in tracking the spread of SARS-CoV-2. Wastewater surveillance not only captures short-term fluctuations but also reveals long-term trends in viral concentration across various locations, providing valuable insights into the virus's behavior over time. This approach enables early detection of potential outbreaks and supports more informed public health responses.

Graph 2. Monthly trend of SARS-CoV-2 concentration (c.g./L) in wastewater across three distinct sites from 2023 to 2024.

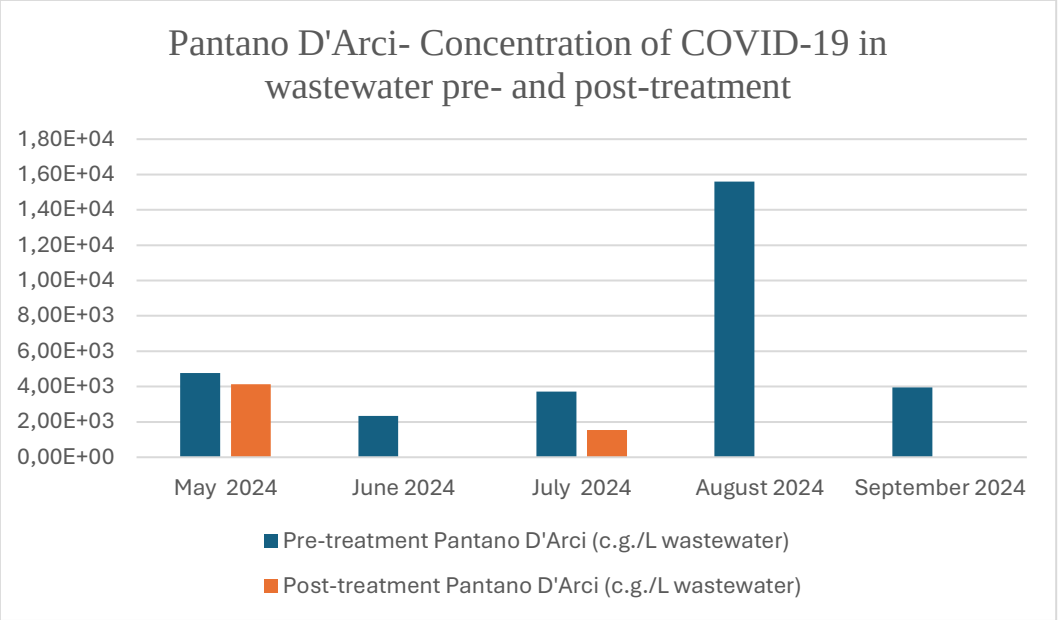


As previously noted, the analysis was also performed on the monthly data. However, no significant joinpoints were detected at any of the sites. Furthermore, to evaluate the effectiveness of wastewater treatment systems, samples were collected both before and after treatment at the Pantano D'Arci site starting from May 2024. For the Giarre and Siracusa sites, sampling occurred from June 2024. The results indicated that the treatment process at Pantano D'Arci is generally effective in lowering the concentration of COVID-19 in wastewater, as evidenced by consistently lower post-treatment levels compared to pre-treatment levels. Similarly, data from the Giarre and Siracusa plants confirmed that their wastewater treatment processes are reliably effective in reducing the viral concentration, even when confronted with higher pre-treatment loads, as observed in August 2024. Specifically, at Pantano D'Arci in May, the concentration of COVID-19 in the wastewater before treatment was approximately 5,000 c.g./L, which reduced to around 2,500 c.g./L after treatment, indicating a significant reduction in viral load due to the purification process. In June, the pre-treatment concentration decreased further compared to May, and the post-treatment levels were undetectable, highlighting the treatment's continued effectiveness in removing the virus. In July, there was a slight increase in the pre-treatment concentration compared to June, but post-treatment levels remained consistently low, in line with previous months. August saw a significant spike in the pre-treatment concentration, reaching approximately 15,000 c.g./L—substantially higher than in the preceding months. However, the post-treatment concentration remained undetectable, demonstrating the treatment process's ability to handle the increased viral load. Finally, in September 2024, the pre-treatment concentration dropped back to around 5,000 c.g./L, with

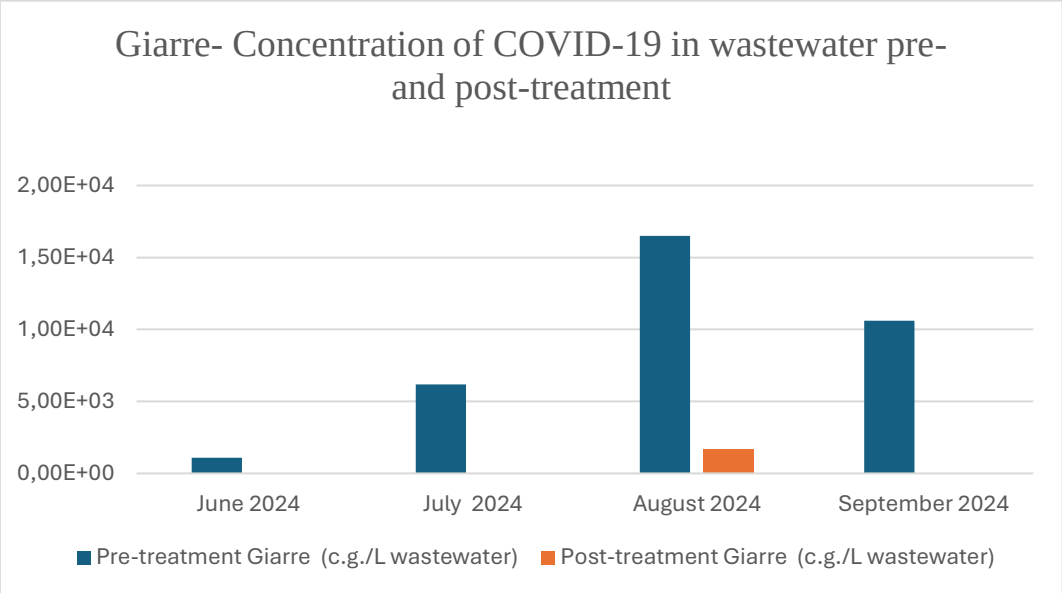
post-treatment levels still undetectable, further confirming the reliability and effectiveness of the wastewater treatment throughout the observed period. In June 2024 at Giarre, the pre-treatment concentration is quite low, around 500 c.g./L, with post-treatment levels undetectable, highlighting the effectiveness of the treatment process in removing the virus from the wastewater. In July, the pre-treatment concentration of the virus rose significantly to around 5,000 c.g./L compared to June. However, the post-treatment levels remained undetectable, indicating that the treatment process maintained its effectiveness even with the increased viral load. In August, there was a significant spike in the pre-treatment concentration, reaching nearly 15,000 c.g./L, a considerable increase compared to previous months. Despite this sharp rise, the post-treatment concentration remained substantially low, though not as low as in June or July. By September, the pre-treatment concentration stabilized at around 10,000 c.g./L, with post-treatment levels once again undetectable. Overall, the consistent reduction in post-treatment viral concentrations throughout all months underscores the robustness of the facility's treatment methods. However, the dramatic rise in pre-treatment levels in August suggests an emerging challenge that may require continued vigilance and potentially enhanced treatment strategies to maintain long-term effectiveness. A similar trend is observed at the Siracusa site, where data indicate the purifier is performing effectively. In June, the pre-treatment concentration of COVID-19 in the wastewater was relatively low (below 5,000 c.g./L), with post-treatment levels undetectable, demonstrating the treatment's ability to significantly reduce the viral load. In July 2024, the pre-treatment concentration remained consistently low, similar to June, and the post-treatment levels continued to be undetectable, suggesting stable and effective treatment performance. However, in August, there was a sharp increase in the pre-treatment concentration, reaching around 25,000 c.g./L—the highest among the three locations. Despite this substantial rise in pre-treatment viral load, the post-treatment concentration remained significantly lower, although it was slightly higher than in June and July. This surge in pre-treatment levels indicates a notable spike in incoming viral concentrations, but the treatment process still managed to reduce the viral load effectively. By September, the pre-treatment concentration decreased to approximately 10,000 c.g./L, with post-treatment levels remaining minimal. Across all three locations the wastewater treatment consistently proves effective in significantly reducing COVID-19 concentrations, even during the peak levels observed in August 2024 (See graph 3a, b, and c.).

Graph 3. Concentration (c.g./L) of COVID-19 in wastewater pre- and post-treatment

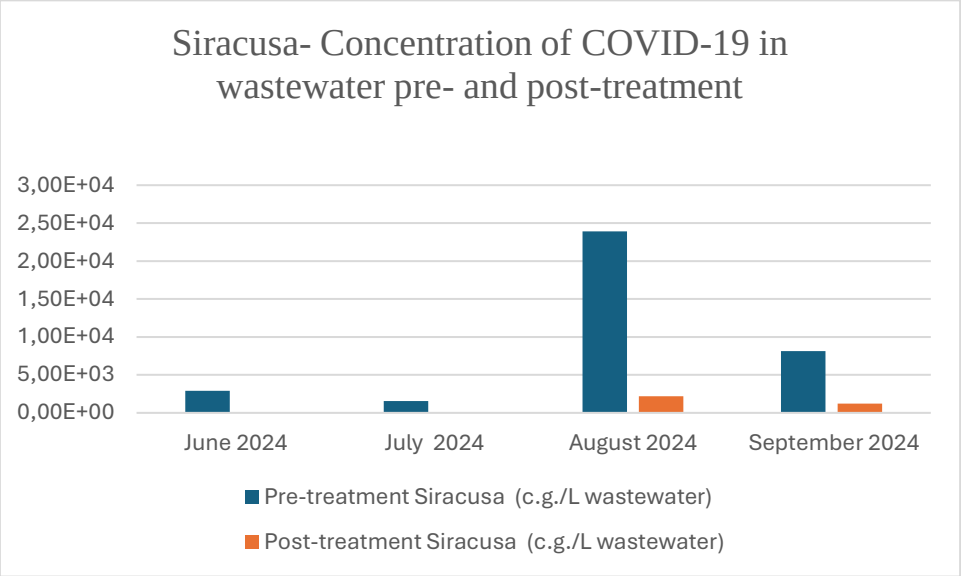
a.



b.

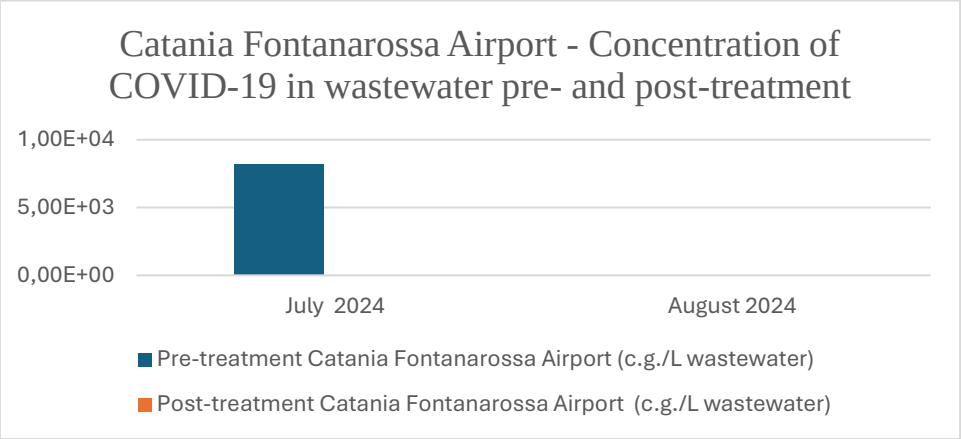


c.



In the four samples collected from the wastewater at Catania Fontanarossa Airport in July 2024, COVID-19 was detected in the untreated wastewater but was absent after treatment. In August 2024, no traces of the virus were found in either the pre-treatment or post-treatment wastewater samples, indicating a complete absence or significant reduction of the virus during that time. This data may be attributed to the days the airport was closed in August due to ash from Mount Etna, but further analysis will be conducted to confirm this (Graph 4.)

Graph 4. Concentration (c.g./L) of COVID-19 in Catania Fontanarossa Airport wastewater pre- and post-treatment



7.3.2. Relative abundance of ARGs in wastewater samples

The relative abundance of ARGs in wastewater samples has been comprehensively assessed using 42 monthly composite samples collected from three WWTPs located in Pantano D'Arci, Siracusa and Giarre. These samples were gathered between February 2022 and March 2023. For each month, a composite sample was created by combining, for each site, a sample portion from each of the 4 weeks of the month. Specifically, the abundance of eight ARGs was evaluated in each sample, providing valuable insight into the prevalence of these genes in wastewater. Among the detected ARGs, four—*blaERM_B*, *blaOXA*, *blaTEM*, and *blaCTX-M*—were found in 100% of the samples, although with different relative abundances. The genes with the highest relative abundance across the samples were *blaERM_B*, *blaSHV*, and *blaTEM*. In the wastewater samples collected from Catania, *blaOXA* was also consistently prevalent, alongside *blaERM-B*, *blaSHV*, and *blaTEM*.

When analyzing the entire dataset of 42 samples, the following ARGs were consistently present:

- The *blaERM_B* gene was detected in all 42 samples (100%), reflecting its widespread occurrence.
- The *blaOXA* gene was also present in 100% of the samples, emphasizing its ubiquitous nature.
- Similarly, the *blaTEM* gene was found in all 42 samples, indicating its high prevalence.
- The *blaCTX-M* gene, which was examined using four different probes, showed varying detection rates. The first probe identified *blaCTX-M* in 45.2% of samples (19 out of 42), while the second, third, and fourth probes detected this gene in 97.7% (41 out of 42) and 100% (42 out of 42) of samples, respectively.

Other notable ARGs included:

- *blaSHV*, detected in 95.2% of the samples (40 out of 42).
- *blaERM_A*, present in 81% of the samples (34 out of 42).
- *blaNDM*, identified in 69% of samples (29 out of 42) when analyzed with the first probe and in 57.1% (24 out of 42) with the second probe.
- *blaVIM*, which was detected in 97.7% of the samples (41 out of 42).

The overall pattern of ARG prevalence across the samples can be visualized through a heatmap, which displays the relative abundance of each detected gene. The heatmap allows the

visualization of hundreds of data points in a single figure. When Real-time PCR data needs to be presented as individual points, it should be displayed as 2^(-ΔCT) or 2^(-CT) instead of the raw CT value. In presenting data from gene expression profiling studies, it is always recommended to normalize the data to an internal control. These abundances were calculated using the 2^(-ΔCT) method, where ΔCT = (CT gene of interest - CT internal control), which compares each ARG to a 16S rRNA gene control to quantify their relative levels. The final results are expressed after being scaled by a factor of 10^5, providing a clearer understanding of the comparative abundance of these ARGs in the wastewater environment, as determined by real-time PCR analyses (see Figure 7).

Figure 7. Heatmap of Relative abundance of antimicrobial resistance genes (ARGs) in wastewater samples

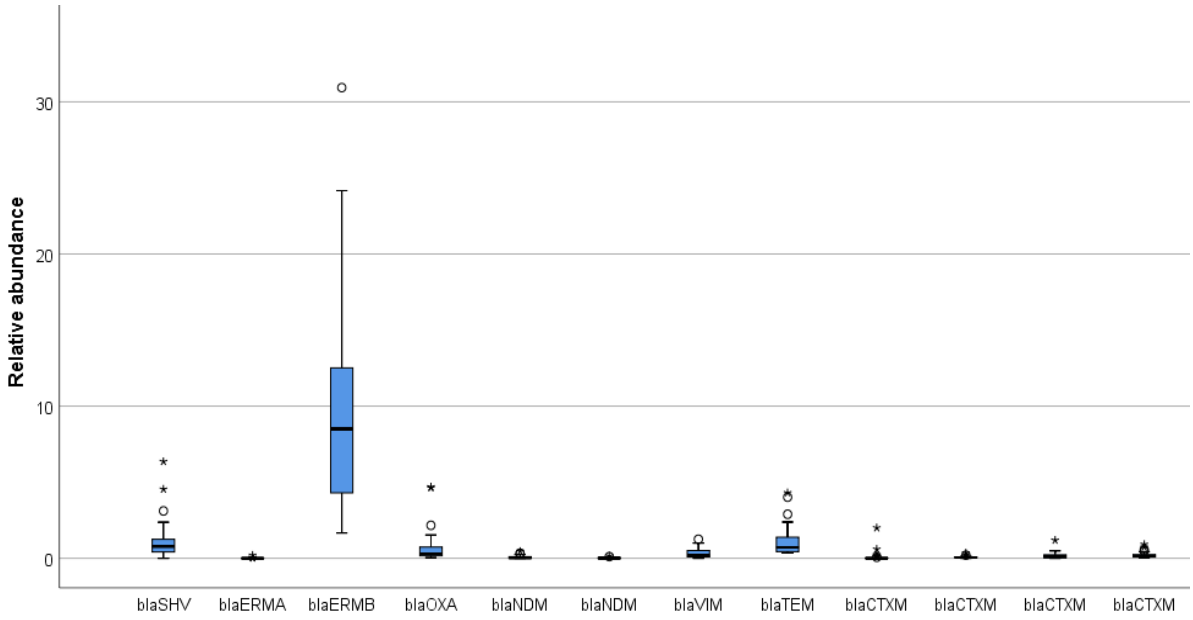
WWTP site	Month	blaSHV	blaERM-A	blaERM-B	blaOXA	blaNDM	blaNDM	blaVIM	blaTEM	blaCTXM	blaCTXM	blaCTXM	blaCTXM
Pantano D'Arci (Catania)	February 2022	0.65	0.00	1.97	0.04	0.02	0.00	0.71	0.40	0.00	0.04	0.47	0.07
	March 2022	0.70	0.00	3.28	0.08	0.22	0.07	1.00	0.67	0.00	0.02	0.43	0.05
	April 2022	0.42	0.00	8.32	0.34	0.32	0.06	0.49	0.81	0.00	0.05	0.23	0.14
	May 2022	0.00	0.00	3.19	0.34	0.08	0.02	0.47	0.53	0.00	0.06	0.23	0.09
	June 2022	0.68	0.01	2.05	1.14	0.13	0.04	0.82	0.58	0.00	0.07	0.17	0.08
	July 2022	0.45	0.01	3.42	0.85	0.03	0.01	0.61	0.40	0.00	0.04	0.14	0.05
	August 2022	1.06	0.20	8.01	2.17	0.23	0.02	0.78	1.49	0.00	0.08	0.34	0.91
	September 2022	1.36	0.04	13.56	4.69	0.15	0.00	1.26	2.03	0.00	0.20	0.36	0.37
	October 2022	1.64	0.04	12.51	4.63	0.25	0.11	0.88	1.46	0.00	0.12	0.19	0.22
	November 2022	0.76	0.01	8.45	0.74	0.09	0.05	0.53	0.74	0.00	0.06	0.20	0.09
	December 2022	0.99	0.02	10.39	0.81	0.07	0.03	0.51	1.14	0.01	0.05	0.21	0.21
	January 2023	0.80	0.00	6.30	0.16	0.08	0.02	0.51	1.01	0.00	0.00	0.12	0.13
	February 2023	0.93	0.00	7.51	1.43	0.18	0.07	0.69	0.95	0.00	0.07	0.19	0.13
	March 2023	0.79	0.00	5.85	0.09	0.10	0.03	0.45	0.75	0.00	0.03	0.09	0.13
Siracusa	February 2022	0.29	0.00	3.67	0.18	0.02	0.01	0.09	0.45	0.02	0.05	0.03	0.24
	March 2022	0.25	0.00	6.32	0.20	0.03	0.00	0.08	0.43	0.00	0.04	0.09	0.11
	April 2022	0.12	0.00	4.29	0.12	0.03	0.02	0.09	0.36	0.01	0.04	0.15	0.23
	May 2022	0.27	0.00	3.70	0.10	0.01	0.01	0.13	0.44	0.12	0.05	0.18	0.13
	June 2022	0.00	0.00	8.66	0.09	0.05	0.01	0.16	0.56	0.09	0.07	1.19	0.17
	July 2022	0.48	0.00	3.72	0.68	0.03	0.01	0.44	0.34	0.00	0.03	0.49	0.07
	August 2022	0.28	0.00	3.27	0.05	0.03	0.03	0.10	0.40	0.00	0.03	0.33	0.10
	September 2022	1.25	0.00	13.89	0.31	0.00	0.00	0.00	0.91	0.00	0.09	0.01	0.34
	October 2022	1.09	0.01	1.67	0.55	0.45	0.06	0.36	0.85	0.01	0.05	0.05	0.23
	November 2022	0.32	0.00	8.56	0.13	0.00	0.00	0.18	0.41	0.02	0.01	0.02	0.14
	December 2022	0.50	0.00	10.26	0.14	0.01	0.00	0.09	0.34	0.02	0.03	0.03	0.08
	January 2023	1.61	0.00	13.72	0.24	0.02	0.03	0.15	0.53	0.12	0.03	0.01	0.21
	February 2023	1.15	0.00	11.55	0.24	0.31	0.16	0.05	0.69	0.24	0.05	0.01	0.15
	March 2023	1.43	0.00	14.74	0.17	0.02	0.00	0.17	1.15	2.00	0.11	0.04	0.73
Giarre	February 2022	1.00	0.00	10.18	0.18	0.00	0.00	0.08	0.37	0.00	0.08	0.01	0.25
	March 2022	0.93	0.00	16.22	0.32	0.00	0.00	0.07	0.60	0.00	0.07	0.04	0.46
	April 2022	0.51	0.00	8.88	0.28	0.00	0.01	0.08	2.18	0.00	0.04	0.03	0.22
	May 2022	0.65	0.00	10.99	0.27	0.00	0.02	0.12	0.70	0.01	0.06	0.05	0.21
	June 2022	0.65	0.00	10.68	0.26	0.00	0.00	0.10	1.07	0.01	0.05	0.09	0.18
	July 2022	2.37	0.00	19.08	1.53	0.00	0.00	0.39	2.39	0.01	0.13	0.24	0.26
	August 2022	1.01	0.00	12.19	0.35	0.00	0.00	0.18	1.38	0.02	0.11	0.04	0.13
	September 2022	4.54	0.00	24.16	0.62	0.09	0.00	0.24	2.89	0.07	0.34	0.16	0.58
	October 2022	6.36	0.00	30.93	0.98	0.14	0.03	0.40	4.02	0.16	0.13	0.25	0.43
	November 2022	3.11	0.00	14.63	0.78	0.07	0.00	0.20	4.30	0.59	0.06	0.03	0.07
	December 2022	2.04	0.00	12.80	0.41	0.00	0.00	0.39	1.61	0.02	0.22	0.00	0.23
	January 2023	1.94	0.00	7.73	0.36	0.00	0.00	0.08	0.66	0.00	0.07	0.02	0.07
	February 2023	0.16	0.00	8.00	0.29	0.00	0.00	0.08	1.51	0.00	0.04	0.01	0.08
	March 2023	0.24	0.00	4.44	0.05	0.00	0.00	0.05	0.44	0.00	0.02	0.01	0.05

Graph 5 provides a detailed view of the relative abundance of ARGs, considering the three reference sites as a whole. For each ARG, the median value, along with the minimum and maximum values, are represented. Confirming the previously mentioned findings, the ARGs with the highest relative abundance are blaERM-B, blaSHV, and blaTEM.

Specifically:

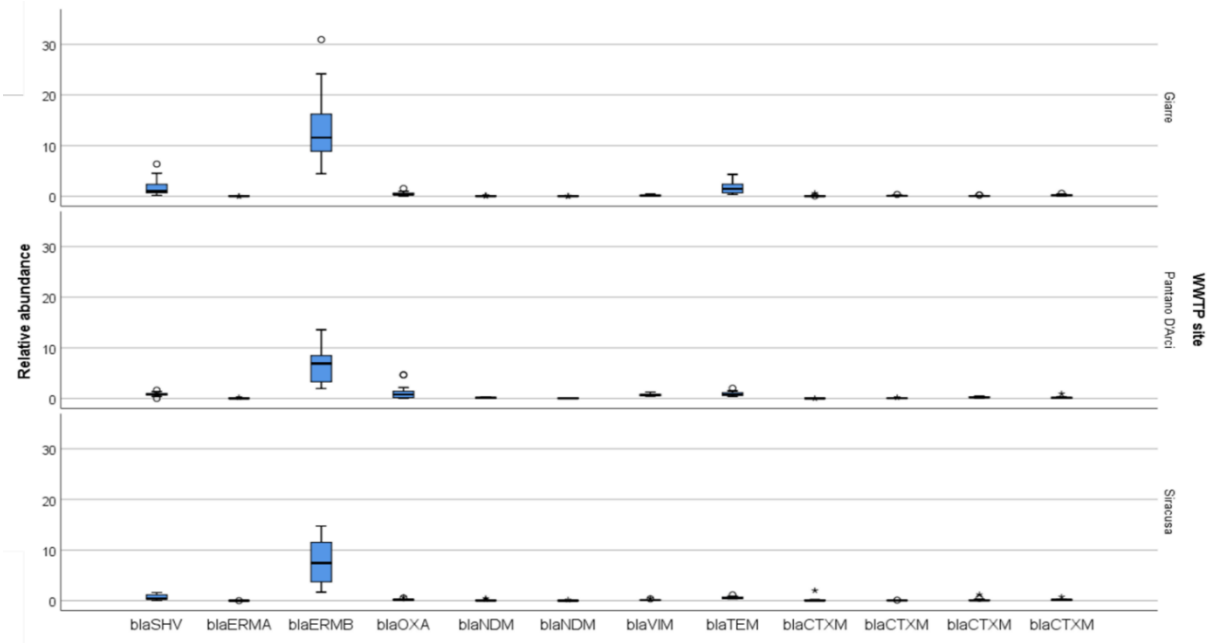
- blaERM-B, with a median of 8,51 (range: 1,67 - 30,93)
- blaSHV, with a median of 0,78 (range: 0,00 - 6,36)
- blaTEM, with a median of 0,72 (range: 0,34 -4,30)
- blaERM-A, with a median of 0; (range: 0 - 0,20)
- blaOXA: with a median of 0,30; (range: 0,04 - 4,69)
- blaNDM: with a median of 0,03; (range: 0 – 0,45)
- blaNDM: with a median of 0,01; (range: 0 - 0,16)
- blaVIM: with a median of 0,19; (range: 0 - 1,26)
- blaCTXM: with a median of 0; (range: 0 – 2,00)
- blaCTXM: with a median of 0,05; (range: 0 – 0,34)
- blaCTXM: with a median of 0,11; (range: 0 - 1,19)
- blaCTXM: with a median of 0,15; (range: 0,05 - 0,91)

Graph 5: ARGs and their relative abundance



When considering each of the three sites individually, the analyses confirm that the genes with the highest relative abundance are blaERM-B, blaSHV, and blaTEM. Furthermore, at the Catania site, in addition to the aforementioned three genes, the blaOXA gene also shows a higher relative abundance (see graph 6).

Graph 6: Relative abundance of ARGs according to WWTP sites

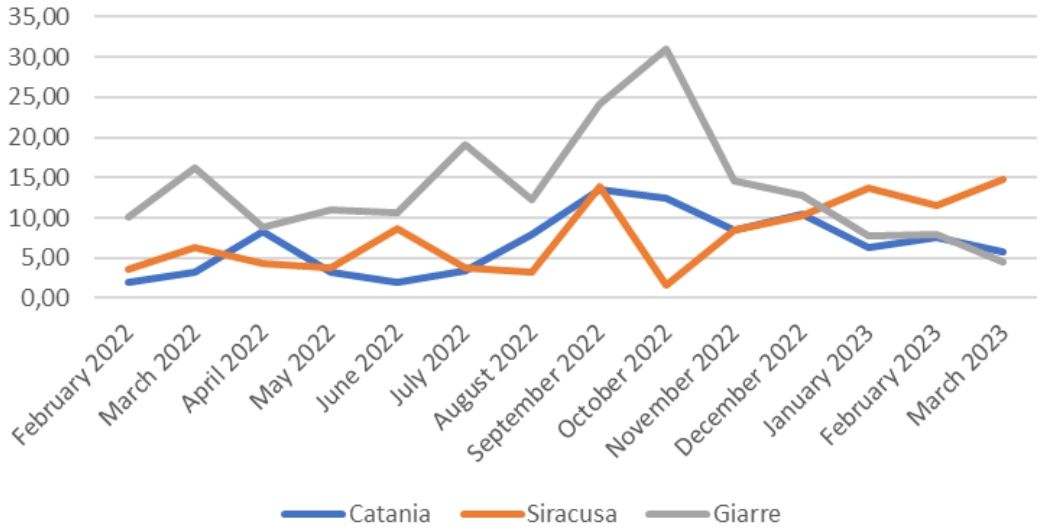


In addition, considering the relative abundance of the blaERM-B gene:

- At the Catania and Giarre sites, a higher relative abundance is observed in September and October 2022.
- At the Siracusa site, the relative abundance increases in September 2022, decreases in October 2022, and then rises again through March 2023.

(see graph 7).

Graph 7: Temporal trend of blaERM-B relative abundance according to WWTP sites

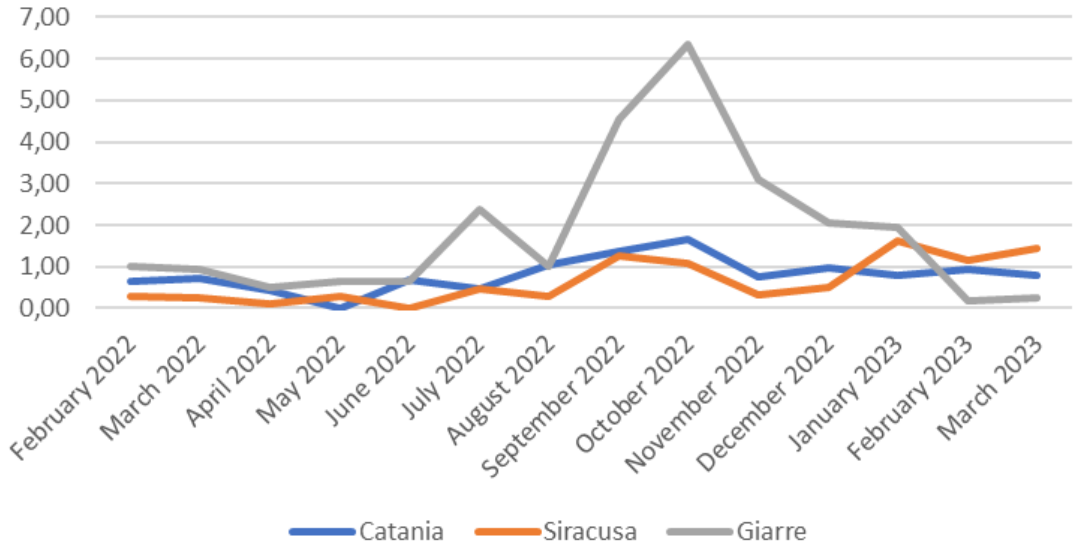


Considering the relative abundance of the blaSHV gene:

- At the Catania and Giarre sites, a higher relative abundance is observed in September and October 2022.
- At the Siracusa site, the relative abundance remains more or less stable until December 2022, with a slight increase from January 2023

(see graph 8).

Graph 8: Temporal trend of blaSHV relative abundance according to WWTP sites

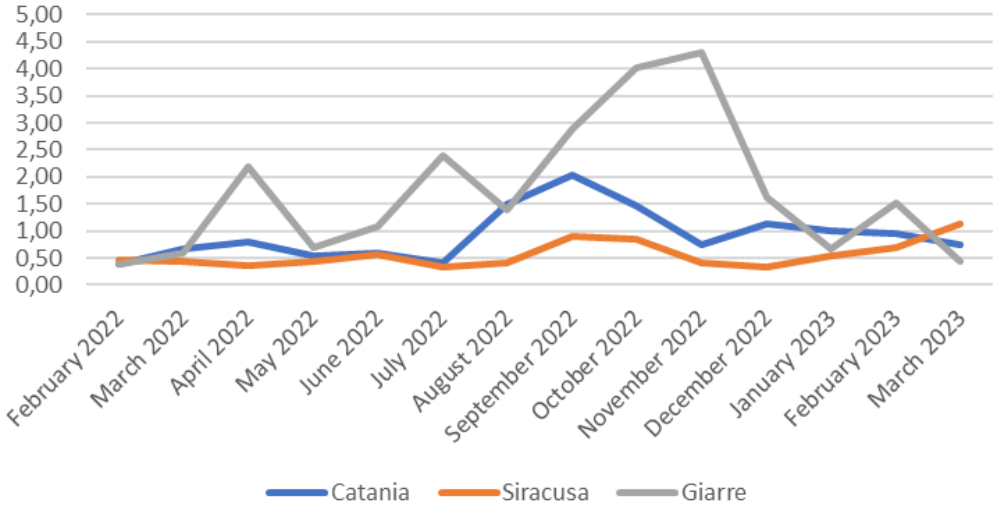


Considering the relative abundance of the blaTEM gene:

- At the Catania site, an increasing trend is observed starting in August 2022, peaking in September, followed by a progressive decrease in October and November 2022.
- At the Siracusa site, the relative abundance remains more or less stable until December 2022, with a slight increase in March 2023.
- At the Giarre site, an increasing trend is observed starting in September 2022, reaching its maximum peak in November 2022.

(see graph 9).

Graph 9: Temporal trend of blaTEM relative abundance according to WWTP sites

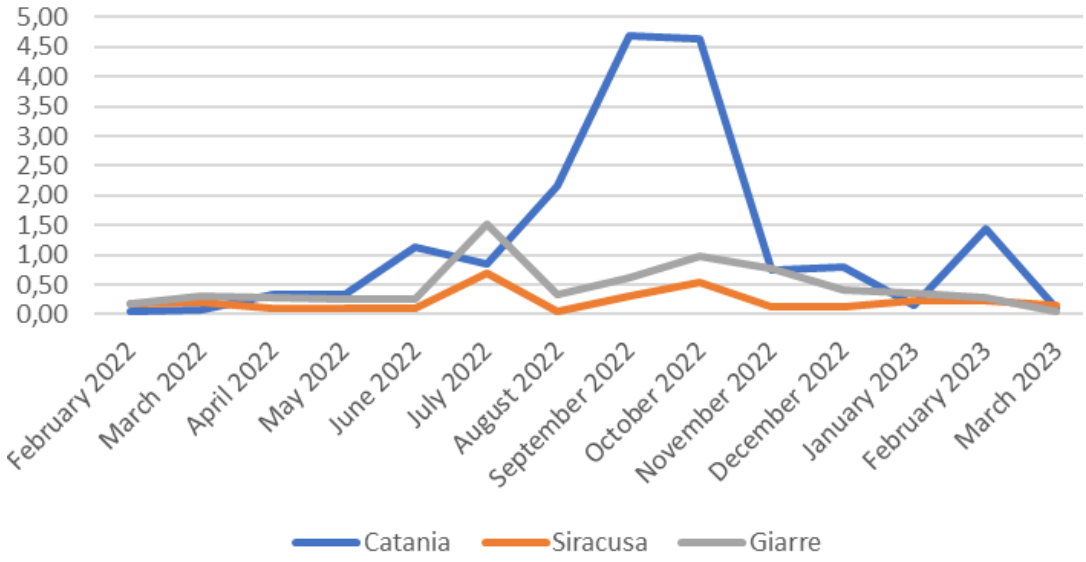


Considering the relative abundance of the blaOXA gene:

- At the Catania site, an increasing trend is observed starting in August 2022, peaking in September and October, before declining again until February 2023.
- At the Siracusa and Giarre sites, the maximum values were recorded in July and October 2022.

(see graph 10).

Graph 10: Temporal trend of blaOXA relative abundance according to WWTP sites



Additionally, in the 42 composite samples analyzed, a negative correlation was found between the concentration of SARS-CoV-2 and the CT value of 16S rRNA ($r = –0.506$, $p = 0.001$). Specifically, this relationship was significant for the ERMB genes ($r = –0.345$, $p = 0.025$), OXA ($r = –0.356$, $p = 0.021$), TEM ($r = –0.350$, $p = 0.023$), CTXM-2 ($r = –0.464$, $p = 0.002$), and CTXM-4 ($r = –0.424$, $p = 0.005$). These results indicate a higher abundance of ARGs with increasing concentrations of SARS-CoV-2 in the analyzed wastewater. However, further analyses are needed to clarify the mechanisms underlying this relationship.

8. STUDY AND RESEARCH EXPERIENCE ABROAD

In line with the Public Competition Notice for admission to the PhD programs - XXXVII Cycle at the University of Catania, published on September 30, 2021, I undertook a six-month study and research period from June 2023 to December 2023 at the Institute for Water Research and the Department of Microbiology, University of Granada, Spain. This research was conducted under the guidance of Prof. Jesus González-López, Professor of Microbiology and head of the RNM-270 "Microbiology and Environmental Technologies" research group within the Department of Microbiology, Faculty of Pharmacy. As outlined in my training program, my work during this period focused on three key areas:

- Application of omics methodologies for the analysis of microbial communities in environmental;
- Mastery of bioinformatics tools: I acquired proficiency in various bioinformatic tools essential for studying complex ecological systems, enabling a deeper understanding of microbial interactions and their environmental impact;
- Molecular protocols for antibiotic resistance: I applied molecular techniques to detect ARGs in environmental samples. This involved the extraction of nucleic acids from environmental matrices in pristine areas, followed by cloning reactions, plasmid extraction, evaluation of the quantity and quality of plasmidic DNA (using a NanoDrop 2000), and the subsequent application of Polymerase Chain Reaction (PCR) and qPCR for the quantification of antibiotic resistance genes.

Through these activities, I contributed to the understanding of antibiotic resistance in natural ecosystems, a crucial issue in public health. Most initiatives aimed at understanding and combating antibiotic resistance focus exclusively on the human and animal sectors, while the extent of antibiotic resistance in the environment, particularly in pristine areas, remains poorly understood. Therefore, it is necessary to address the effects of the increasing selection pressures derived from the surrounding human activities in the enrichment of ARGs in pristine environments. In addition, the evolutionary and ecological processes leading to the dispersal of ARGs in these environments are still lacking. Pristine and remote locations are considered benchmarks for human-caused Earth contamination, mainly, high mountains are target locations for monitoring human impacts on natural ecosystems [622,623]. In this context, the Sierra Nevada in southern Spain, with its numerous lakes, is an ideal place to monitor human impact in pristine locations. During my research, we investigated ten ARGs in fourteen biomass

samples collected from seven pristine lakes near the Sierra Nevada between 2021 and 2022. Samples were taken once a year over two years from the following lakes: Caldera, Rio Seco, Rio Mulhacén, Aguas Verdes, Virgen Grande, Lagunillo Grande, and Lagunillo Alto. The selection of these ARGs was based on their reported clinical significance and the growing resistance to corresponding antibiotics in various environmental contexts [624]. Specifically, we focused on the genes aadA and aadB (resistance to aminoglycosides), ampC, blaSHV, blaTEM, and mecA (resistance to beta-lactams), dfrA1 (trimethoprim resistance), ermB (macrolide resistance), qnrS (quinolone resistance), and tetA(A) (tetracycline resistance).

DNA Extraction

Nucleic acids were extracted from the samples using the FastDNA SPIN Kit for Soil and the FastPrep-24 Instrument (MP Biomedicals, Germany), following the manufacturer's protocol. Specifically, total DNA was extracted from 0.5 g of each biomass sample, as detailed in Correa-Galeote et al. 2021 [625].

The extraction process involved the following steps:

1. Sodium Phosphate Buffer Addition: 978 µL of Sodium Phosphate Buffer was added to the sample contained in a Lysing Matrix E tube.
2. MT Buffer Addition: 122 µL of MT Buffer was then added to solubilize external contaminants.
3. Homogenization: The sample was homogenized using the FastPrep-24 instrument for 40 seconds at a speed of 6.0 m/s to disrupt the cell wall and release nucleic acids.
4. Centrifugation: The homogenized sample was centrifuged at 14,000 x g for 10 minutes to pellet insoluble debris, including cellular fragments and the lysing matrix.
5. Supernatant Transfer: The supernatant was carefully transferred to a clean 2.0 mL microcentrifuge tube.
6. PPS Addition: 250 µL of PPS was added to the supernatant to precipitate proteins and further separate solubilized nucleic acids from any remaining debris. The tube was mixed by inverting it 10 times.
7. Centrifugation: The mixture was centrifuged again at 14,000 x g for 5 minutes to pellet the precipitate, thus removing the cellular debris and lysing matrix.

8. Supernatant Transfer: The clear supernatant was transferred to a clean 15 mL microcentrifuge tube.
9. Binding Matrix Suspension: The Binding Matrix suspension was resuspended, and 1.0 mL was added to the supernatant in the 15 mL tube.
10. DNA Binding: The tube was placed on a rotator or inverted by hand for 2 minutes to allow DNA binding to the Binding Matrix. The tube was then placed on a rack for 3 minutes to allow the Binding Matrix to settle.
11. Supernatant Removal: 500 µL of supernatant was removed and discarded, taking care to avoid disturbing the settled Binding Matrix.
12. Resuspension: The Binding Matrix was gently resuspended in the remaining supernatant.
13. SPIN Filter Transfer: Approximately 600 µL of the mixture was transferred to a SPIN filter and centrifuged at 14,000 x g for 1 minute. The catch tube was emptied and reused, and the process was repeated with the remaining mixture.
14. SEWS-M Addition: 500 µL of prepared SEWS-M (with the appropriate amount of ethanol added) was added to further purify the sample by solubilizing impurities. The pellet was gently resuspended using the liquid from the pipette tip.
15. Centrifugation: The sample was centrifuged at 14,000 x g for 1 minute to remove impurities. The catch tube was emptied and reused.
16. Drying: A second centrifugation at 14,000 x g for 2 minutes, without the addition of any liquid, was performed to dry the Binding Matrix of residual wash solution. The catch tube was replaced with a new, clean one.
17. Air Drying: The SPIN Filter was air-dried for 5 minutes at room temperature.
18. DNA Elution: The Binding Matrix (above the SPIN Filter) was gently resuspended with 200 µL of DES.
19. Final Centrifugation: The mixture was centrifuged at 14,000 x g for 1 minute to elute the DNA into a clean catch tube. The SPIN Filter was then discarded.

The extracted DNA was eluted in 200 µL of DNase/pyrogen-free water. DNA quality was assessed by electrophoresis on a 1% agarose gel, and the DNA concentration was measured

spectrophotometrically at 260 nm using a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, USA). After extraction, all DNA samples were immediately frozen and stored at -20°C.

Quantitative polymerase chain reaction

After DNA extraction, the quantification of total bacterial targets and the copy numbers of ten ARGs was conducted using qPCR on a QuantStudio-3 Real-Time PCR system (Applied Biosystems, USA). The bacterial 16S rRNA genes were amplified using the primers 341F and 534R to estimate the overall abundance of the bacterial population. SYBR Green I dye (Thermo Scientific, USA) was employed as dye method for real-time fluorescence detection. The thermal profiles are described in Table 4 (Perez-Bou et al.,2023). Each qPCR reaction was performed in quadruplicate for each DNA sample, with non-template controls included, in a final reaction volume of 25 µL. The reaction mixture comprised 2.5 µL of 10× DreamTaq buffer (Thermo Scientific, USA), 0.5 µL of TruePure dNTPs (8 mM) (Canvax, Spain), 0.15 µL of each primer (10 µM), 0.125 µL of 5 U/µL DreamTaq Hot Start DNA Polymerase (Thermo Scientific, USA), 0.125 µL of 20× SYBR Green I (Thermo Scientific, USA), 0.0625 µL of 20 mg/mL bovine serum albumin (New England Biolabs, USA), 19.3875 µL of ultrapure water, and 2 µL of template DNA. For absolute quantification, serial dilutions of linearized plasmids containing the target genes, ranging from 10^8 to 10 copies/µL, were used as standards [626,627]. The cloning of the genes into plasmids was performed using the "TOPO TA Cloning Kit for Sequencing". Initially, the amplified DNA fragments were purified with the QIAquick PCR Purification Kit (QIAGEN, Germany). The purified products were then ligated into the PCR 4-TOPO vector (Invitrogen, USA) and used to transform Escherichia coli DH5α competent cells, adhering to the protocol specified by the TOPO TA Cloning System (Invitrogen, USA). All qPCR assays exhibited correlation coefficients (r^2) > 0.99, with PCR efficiencies ranging from 80% to 105%. The total number of ARG copies was quantified per gram of environmental sample, based on the predicted concentrations derived from standard amplification curves. Quantification of gene copy numbers by qPCR relied on the linear correlation between the logarithm of the initial template quantity and the threshold cycle (Ct) value during amplification [628].

Results

With the exception of the ampC, blaSHV, and mecA genes, all other ARGs were detected in every sample. The abundances of different ARGs varied from undetectable levels to high concentrations. Among the samples, the blaTEM gene was the most abundant, followed by

tetA(A), dfrA1, aadA, aadB, qnrS, blaSHV, mecA, ermB, and ampC. This study reinforces the growing body of evidence that ARGs are spreading globally, even reaching pristine environments. These findings confirm that antibiotic resistance is an escalating threat to public health worldwide. The widespread detection of ARGs associated with commonly used antibiotics, including beta-lactams, fluoroquinolones, tetracyclines, macrolides, and sulfonamides, in natural ecosystems underscores a significant public health concern. This issue is largely attributed to the presence of sub-minimal concentrations of antibiotics in these environments, which can drive the selection and persistence of resistant bacteria [199,629,630]. The results reveal significant variability in gene distribution, with some locations showing a broad spread of values and others exhibiting more tightly clustered data. This research provides strong evidence that the global spread of ARGs is an escalating phenomenon, reaching even remote and pristine environments with minimal urban presence.

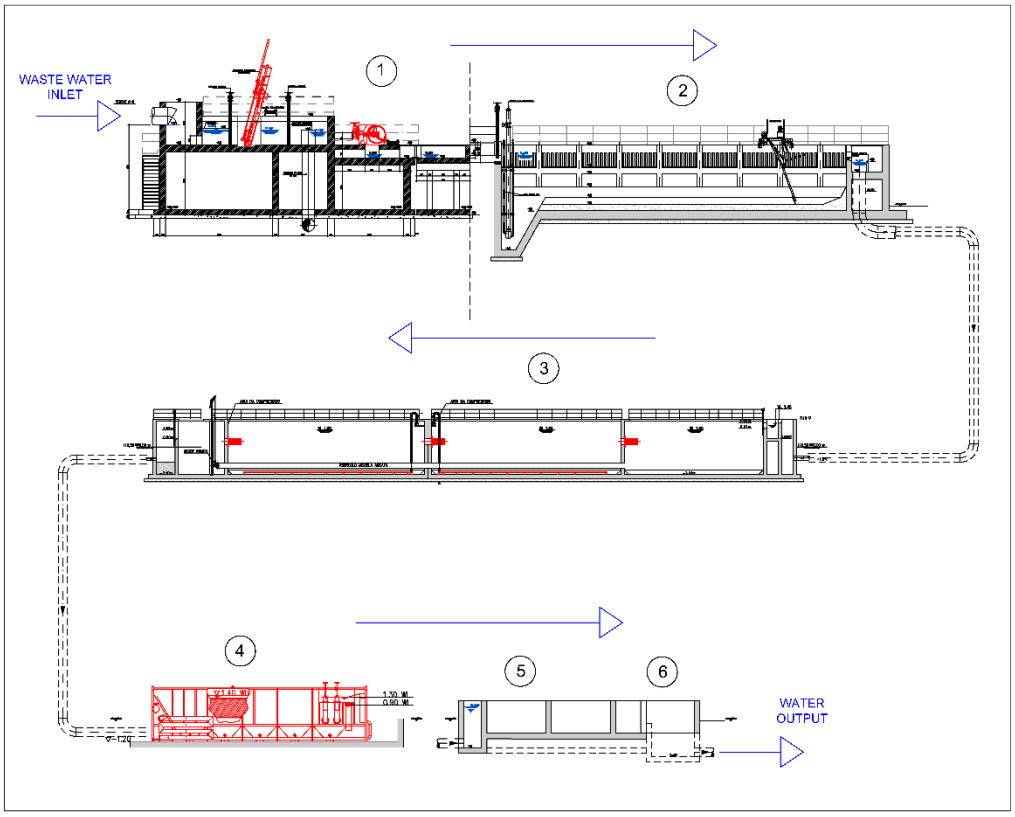
9. STUDY AND RESEARCH ACTIVITIES AT WTC SRL

In accordance with the Public Competition Notice for admission to the PhD programs (XXXVII Cycle) at the University of Catania, published on September 30, 2021, I completed a six-month research and study period at WATER TECHNOLOGY COMPANY (WTC) S.R.L. This experience was conducted under the supervision of Dr. Francesco Bizzini and Dr. Amedeo Ferracane. As part of my training program, my research has focused on studying the different types of wastewater treatment plants and the latest measures to address water scarcity, including efforts to strengthen and adapt water infrastructure. Specifically, we focused on Regulation (EU) 2020/741, the Italian Ministerial Decree No. 185 of June 12, 2003, and the provisions of D.A. No. 6/GAB of February 6, 2024, in Sicily (Decree implementing Regional Law No. 4 of March 22, 2022, regarding regulations on the reuse of urban wastewater, and amendments to Regional Law No. 20 of July 29, 2021). Regulation 2020/741/EU establishes, for the first time at the European level, the minimum requirements for the reuse of urban wastewater for agricultural purposes. The draft Italian Presidential Decree (DPR) both "transposes" and implements the European regulation, while also, in line with provisions in place for nearly 20 years (Ministerial Decree of June 12, 2003, No. 185), defining the rules for the reuse of wastewater not only for irrigation but also for civil and industrial purposes. The provision put forward for consultation by the Ministry of the Environment applies to the treatment and reuse of urban, domestic, and industrial wastewater (with certain limits and exclusions for the latter). Industrial wastewater produced at the same site subject to Integrated Environmental Authorization (AIA) or Unified Environmental Authorization (AUA) is excluded. The regulation outlines the procedures for requesting authorization to produce and deliver treated wastewater, as well as for drafting the Risk Management Plan (in line with Regulation 2020/741/EU), which is a prerequisite for obtaining authorization. It also specifies the obligations of the operators of water treatment plants and their relations with distribution managers. The regulation includes the physical-chemical quality parameters to be met for different uses and details the compliance checks to be carried out by the operator and the competent authority. As in current regulations, regional legislation plays a significant role in planning water reuse activities, ensuring they align with the specific needs of the territory [631]. During this period, I had the opportunity to study the Castelvetrano wastewater treatment plant, which is the first facility in Sicily that enables the reuse of treated wastewater for agricultural purposes. Through an output pipeline, and following stringent checks to ensure the water meets the required parameters for irrigation, the treated water is directed to the pipeline of the nearby reclamation consortium. From there, it is

distributed to meet the needs of local farmers. Specificall, the sizing of the plant followed the best guidelines from specialized literature (see figure 8). The pretreatments include an initial stage of coarse screening to protect the rotating sieves, performed with a hydraulic screen with a spacing of 40 mm between bars. Fine screening, on the other hand, is carried out using 2 rotating wedge wire screens, with a spacing of 1 mm between the bars of the rotating cylinder. The sand and oil removal basin completes the pretreatment process. The biological treatment system utilizes MBBR technology, which, as is well known, takes advantage of the insertion of a random-type plastic media in the reaction basins, allowing for excellent process efficiency, reducing the volume of the biological tanks, and minimizing the overall environmental impact of these purification works.The final sludge separation system from the MBBR compartments consists of pressurized flotation systems.This is a well-tested technology, particularly in similar applications, which has replaced the use of final sedimentation tanks. Being essentially a physical-chemical treatment, the final flotation can independently reduce the solids in the effluent to well below the 10 mg/l required by regulations, with average values even below 5 mg/l if operational control is properly maintained. Following the biological and physical-chemical treatments, the recovery of effluent water for irrigation use is planned. Downstream of the 4 planned flotation units (2 for each biological line), which use pressurized recirculation and are fully constructed in AISI 304L, each equipped with all necessary auxiliary equipment (chains, pressurization recirculation systems, purge valves, sludge extraction, etc.), there are two rotating disc filters. These filters provide a finishing treatment to the wastewater exiting the flotation units, allowing for suspended solids reduction to values below 10 mg/l, even in the event of a temporary malfunction in one of the pressurized flotation units. The installation of the final disc filters ensures a high efficiency of the effluent, even in the subsequent final UV disinfection phase, in cases of possible flotation unit failure. Additionally, it is worth noting that the final filtration system is designed to handle a flow rate of 850 m^3/h. The hydraulic overflow system, which is typically foreseen (and in any case indispensable), manages the excess flow of 400 m^3/h, resulting from the difference between 1250 m^3/h (the flow rate from the biological section and, consequently, the flotation units) and the aforementioned 850 m^3/h. Consequently, for irrigation reuse, the flow rate of 850 m^3/h is fully 'protected' by the filters. This is also considering that exceeding this flow rate occurs only in specific cases of intense meteorological events. A suitably sized overflow system, positioned both upstream and downstream of the two disc filters, ensures that only the treated flow is directed for reuse, while the flow from the screening and/or sand removal is discharged directly into the final receiving body, as specified by the system. Therefore, it is hoped that an increasing number of wastewater treatment plants

will be designed and developed with the same innovative approach as the Castelvetrano plant, which stands as the first in Sicily to successfully enable the reuse of treated wastewater for agricultural purposes. Such advancements are crucial for promoting sustainable water management and addressing the growing challenges of water scarcity.

Figure 8. Diagram of the Castelvetrano sewage treatment plant.



LEGEND

1. Coarse and fine screening section (rotating screens);
2. Sand and oil removal;
3. Biological treatment and coagulation: a. Denitrification; b. Oxidation and nitrification compartments (MBBR); c. Coagulation;
4. Flocculation and flotation;
5. Polishing filtration (tertiary);
6. Disinfection with UV lamps.

10. DISCUSSION AND CONCLUSION

This research project has redefined wastewater from a discarded by-product into a valuable resource for assessing and monitoring public health within a One Health framework. Through the advanced technique of WBE, we can capture a dynamic snapshot of community health, including levels of microbial contamination and environmental exposure. This innovative approach establishes WBE as an eco-friendly strategy for public health, enabling early detection and sustainable monitoring of both environmental and health threats. WBE offers not only a comprehensive understanding of population health but also serves as a powerful tool for indirect epidemic monitoring, providing real-time data that can guide timely public health interventions. By analyzing the chemical and biological markers in wastewater—such as those found in urine, feces, and other bodily substances—WBE allows for continuous monitoring of health trends and environmental impacts at the community level [8-10]. Over the years, WBE has proven to be adaptable across various public health domains, addressing a wide range of concerns from infectious disease outbreaks to environmental contaminants and illicit drug use. Unlike traditional methods that rely on individual blood or urine samples, WBE assesses the health status of entire populations through wastewater analysis, providing a non-invasive and scalable method that does not require personal consent—only approval from relevant wastewater authorities. These findings can be cross-referenced with population surveys or used to estimate public health metrics in the absence of direct data, as demonstrated in studies of drug consumption patterns [27,316]. By enabling early detection of emerging threats, WBE allows for timely public health interventions that can prevent widespread disease transmission. Recent advances in environmental pathogen detection technologies, such as gene amplification and sequencing, have significantly expanded the capabilities of WBE, allowing it to monitor a wide range of bacteria, parasites, and viruses that are commonly excreted in human waste. One of the key advantages of wastewater monitoring is that many pathogens of interest can remain viable in the sewer environment for several days. Additionally, molecular detection methods allow for the identification of pathogens even when they are no longer viable, significantly extending the window for their detection in wastewater. In some cases, simply detecting the presence of high-risk pathogens in the sewage system can be enough to trigger further investigation [36]. However, when the pathogen load per infected individual is low, a large number of infected individuals may be necessary to detect the pathogen in wastewater. The COVID-19 pandemic, caused by SARS-CoV-2 [99], has highlighted the urgent need for innovative and scalable surveillance methods. The magnitude of the crisis has reinforced the

value of tracking SARS-CoV-2 prevalence in communities and developing dynamic models for monitoring infections [11]. WBE has proven to be an indispensable tool for proactive, population-wide monitoring of viral presence. Furthermore, wastewater surveillance has been instrumental in identifying dominant viral variants circulating within communities [103-105]. Multiple studies have demonstrated the importance of environmental surveillance as an early warning system, capable of detecting rising viral levels and impending outbreaks before they are reflected in healthcare reports [106,107]. Numerous studies have demonstrated the detection of SARS-CoV-2 RNA in wastewater samples from various cities even before the first COVID-19 cases were officially reported by public health authorities. These findings underscore the value of WBE as a symptom-independent method for tracking SARS-CoV-2 transmission trends within communities over extended periods, offering a broader and more continuous perspective compared to individual laboratory testing [81]. Indeed, wastewater serves as a collective reservoir of genetic material from an entire population, offering insight into the prevalence of SARS-CoV-2, including cases from asymptomatic and presymptomatic individuals [103,104,109]. This is particularly crucial as research shows that the viral loads of asymptomatic individuals are often comparable to those of symptomatic patients [110,111]. It is estimated that 48–67% of infected individuals excrete SARS-CoV-2 in their feces [113-115], and the extent of fecal RNA shedding does not correlate with the severity of COVID-19 symptoms [116]. Notably, SARS-CoV-2 RNA has been detected in stool samples even when nasopharyngeal swabs yield negative results [117,118], with viral RNA persisting in stool for 9–16 days post-symptom onset, compared to 6–11 days for nasopharyngeal swabs. This highlights the potential of fecal monitoring as a valuable tool in cases where swab tests may fail to detect active infections [119]. WBE also proves to be significantly more cost-effective than widespread individual testing campaigns, making it an attractive option for large-scale public health monitoring. The structured sampling methodology used in wastewater monitoring enhances the likelihood of detecting viral signals, mimicking the principles of random population surveys. This reduces biases and challenges associated with individual testing, providing a more accurate representation of community-wide infection levels [11]. Real-time data on COVID-19 cases and SARS-CoV-2 RNA levels in wastewater can empower individuals to make informed decisions regarding personal safety, such as wearing masks or avoiding crowded areas. As COVID-19 transitions to an endemic stage, wastewater surveillance remains a future-ready tool, capable of adapting to new public health priorities, tracking emerging SARS-CoV-2 variants, and monitoring unknown pathogens under the WHO's 'disease X' framework [124]. Surveillance of pathogens through WBE, aligned with the One Health

framework, is critical for the early detection and prevention of future pandemics [35]. This approach has proven particularly effective in tracking the spread of ARB, especially those originating from healthcare facilities and agricultural settings [33,34]. Over the past two decades, bacterial resistance has surged globally, driven by the widespread development and dissemination of ARB and ARGs [211,239]. Initially associated with healthcare-acquired infections [267,268], AMR is now increasingly linked to sectors such as animal farming, aquaculture, and urban WWTPs. These industries contribute significantly to the spread of ARB, ARGs, and residual antibiotics into surrounding environments [240,241,255,258]. Antibiotics discharged into sewage systems eventually enter aquatic and terrestrial ecosystems, turning these environments into both pathways for the spread of ARB and reservoirs for the development of new ARGs [204,246-248]. The presence of antimicrobials in these ecosystems exerts selective pressure, intensifying resistance and promoting the emergence of novel resistance mechanisms. Identifying hotspots and reservoirs of ARB is crucial to mitigating the impact of AMR [251]. Furthermore, monitoring the prevalence of ARGs in environmental bacterial communities is essential to understand the impact of human activities on the spread of resistant bacteria [200,252,255]. In particular, the WHO has highlighted the growing concern over the presence of ARGs in aquatic ecosystems, with urban water systems serving as major pathways for the spread of AMR due to wastewater discharges, agricultural runoff, and other human activities [253,254]. Aquatic ecosystems are especially vulnerable as they are continuously exposed to antimicrobial compounds. WWTPs, identified as hotspots for antibiotic resistance, play a central role in the concentration and spread of multidrug-resistant strains [145,259]. Effective wastewater treatment is vital for protecting public health from these resistance epidemics [145]. AMR surveillance is essential for tracking resistance patterns, identifying new or rare resistance traits, and monitoring emerging trends. This data is crucial for informing interventions, guiding treatment protocols, and developing new antimicrobial drugs [306]. WBE offers a cost-effective and comprehensive method for monitoring AMR dynamics across populations. It provides valuable insights at a fraction of the cost of traditional individual sampling approaches, and unlike conventional clinical surveillance, WBE can track resistance on a much broader scale [246,312]. In addition, modern societal needs are reshaping wastewater management, driving paradigm shifts to address both current challenges and future demands. The depletion of freshwater resources highlights the urgent need for innovative solutions, such as wastewater reclamation and reuse, to meet increasing demand [342,343]. The shift toward a circular economy, driven by growing environmental concerns and social awareness, has prompted a reimagining of wastewater as a valuable resource rather than a waste

product [334]. This approach aligns with sustainable practices, focusing on reducing human impact by reclaiming and reusing wastewater. Efficient wastewater management—including collection, treatment, and discharge with minimal environmental impact—remains essential to protecting public health and the environment. However, despite advancements, it continues to be a pressing issue, necessitating innovative and evolving strategies. Numerous studies confirm that current consumption and production systems are unsustainable and pose a significant threat to global well-being [373]. In the context of rising water demand—driven by population growth, industrialization, and climate change—this circular model enhances water use efficiency, promoting resilience and sustainability in water availability [342,343]. The mismanagement of water resources has exacerbated pollution and water stress globally, leading to severe local water crises that affect public health, food security, and economic development. Water quality, safety, and availability are critical to human health. Target 6.3 of the United Nations Sustainable Development Goal (SDG) 6 aims to improve water quality by 2030 through reducing pollution, halving untreated wastewater, and increasing recycling and safe reuse. A major barrier to water reuse is microbiological contamination, as wastewater often harbors harmful microorganisms, nutrients that cause eutrophication, and pollutants [370-372]. Ensuring access to safe water, particularly by reducing microbial contamination, remains a critical challenge [381]. According to the SDG Synthesis Report on Water and Sanitation, the targets for SDG 6 are far from being met. This highlights the need for greater efforts to improve water monitoring and management. To mitigate health risks from wastewater reuse, stringent safety measures and effective treatment processes are crucial, emphasizing the importance of proper wastewater management to prevent adverse health outcomes. The results of the scoping review, "The Impact of Wastewater on Antimicrobial Resistance: A Scoping Review of Transmission Pathways and Contributing Factors," show that to minimize the incidence and spread of AMR, and its risks to human and animal health, it is crucial to identify hotspots and transmission pathways for ARGs. A key factor in addressing this challenge is understanding the role of MGEs in facilitating the development and transfer of antibiotic resistance. Insight into how ARGs are transferred and the mechanisms driving their movement is essential for devising strategies to curb the spread of resistance, both in healthcare settings and environmental contexts. WWTPs are recognized as major sources of ARB and ARGs in aquatic ecosystems. The high microbial density, nutrient-rich environment, and favorable conditions for bacterial growth, such as optimal temperature and pH, in WWTPs make them ideal hotspots for the transfer of ARGs and the emergence of multi-resistant bacteria [303,514,516,522,527,532,534,549,550,552,553]. Additionally, the co-selection of ARGs is exacerbated when bacterial communities are exposed

to chemical stressors, including heavy metals and antibiotics, in the ever-changing WWTP environment [518,519,523]. One of the challenges is that current wastewater treatment technologies are often insufficient to fully eliminate ARB and ARGs from effluents. While methods like UV irradiation and chlorination can be effective, they may require enhancements, such as combining both processes or integrating oxidizing agents and pretreatments, to maximize efficiency [201,543-545]. A comprehensive understanding of the risks posed by ARB and ARGs in WWTPs, particularly in relation to wastewater reuse, is essential for protecting human health and ecosystems. Additionally, research is needed to assess how technological interventions can improve treatment efficacy while considering the economic feasibility of wastewater reuse applications. In addition, the results of the scoping review, "Updates on developing and applying biosensors for the detection of microorganisms, antimicrobial resistance genes and antibiotics: a scoping review" show that the detection and identification of bacterial pathogens, antibiotics, and ARGs is a public health priority to combat the spread of AMR. A One Health approach is essential, involving measures such as antibiotic use surveillance, detection of resistance in humans and animals, public awareness campaigns on proper antibiotic use, and stewardship programs in healthcare settings [140,632]. Traditionally, bacterial identification has relied on labor-intensive methods requiring specialized labs and expensive equipment. This has driven interest in faster, cost-effective, and more sensitive techniques that can identify bacteria, ARGs, and antibiotics in clinical and environmental samples. Biosensors are emerging as a promising alternative to traditional methods in the context of AMR detection. They offer high sensitivity, specificity, and reproducibility for identifying microorganisms and antibiotics. The specificity of biosensors is influenced by the interaction between the biological component and the transducer, while their sensitivity depends on the bioreceptor, immobilization procedure, and transduction method. Biosensors are advantageous due to their low cost and rapid detection capabilities, enabling quick identification of resistant genes, pathogens, and antibiotics [633,634]. A key component in biosensor function is the conversion of biological interactions into measurable signals via a transduction system. Our scoping review highlights electrochemical [581,587,589-591,594-601,606,607,611,615,618-620], optical [573-575,577-580,582-586,588,593,602-605,608-610,612-614,616,617], and mechanical [592] transducers as the most commonly used platforms. Among these, plasmonic biosensors, particularly those utilizing surface plasmon resonance (SPR) and surface-enhanced Raman spectroscopy (SERS), have proven effective for detecting pathogens and antibiotics [586,588,602,613]. These transducers are often combined with bioreceptors like antibodies specific to AMR-related proteins (e.g., anti-penicillin-binding

protein 2A, PBP2a). Colorimetric assays also present a user-friendly alternative, as they enable detection through simple color changes visible to the naked eye, without expensive equipment [576,581,583,585,609,610,616]. However, electrochemical detection methods offer distinct advantages, such as better sensitivity and the ability to classify biosensors as amperometric, potentiometric, or impedimetric, based on the electrical parameters measured [581,587,589-591,594-601,606,607,611,615,618-620]. Nanomaterials like graphene-based materials, magnetic nanoparticles, and metal/metal oxide nanoparticles (e.g., AuNPs) have further enhanced biosensor sensitivity. These nanomaterials are valued for their excellent optical and electrical properties, high conductivity, photoelectrochemical activity, and large surface-to-volume ratio [585,586,594,595,599,618,619]. Capacitive sensors, especially interdigitated electrodes (IDEAs), have garnered particular interest due to their rapid detection kinetics, high signal-to-noise ratio, quick steady-state response, affordability, and ease of miniaturization [591].Overall, the development of advanced biosensors represents a crucial step forward in the fight against AMR, offering innovative and efficient tools for detecting resistance and monitoring its spread across clinical and environmental settings. The collection of wastewater samples over the course of these three years of doctoral research enabled the monitoring and detection of the spread of SARS-CoV-2, as well as the tracking of antimicrobial resistance genes, while assessing the environmental benefits and risks associated with wastewater reuse within a circular economy framework. Specifically, SARS-CoV-2 was detected in 293 out of the 316 wastewater samples collected at the Pantano D'Arci, Giarre, and Siracusa sites. The weekly sampling results from February 2022 to March 2023 revealed that the trend in SARS-CoV-2 concentration in wastewater from the three sites showed significant fluctuations. In Pantano D'Arci, SARS-CoV-2 levels showed multiple peaks followed by sharp declines, with a slight downward trend over the year. Giarre also experienced several peaks, with high variability in viral load, yet exhibited a similar overall decrease. Siracusa mirrored this pattern, displaying frequent fluctuations and a slight reduction in concentrations over time. Despite these fluctuations, all three sites demonstrated a gradual decline in SARS-CoV-2 levels throughout the year, indicating a general decrease in viral presence in wastewater. Significant spikes occurred in May 2022 and January 2023, potentially driven by public health events or seasonal factors. The peaks in viral load closely aligned with surges in reported COVID-19 cases during these periods, reinforcing the reliability of wastewater data as a reflection of community infection levels. For example, a notable spike in June 2022 coincided with a documented increase in COVID-19 cases in the region, as reported by the Sicilian authorities (www.regione.sicilia.it). This association underscores the importance of wastewater monitoring

as a tool for tracking pandemic dynamics. Wastewater data can act as an early warning system, detecting viral presence before clinical cases are reported, as individuals shed the virus in their stool even before showing symptoms or undergoing testing. Furthermore, wastewater surveillance captures information from the entire population, including asymptomatic individuals, providing a more comprehensive picture of the virus's spread than clinical reports alone. With containment measures and widespread vaccination, SARS-CoV-2 began stabilizing. As a result, wastewater surveillance shifted from weekly to monthly sampling from April 2023 to September 2024, reflecting the need for a more sustainable monitoring approach during this phase. During this period, viral concentrations remained relatively stable across the three sites, fluctuating between 1,500 and 2,500 c.g./L, with a slight decrease from May to June, followed by stabilization. However, a significant spike was observed from December 2023 to January 2024, particularly in Giarre, where concentrations surged to nearly 50,000 c.g./L. Pantano D'Arci and Siracusa also experienced increases, though less pronounced. This spike likely corresponded to a winter surge in COVID-19 cases, driven by factors like indoor gatherings. Following this peak, concentrations dropped significantly and stabilized by April 2024, with viral loads remaining below 1,000 c.g./L from April to July 2024, suggesting a reduction in virus transmission. A gradual increase was observed from August to September 2024, possibly signaling the onset of another wave of infections as summer ended. The data underscores the importance of continuous wastewater monitoring, as it captures both short-term fluctuations and long-term trends in viral concentrations. This approach enables early detection of potential outbreaks, providing valuable insights for public health responses. From May 2024 onwards, wastewater samples were collected both before and after treatment at Pantano D'Arci, with Giarre and Siracusa following suit in June 2024. The results demonstrated that treatment processes at all three sites were effective in reducing SARS-CoV-2 concentrations. At Pantano D'Arci, post-treatment viral levels were consistently lower than pre-treatment levels. Similarly, Giarre and Siracusa exhibited effective treatment, even when confronted with higher viral loads in August 2024. This consistent reduction in post-treatment concentrations across all months highlights the robustness of the wastewater treatment processes at these sites. Even during peak viral levels, the facilities proved effective in significantly lowering SARS-CoV-2 concentrations, underscoring the reliability of their treatment methods. Concerning the study on the presence of ARGs, their relative abundance in wastewater was assessed using 42 monthly composite samples collected from three WWTPs in Pantano D'Arci, Siracusa, and Giarre, spanning the period from February 2022 to March 2023. Each sample was analyzed for the following ARGs: blaSHV, blaOXA, blaNDM, blaVIM, blaTEM, blaCTX-M, ermA, ermB.

Specifically, for the blaNDM and blaCTX-M genes, more than one probe was used, capable of identifying different sequences within the same gene. The 16S rRNA gene was used as a reference for qPCR measurements to normalize the relative abundances of ARGs in the wastewater samples. Among the detected ARGs, four genes—blaERM_B, blaOXA, blaTEM, and blaCTX-M—were consistently present in 100% of the samples, though with varying relative abundances. Of these, blaERM_B, blaSHV, and blaTEM exhibited the highest relative abundance across all samples. Notably, in wastewater samples from Catania, blaOXA was also found to be consistently prevalent, alongside blaERM-B, blaSHV, and blaTEM. The consistent presence of these genes in 100% of the samples suggests that they are widely distributed among the environmental microbes found in wastewater. This finding could indicate a common resistance in both pathogenic and environmental bacteria, likely due to the widespread use of the corresponding antibiotics. The variations in relative abundance observed among the different genes may reflect seasonal fluctuations or changes in the antibiotic loads in wastewater, such as the more intensive use of specific drugs during certain periods of the year. Further studies will be necessary to better understand the transmission dynamics of these genes and their potential correlations with human and environmental factors. In addition to the ARG analysis, a significant negative correlation was observed between the concentration of SARS-CoV-2 in the wastewater and the CT value of 16S rRNA ($r = –0.506$, $p = 0.001$). Specifically, this negative relationship was significant for blaERM_B ($r = –0.345$, $p = 0.025$), blaOXA ($r = –0.356$, $p = 0.021$), blaTEM ($r = –0.350$, $p = 0.023$), blaCTXM-2 ($r = –0.464$, $p = 0.002$), and blaCTXM-4 ($r = –0.424$, $p = 0.005$). This suggests that an increase in SARS-CoV-2 concentrations is associated with a higher relative abundance of these ARGs in wastewater. The findings raise important questions regarding the mechanisms driving the observed correlation between viral load and ARG abundance. One possible explanation could involve the selective pressure induced by increased antibiotic use during the pandemic. However, further research is required to elucidate the exact processes underlying this relationship and to determine whether these trends are specific to the conditions studied or reflect a broader environmental phenomenon. Furthermore, the six-month research period conducted from June 2023 to December 2023 at the Water Research Institute and the Department of Microbiology, University of Granada, Spain, allowed me to study antibiotic resistance, with a particular focus on the presence of ARGs in pristine areas. Most efforts to understand and combat antibiotic resistance have traditionally focused on the human and animal sectors, while the prevalence of antibiotic resistance in the environment, particularly in pristine and remote areas, remains insufficiently explored. Addressing this gap is crucial, as increasing selection pressures from human activities

may be contributing to the enrichment of ARGs in these supposedly untouched environments. Additionally, the evolutionary and ecological processes driving the dissemination of ARGs in these locations are still poorly understood. Pristine and remote areas, such as high mountains, are often viewed as benchmarks for assessing human-induced environmental contamination. In this context, the Sierra Nevada in southern Spain, with its numerous remote lakes, serves as an ideal location for studying human impacts on pristine ecosystems. During our research, we analyzed ten ARGs in fourteen biomass samples collected from seven pristine lakes in the Sierra Nevada between 2021 and 2022. Excluding *ampC*, *blaSHV*, and *mecA*, seven other ARGs were detected in all samples, highlighting the pervasive nature of antibiotic resistance even in isolated environments. These findings contribute to the growing body of evidence that ARGs are spreading globally, even reaching ecosystems previously considered untouched by human influence. The widespread detection of ARGs associated with commonly used antibiotics—such as beta-lactams, fluoroquinolones, tetracyclines, macrolides, and sulfonamides—in natural ecosystems underscores the severity of this public health threat. This widespread contamination is largely attributed to the presence of sub-minimal concentrations of antibiotics in these environments, which can drive the selection and persistence of resistant bacteria [199,629,630]. Our data also reveal significant spatial and temporal fluctuations in the abundance of ARGs, underscoring the dynamic nature of antibiotic resistance in pristine environments. These fluctuations may be influenced by a range of factors, including environmental conditions, microbial community interactions, and anthropogenic pressures. Similarly, we observed substantial variations in total bacterial load across sediment samples, likely driven by these same factors. This emphasizes the complexity and variability of bacterial populations in these ecosystems.

In conclusion, this research highlights the alarming spread of ARGs to remote and pristine environments, reinforcing the need for ongoing monitoring. Understanding the factors driving these changes and their potential consequences is essential for addressing the global antibiotic resistance crisis, which now appears to be affecting even the most isolated ecosystems. The findings of this research emphasize the critical role of WBE as a powerful and sustainable tool in the monitoring and prevention of public health threats, particularly AMR and pandemics. As global health continues to face complex challenges, including the rise of AMR and the ongoing threat of emerging infectious diseases, the need for innovative, scalable, and eco-friendly approaches to surveillance has never been more evident. WBE provides an early warning system for the spread of infectious diseases and antimicrobial-resistant organisms, allowing for timely interventions before these threats become unmanageable. Unlike traditional surveillance

methods, WBE is able to capture data from entire populations, including individuals who may not seek medical care, which significantly enhances the accuracy and comprehensiveness of health data. Moreover, its cost-effectiveness and ability to operate on a large scale make WBE a viable and green solution to global health surveillance challenges.

This thesis demonstrates that incorporating WBE into public health strategies can bridge the gap between environmental sustainability and health protection. By combining technological innovation with ecological responsibility, WBE not only contributes to safeguarding public health but also supports broader environmental objectives. For instance, a major paradigm shift in wastewater management is essential to more effectively safeguard drinking water resources, protect aquatic ecosystems, and support sustainable development, while also contributing to both climate change mitigation and adaptation efforts. Therefore, future research and policy should focus on further developing WBE methodologies and integrating them into national and international health surveillance programs.

11. APPENDIX

Figure 1. Map showing the locations of the wastewater treatment plants included in the study

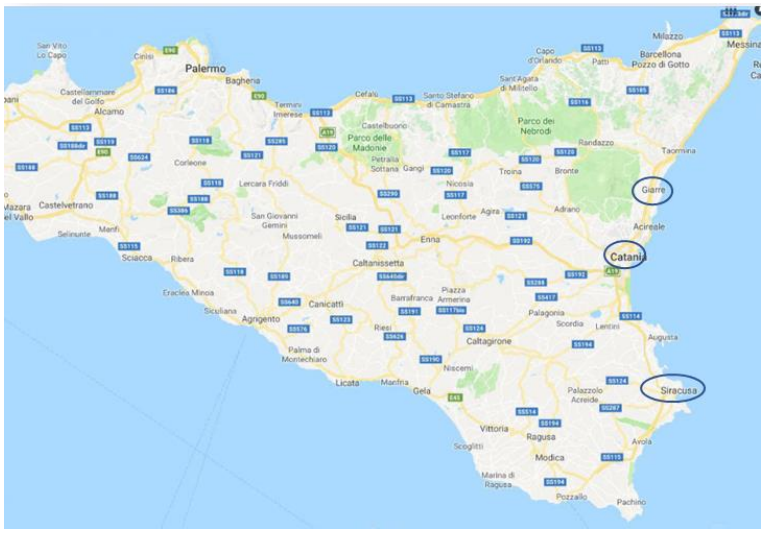


Table 1. Characteristics of the wastewater treatment plants in place

Wastewater Treatment Plant	Served Population (N)	Type of treatment
Catania (Pantano D'Arci)	68434	Tertiary
Giarre (Mascali)	47600	Secondary
Siracusa	180000	Tertiary

Table 2. Results of SARS-CoV-2 Detection by Real-time RT-PCR

Sample collection date	Collection site	Sewage treatment plant flow rate (m3/24h)	Total Suspended Solids (mg/l)	Frozen/Refrigerated (fresh)	Sample storage temperature until analysis	Result	Result (c.g./microL RNA)	Result (c.g./L wastewater)	Inhibition value (delta Ct)	Recovery percentage value (%)
1/2/22	Pantano D'Arci	50112 m3/24h	10 mg/L	Refrigerated (fresh)	5±3°C	non detected				
3/2/22	Pantano D'Arci	49.101,12 m3/24h	10 mg/L	Refrigerated (fresh)	5±3°C	detected	1.58	3.94E+03	0.44	4.6
3/2/22	Giarre	2.611,33 m3/24h	90 mg/L	Refrigerated (fresh)	5±3°C	detected	4.32	1.08E+04	0.60	14.9
3/2/22	Siracusa	37.595 m3/24h	212 mg/L	Refrigerated (fresh)	5±3°C	detected	1.32	3.30E+03	0.43	1.1
8/2/22	Pantano D'Arci	52.202,88 m3/24h	10 mg/L	Refrigerated (fresh)	5±3°C	non detected				
8/2/22	Giarre	2.812 m3/24h	170 mg/L	Refrigerated (fresh)	5±3°C	detected	4.36	1.09E+04	1.99	15.1
8/2/22	Siracusa	35.239 m3/24h	148 mg/L	Refrigerated (fresh)	5±3°C	detected	2.83	7.08E+03	1.97	5.6
10/2/22	Pantano D'Arci	49.593,6 m3/24h	22 mg/L	Refrigerated (fresh)	5±3°C	non detected				
10/2/22	Giarre	2.755,33 m3/24h	120 mg/L	Refrigerated (fresh)	5±3°C	non detected				
10/2/22	Siracusa	37.445 m3/24h	168 mg/L	Refrigerated (fresh)	5±3°C	detected	4.99	1.25E+04	1.98	18
15/2/22	Pantano D'Arci	48.816 m3/24h	20 mg/L	Refrigerated (fresh)	5±3°C	detected	1,23	3,08E+03	0,22	1,1
15/2/22	Giarre	3.538,33 m3/24h	110 mg/L	Refrigerated (fresh)	5±3°C	detected	3,38	8,45E+03	1,33	8,1
15/2/22	Siracusa	37.364 m3/24h	260 mg/L	Refrigerated (fresh)	5±3°C	detected	10,89	2,72E+04	0,56	26,07
17/2/22	Pantano D'Arci	48.902,4 m3/24h	38 mg/L	Refrigerated (fresh)	5±3°C	non detected				
17/2/22	Giarre	3.816 m3/24h	200 mg/L	Refrigerated (fresh)	5±3°C	non detected				
17/2/22	Siracusa	40.933 m3/24h	216 mg/L	Refrigerated (fresh)	5±3°C	non detected				
22/2/22	Pantano D'Arci	48.666,76 m3/24h	11 mg/L	Refrigerated (fresh)	5±3°C	non detected				

22/2/22	Giarre	3997 m3/24h	20 mg/L	Refrigerated (fresh)	5±3°C	detected	5,88	1,47E+04	1,43	26,6
22/2/22	Siracusa	36.219 m3/24h	184 mg/L	Frozen	-20°C	detected	6,97	1,74E+04	0,72	42,9
24/2/22	Pantano D'Arci	49.101,12 m3/24h	34 mg/L	Refrigerated (fresh)	5±3°C	non detected				
24/2/22	Giarre	3484 m3/24h	80 mg/L	Refrigerated (fresh)	5±3°C	detected	6,04	1,51E+04	1,35	27,7
24/2/22	Siracusa	36.791 m3/24h	416 mg/L	Refrigerated (fresh)	5±3°C	detected	10,63	2,66E+04	0,92	37
1/3/22	Pantano D'Arci	50.250,24 m3/24h	27 mg/L	Refrigerated (fresh)	5±3°C	non detected				
1/3/22	Giarre	3.950,66 m3/24h	100 mg/L	Refrigerated (fresh)	5±3°C	detected	10,67	2,67E+04	0,31	71,7
1/3/22	Siracusa	43.778 m3/24h	252 mg/L	Frozen	-20°C	detected	7,15	1,79E+04	0,21	40,1
3/3/22	Pantano D'Arci	47.174,4 m3/24h	10 mg/L	Refrigerated (fresh)	5±3°C	detected	3,2	8,00E+03	0,24	5,1
3/3/22	Giarre	3.934 m3/24h	30 mg/L	Refrigerated (fresh)	5±3°C	detected	5,15	1,29E+04	1,43	18,9
3/3/22	Siracusa	38.607 m3/24h	200 mg/L	Refrigerated (fresh)	5±3°C	detected	7,12	1,78E+04	0,6	28,8
8/3/22	Pantano D'Arci	47.088 m3/24h	9 mg/L	Refrigerated (fresh)	5±3°C	non detected				
8/3/22	Giarre	2.970,33 m3/24h	50 mg/L	Refrigerated (fresh)	5±3°C	detected	19,19	4,80E+04	1,98	34,8
8/3/22	Siracusa	40.853 m3/24h	92 mg/L	Frozen	-20°C	detected	7,42	1,86E+04	1,16	37,9
10/3/22	Pantano D'Arci	45.532,8 m3/24h	10mg/L	Refrigerated (fresh)	5±3°C	non detected				
10/3/22	Giarre	5.568 m3/24h	30 mg/L	Refrigerated (fresh)	5±3°C	detected	6,37	1,59E+04	1,26	32,1
10/3/22	Siracusa	36.674 m3/24h	168 mg/L	Refrigerated (fresh)	5±3°C	detected	6,12	1,53E+04	1,67	27,5
15/3/22	Pantano D'Arci	41.558,4 m3/24h	10 mg/L	Refrigerated (fresh)	5±3°C	detected	5,34	1,33E+04	1,04	18,2
15/3/22	Giarre	4002,3 m3/24h	40 mg/L	Refrigerated (fresh)	5±3°C	detected	26,39	6,60E+04	1,04	49,3
15/3/22	Siracusa	35.642 m3/24h	228 mg/L	Frozen	-20°C	detected	8,05	2,01E+04	1,02	46,5
17/3/22	Pantano D'Arci	47.692,8 m3/24h	26 mg/L	Refrigerated (fresh)	5±3°C	detected	2,43	6,08E+03	0,48	1,9

17/3/22	Giarre	3.871 m3/24h	42 mg/L	Refrigerated (fresh)	5±3°C	detected	8,44	2,11E+04	0,92	72,5
17/3/22	Siracusa	36.263 m3/24h	176 mg/L	Refrigerated (fresh)	5±3°C	detected	4,76	1,19E+04	0,63	13,3
22/3/22	Pantano D'Arci	47.692,8 m3/24h	10 mg/L	Refrigerated (fresh)	5±3°C	detected	4,73	1,18E+04	1,69	7,3
22/3/22	Giarre	9.307 m3/24h	40 mg/L	Refrigerated (fresh)	5±3°C	detected	18,39	4,60E+04	1,37	50,3
22/3/22	Siracusa	38.155 m3/24h	292 mg/L	Frozen	-20°C	detected	23,93	5,98E+04	1,36	37,9
24/3/22	Pantano D'Arci	44.496 m3/24h	14 mg/L	Refrigerated (fresh)	5±3°C	detected	3,43	8,58E+03	1,01	6,2
24/3/22	Giarre	3.740,66 m3/24h	31 mg/L	Refrigerated (fresh)	5±3°C	detected	12,39	3,10E+04	0,93	34,6
24/3/22	Siracusa	36.367 m3/24h	112 mg/L	Refrigerated (fresh)	5±3°C	detected	25,92	6,48E+04	0,08	87,1
29/3/22	Pantano D'Arci	46.915,2 m3/24h	20 mg/L	Refrigerated (fresh)	5±3°C	non detected				
29/3/22	Giarre	3.664,33 m3/24h	90 mg/L	Refrigerated (fresh)	5±3°C	detected	4,472	1,12E+04	1,41	15,2
29/3/22	Siracusa	35.244 m3/24h	112 mg/L	Frozen	-20°C	detected	1,646	4,12E+03	0,94	1,7
31/3/22	Pantano D'Arci	47.001,6 m3/24h	8 mg/L	Refrigerated (fresh)	5±3°C	detected	6,39	1,60E+04	1,19	66,5
31/3/22	Giarre	4.102,66 m3/24h	14 mg/L	Refrigerated (fresh)	5±3°C	detected	3,495	8,74E+03	1,45	12,1
31/3/22	Siracusa	36.466 m3/24h	136 mg/L	Refrigerated (fresh)	5±3°C	detected	5,408	1,35E+04	1,01	36,6
5/4/22	Pantano D'Arci	43.113,6 m3/24h	32 mg/L	Refrigerated (fresh)	5±3°C	detected	2,154	5,39E+03	1,37	3,5
5/4/22	Giarre	3.671 m3/24h	45 mg/L	Refrigerated (fresh)	5±3°C	detected	1,724	4,31E+03	1,17	1,9
5/4/22	Siracusa	36.664 m3/24h	284 mg/L	Frozen	-20°C	detected	3,524	8,81E+03	1,53	10,9
7/4/22	Pantano D'Arci	43.856 m3/24h	10 mg/L	Refrigerated (fresh)	5±3°C	detected	1,262	3,16E+03	1,06	0,6
7/4/22	Giarre	3.769 m3/24h	50 mg/L	Refrigerated (fresh)	5±3°C	detected	1,131	2,83E+03	1,28	0,4
7/4/22	Siracusa	37.332 m3/24h	220 mg/L	Refrigerated (fresh)	5±3°C	detected	4,305	1,08E+04	1,16	21,6
12/4/22	Pantano D'Arci	41.558,4 m3/24h	12 mg/L	Frozen	-20°C	detected	5,438	1,36E+04	1,53	6,3

14/4/22	Pantano D'Arci	43.683,84 m3/24h	60 mg/L	Refrigerated (fresh)	5±3°C	detected	2,389	5,97E+03	1,45	0,9
14/4/22	Giarre	2.718,5 m3/24h	60 mg/L	Refrigerated (fresh)	5±3°C	detected	10,092	2,52E+04	1,75	41,4
14/4/22	Siracusa	37.688 m3/24h	414 mg/L	Refrigerated (fresh)	5±3°C	detected	25,916	6,48E+04	1,72	73,4
19/4/22	Pantano D'Arci	42.327,36 m3/24h	380 mg/L	Frozen	-20°C	detected	3,982	9,96E+03	0,77	3,6
21/4/22	Pantano D'Arci	43.614,72 m3/24h	18 mg/L	Refrigerated (fresh)	5±3°C	detected	10,193	2,55E+04	1,02	45,3
19/4/22	Giarre	2.772,25 m3/24h	10 mg/L	Frozen	-20°C	detected	2,488	6,22E+03	0,8	0,9
21/4/22	Siracusa	37.620 m3/24h	332mg/L	Refrigerated (fresh)	5±3°C	detected	9,257	2,31E+04	0,95	20,8
26/4/22	Pantano D'Arci	44.496 m3/24h	7 mg/L	Frozen	-20°C	detected	22,544	5,64E+04	0,98	72
28/4/22	Pantano D'Arci	42.595,2 m3/24h	9 mg/L	Refrigerated (fresh)	5±3°C	detected	9,863	2,47E+04	0,63	41,4
28/4/22	Giarre	3.334,25 m3/24h	20 mg/L	Refrigerated (fresh)	5±3°C	detected	13,568	3,38E+04	1,19	96,3
28/4/2022	Siracusa	37.956 m3/24h	540 mg/L	Refrigerated (fresh)	5±3°C	detected	17,675	4,42E+04	1,21	79,8
3/5/22	Pantano D'Arci	42.854,4 m3/24h	16 mg/L	Frozen	-20°C	detected	65,7	1,64E+05	0,1	96,5
5/5/22	Pantano D'Arci	43.545,6 m3/24h	26 mg/L	Refrigerated (fresh)	5±3°C	detected	68,829	1,72E+05	0,79	98,8
5/5/22	Giarre	2.671,25 m3/24h	110 mg/L	Refrigerated (fresh)	5±3°C	detected	113	2,83E+05	0,19	99,5
5/5/22	Siracusa	36.212 m3/24h	104 mg/L	Refrigerated (fresh)	5±3°C	detected	38,674	9,67E+04	0,67	82,7
10/5/22	Pantano D'Arci	42.768 m3/24h	19 mg/L	Frozen	-20°C	detected	64,354	1,61E+05	0,34	98
12/5/22	Pantano D'Arci	49.973,76 m3/24h	10 mg/L	Refrigerated (fresh)	5±3°C	detected	18,822	4,71E+04	0,91	78,8
12/5/22	Giarre	2.290,75 m3/24h	101 mg/L	Refrigerated (fresh)	5±3°C	detected	29,1	7,28E+04	0,53	91,6
12/5/22	Siracusa	38.758 m3/24h	140 mg/L	Refrigerated (fresh)	5±3°C	detected	23,368	5,84E+04	0,61	70,9
17/5/22	Pantano D'Arci	47.701,44 m3/24h	5 mg/L	Frozen	-20°C	detected	9,336	2,33E+04	0,05	66.5
19/5/22	Pantano D'Arci	43.718 m3/24h	2 mg/L	Refrigerated (fresh)	5±3°C	detected	4,048	1,01E+04	0,1	19,5

19/5/22	Giarre	2.666,5 m3/24h	80 mg/L	Refrigerated (fresh)	5±3°C	detected	4,52	1,13E+04	0,46	26,3
19/5/22	Siracusa	37.554,1 m3/24h	336 mg/L	Refrigerated (fresh)	5±3°C	detected	5,262	1,32E+04	0,22	39,7
24/5/22	Pantano D'Arci	47.278,08 m3/24h	100 mg/L	Frozen	-20°C	detected	12,903	3,23E+04	1,15	66,3
26/5/22	Pantano D'Arci	40.685,76 m3/24h	2 mg/L	Refrigerated (fresh)	5±3°C	detected	12,197	3,05E+04	1,95	65
26/5/22	Giarre	4.222,66 m3/24h	38 mg/L	Refrigerated (fresh)	5±3°C	detected	13,947	3,49E+04	1,23	72,3
26/5/22	Siracusa	38.860 m3/24h	592 mg/L	Refrigerated (fresh)	5±3°C	detected	30,867	7,72E+04	1,5	91,3
31/5/22	Pantano D'Arci	48.643,2 m3/24h	11 mg/L	Refrigerated (fresh)	5±3°C	detected	40,366	1,01E+05	0,68	96
31/5/22	Giarre	35.673 m3/24h	66 mg/L	Refrigerated (fresh)	5±3°C	detected	26,611	6,65E+04	1,21	85,5
31/5/22	Siracusa	39.896 m3/24h	152 mg/L	Refrigerated (fresh)	5±3°C	detected	53,287	1,33E+05	0,92	77,6
1/6/22	Pantano D'Arci	54.432 m3/24h	1 mg/L	Frozen	-20°C	detected	15,101	3,78E+04	0,63	83,1
7/6/22	Pantano D'Arci	49.481,28 m3/24h	1 mg/L	Refrigerated (fresh)	5±3°C	detected	6,73	1,68E+04	0,25	49,5
9/6/22	Pantano D'Arci	51.580,8 m3/24h	15 mg/L	Refrigerated (fresh)	5±3°C	detected	25,065	6,27E+04	0,14	73,8
9/6/22	Giarre	31.614,25 m3/24h	73 mg/L	Refrigerated (fresh)	5±3°C	detected	15,689	3,92E+04	0,47	97
9/6/22	Siracusa	39.722 m3/24h	116 mg/L	Refrigerated (fresh)	5±3°C	detected	10,015	2,50E+04	0,67	87,6
14/6/22	Pantano D'Arci	52.237,44 m3/24h	2 mg/L	Frozen	-20°C	detected	40,226	1,01E+05	0,1	65
16/6/22	Pantano D'Arci	48.850,56 m3/24h	7 mg/L	Refrigerated (fresh)	5±3°C	detected	41,636	1,04E+05	1,34	80,7
16/6/22	Giarre	2.775 m3/24h	64 mg/L	Refrigerated (fresh)	5±3°C	detected	74,563	1,86E+05	1,17	76,4
16/6/22	Siracusa	39.187 m3/24h	76 mg/L	Refrigerated (fresh)	5±3°C	detected	52,248	1,31E+05	1,58	77
21/6/22	Pantano D'Arci	51.321,6 m3/24h	6 mg/L	Frozen	-20°C	detected	21,876	5,47E+04	1,41	64,5
23/6/22	Pantano D'Arci	41.523,84 m3/24h	12 mg/L	Refrigerated (fresh)	5±3°C	detected	40,828	1,02E+05	1,28	64,8
23/6/22	Giarre	2.894 m3/24h	51 mg/L	Refrigerated (fresh)	5±3°C	detected	29,111	7,28E+04	1,42	52,2

23/6/22	Siracusa	39.399 m3/24h	296 mg/L	Refrigerated (fresh)	5±3°C	detected	53,449	1,34E+05	1,36	88,9
28/6/22	Pantano D'Arci	48.047,04 m3/24h	15 mg/L	Frozen	-20°C	detected	4,085	1,02E+04	1,99	53,9
30/6/22	Pantano D'Arci	48.504,96 m3/24h	20 mg/L	Refrigerated (fresh)	5±3°C	detected	25,752	6,44E+04	0,54	67
30/6/22	Giarre	3.101,25 m3/24h	81 mg/L	Refrigerated (fresh)	5±3°C	detected	6,564	1,64E+04	1,88	51,4
30/6/22	Siracusa	39.738 m3/24h	580 mg/L	Refrigerated (fresh)	5±3°C	detected	35,178	8,79E+04	0,36	54,4
05/7/22	Pantano D'Arci	51.719,04 m3/24h	16 mg/L	Frozen	-20°C	detected	12,013	3,00E+04	0,52	37,6
07/7/22	Pantano D'Arci	49.438,08 m3/24h	19 mg/L	Refrigerated (fresh)	5±3°C	detected	10,201	2,55E+04	0,23	32,6
06/7/22	Giarre	2.858 m3/24h	58 mg/L	Frozen	-20°C	detected	12,801	3,20E+04	0,55	39,7
07/7/22	Siracusa	38.595 m3/24h	320 mg/L	Refrigerated (fresh)	5±3°C	detected	17,868	4,47E+04	0,07	53,1
12/7/22	Pantano D'Arci	48.885,12 m3/24h	17 mg/L	Frozen	-20°C	detected	16,335	4,08E+04	0,53	46
14/7/22	Pantano D'Arci	46.759,68 m3/24h	25 mg/L	Refrigerated (fresh)	5±3°C	detected	24,72	6,18E+04	-0,12	82,8
14/7/22	Giarre	2.821,75 m3/24h	30 mg/L	Refrigerated (fresh)	5±3°C	detected	11,526	2,88E+04	-0,39	27,6
14/7/22	Siracusa	38.315 m3/24h	144 mg/L	Refrigerated (fresh)	5±3°C	detected	23,374	5,84E+04	-0,15	77,5
19/7/22	Pantano D'Arci	40.020,48 m3/24h	8 mg/L	Frozen	-20°C	detected	7,066	1,77E+04	0,79	13,3
19/7/22	Giarre	2.973,75 m3/24h	70 mg/L	Frozen	-20°C	detected	9,977	2,49E+04	0,93	22,4
21/7/22	Pantano D'Arci	49.967,04 m3/24h	29 mg/L	Refrigerated (fresh)	5±3°C	detected	11,218	2,80E+04	0,67	26,1
21/7/22	Siracusa	38.327 m3/24h	756 mg/L	Refrigerated (fresh)	5±3°C	detected	9,327	2,33E+04	1,07	20,2
26/7/22	Pantano D'Arci	42.230,4 m3/24h	35 mg/L	Frozen	-20°C	detected	11,778	2,94E+04	0,48	29,6
28/7/22	Pantano D'Arci	45.230,4 m3/24h	21 mg/L	Refrigerated (fresh)	5±3°C	detected	9,453	2,36E+04	1,01	24,3
28/7/22	Giarre	2.091,25 m3/24h	70 mg/L	Refrigerated (fresh)	5±3°C	detected	10,309	2,58E+04	-1,87	26,1
28/7/22	Siracusa	39.943 m3/24h	680 mg/L	Refrigerated (fresh)	5±3°C	detected	9,241	2,31E+04	0,57	23,7

02/8/22	Pantano D'Arci	43.087,68 m3/24h	20 mg/L	Frozen	-20°C	detected	14,828	3,71E+04	-1,4	38,3
04/8/22	Pantano D'Arci	43.683,84 m3/24h	20 mg/L	Refrigerated (fresh)	5±3°C	detected	13,088	3,27E+04	-0,22	22,7
03/8/22	Giarre	2.103,25 m3/24h	62 mg/L	Frozen	-20°C	detected	15,536	3,88E+04	0,34	41,4
04/8/22	Siracusa	38.116 m3/24h	164 mg/L	Refrigerated (fresh)	5±3°C	detected	50,487	1,26E+05	-0,56	89
09/8/22	Pantano D'Arci	41.005,44 m3/24h	20 mg/L	Frozen	-20°C	detected	3,93	9,84E+03	0,4	30,3
11/8/22	Pantano D'Arci	43.338,24 m3/24h	22 mg/L	Frozen	-20°C	detected	14,62	3,66E+04	0,11	97,1
11/8/22	Giarre	2.630,75 m3/24h	80 mg/L	Frozen	-20°C	detected	10,81	2,70E+04	-0,02	64
11/8/22	Siracusa	40.262 m3/24h	164 mg/L	Frozen	-20°C	detected	10,63	2,66E+04	-0,13	73,7
16/8/22	Pantano D'Arci	42.111,36 m3/24h	20 mg/L	Frozen	-20°C	detected	9,95	2,49E+04	-0,6	58,1
18/8/22	Pantano D'Arci	39.286,08 m3/24h	60 mg/L	Frozen	-20°C	detected	24,35	6,09E+04	0,01	48,6
16/8/22	Giarre	2.366,50 m3/24h	40 mg/L	Frozen	-20°C	detected	15,65	3,91E+04	0,11	88
18/8/22	Siracusa	38.851 m3/24h	88 mg/L	Frozen	-20°C	detected	12,55	3,14E+04	-0,57	72
23/8/22	Pantano D'Arci	34.758,92 m3/24h	60 mg/L	Frozen	-20°C	detected	14,43	3,61E+04	-0,48	82,1
25/8/22	Pantano D'Arci	39.985,92 m3/24h	70 mg/L	Refrigerated (fresh)	5±3°C	detected	15,78	3,94E+04	-0,38	94,3
25/8/22	Giarre	2.824 m3/24h	70 mg/L	Refrigerated (fresh)	5±3°C	detected	21,81	5,45E+04	0,2	97,2
25/8/22	Siracusa	42.550 m3/24h	424 mg/L	Refrigerated (fresh)	5±3°C	detected	13,5	3,38E+04	-0,16	89,9
30/8/22	Pantano D'Arci	35.968,32 m3/24h	10 mg/L	Frozen	-20°C	detected	14,12	3,53E+04	-0,88	80
01/9/22	Pantano D'Arci	39.009,6 m3/24h	10 mg/L	Refrigerated (fresh)	5±3°C	detected	9,91	2,48E+04	0,43	57,3
01/9/22	Giarre	2.961,5 m3/24h	20 mg/L	Refrigerated (fresh)	5±3°C	detected	15,2	3,80E+04	0,55	85,3
01/9/22	Siracusa	41.359 m3/24h	132 mg/L	Refrigerated (fresh)	5±3°C	detected	22,78	5,69E+04	0,19	85
06/9/22	Pantano D'Arci	37.333,44 m3/24h	50 mg/L	Frozen	-20°C	non detected				

08/9/22	Pantano D'Arci	39.493,44 m3/24h	50 mg/L	Refrigerated (fresh)	5±3°C	non detected				
08/9/22	Giarre	2.623,25 m3/24h	10 mg/L	Refrigerated (fresh)	5±3°C	non detected				
08/9/22	Siracusa	38.330 m3/24h	132 mg/L	Refrigerated (fresh)	5±3°C	non detected				
13/9/22	Pantano D'Arci	38.059,2 m3/24h	50 mg/L	Refrigerated (fresh)	5±3°C	detected	0,74	1,85E+03	-0,6	19
13/9/22	Giarre	2.879,25 m3/24h	20 mg/L	Refrigerated (fresh)	5±3°C	non detected				
13/9/22	Siracusa	30.677 m3/24h	344 mg/L	Refrigerated (fresh)	5±3°C	non detected				
15/9/22	Pantano D'Arci	40.227,84 m3/24h	26 mg/L	Frozen	-20°C	detected	14,7	3,68E+04	-0,07	95,5
20/9/22	Pantano D'Arci	24.304,32 m3/24h	30 mg/L	Frozen	-20°C	detected	2,44	6,10E+03	-0,93	59,8
22/9/22	Pantano D'Arci	25.488 m3/24h	20 mg/L	Refrigerated (fresh)	5±3°C	detected	0,2	5,00E+02	0	9
22/9/22	Giarre	5.139,33 m3/24h	40 mg/L	Refrigerated (fresh)	5±3°C	detected	0,5	1,25E+03	0,23	16,4
22/9/22	Siracusa	46.127 m3/24h	204 mg/L	Refrigerated (fresh)	5±3°C	detected	2,17	5,43E+03	0,44	54,6
27/9/22	Pantano D'Arci	32.019,84 m3/24h	80 mg/L	Frozen	-20°C	detected	0,3	7,50E+02	-0,79	10,6
29/9/22	Pantano D'Arci	27.129,6 m3/24h	20 mg/L	Frozen	-20°C	detected	2,11	5,28E+03	-1,42	53,4
29/9/22	Giarre	3.382 m3/24h	30 mg/L	Frozen	-20°C	detected	0,96	2,40E+03	0,13	29,1
29/9/22	Siracusa	38.783 m3/24h	344 mg/L	Frozen	-20°C	detected	8,88	2,22E+04	-2,09	88,2
4/10/22	Pantano D'Arci	26.576,64 m3/24h	100 mg/L	Frozen	-20°C	detected	0,46	1,15E+03	-0,03	16,4
6/10/22	Pantano D'Arci	25.980,48 m3/24h	30 mg/L	Frozen	-20°C	detected	2,55	6,38E+03	1,13	75,1
6/10/22	Giarre	2.378,6 m3/24h	420 mg/L	Frozen	-20°C	detected	2,93	7,33E+03	0,11	68,9
6/10/22	Siracusa	34.115 m3/24h	200 mg/L	Frozen	-20°C	detected	0,26	6,50E+02	-0,1	10,7
11/10/22	Pantano D'Arci	23.431,68 m3/24h	180 mg/L	Frozen	-20°C	detected	2,29	5,73E+03	-1,61	13,5
13/10/22	Pantano D'Arci	24.131,52 m3/24h	100 mg/L	Refrigerated (fresh)	5±3°C	detected	3,33	8,33E+03	-1,22	76,1

13/10/22	Giarre	2.383,6 m3/24h	198 mg/L	Refrigerated (fresh)	5±3°C	detected	7,88	1,97E+04	-0,79	98,2
13/10/22	Siracusa	38.003 m3/24h	244 mg/L	Refrigerated (fresh)	5±3°C	detected	0,88	2,20E+03	-0,49	27,3
18/10/22	Pantano D'Arci	25.669 m3/24h	200 mg/L	Frozen	-20°C	detected	3,97	9,93E+03	-0,24	87,1
20/10/22	Pantano D'Arci	22.559 m3/24h	120 mg/L	Refrigerated (fresh)	5±3°C	detected	4,18	1,05E+04	-0,2	90,7
20/10/22	Giarre	2.816,3 m3/24h	240 mg/L	Refrigerated (fresh)	5±3°C	detected	2,17	5,43E+03	0,17	54,7
20/10/22	Siracusa	38.519 m3/24h	216 mg/L	Refrigerated (fresh)	5±3°C	detected	1,85	4,63E+03	-1,86	48,3
25/10/22	Pantano D'Arci	27.483,84 m3/24h	80 mg/L	Frozen	-20°C	detected	1,04	2,60E+03	0,19	30,9
27/10/22	Pantano D'Arci	23.604 m3/24h	110 mg/L	Refrigerated (fresh)	5±3°C	detected	0,24	6,00E+02	-0,04	9,8
27/10/22	Giarre	2.813 m3/24h	140 mg/L	Refrigerated (fresh)	5±3°C	detected	1,35	3,38E+03	-0,83	37,9
27/10/22	Siracusa	37.261 m3/24h	184 mg/L	Refrigerated (fresh)	5±3°C	detected	0,54	1,35E+03	-0,28	18,7
2/11/22	Pantano D'Arci	22.731,84 m3/24h	100 mg/L	Frozen	-20°C	detected	0,82	2,05E+03	-0,33	25,8
3/11/22	Pantano D'Arci	26.153,28 m3/24h	10 mg/L	Refrigerated (fresh)	5±3°C	detected	0,31	7,75E+02	-0,41	12,2
3/11/22	Giarre	2.544,33 m3/24h	110 mg/L	Refrigerated (fresh)	5±3°C	detected	4,1	1,03E+04	-0,45	91,8
3/11/22	Siracusa	38.708 m3/24h	108 mg/L	Refrigerated (fresh)	5±3°C	detected	2,73	6,83E+03	-1,01	65,3
8/11/22	Pantano D'Arci	22.351,68 m3/24h	50 mg/L	Frozen	-20°C	detected	4,13	1,03E+04	-0,77	89,8
10/11/22	Pantano D'Arci	24.408 m3/24h	80 mg/L	Refrigerated (fresh)	5±3°C	detected	4,18	1,05E+04	-1,12	91,1
09/11/22	Giarre	2119 m3/24h	50 mg/L	Frozen	-20°C	detected	4,94	1,24E+04	-1,04	98,4
10/11/22	Siracusa	36.820 m3/24h	212 mg/L	Refrigerated (fresh)	5±3°C	detected	1,79	4,48E+03	-0,11	47
15/11/22	Pantano D'Arci	24.062,4 m3/24h	60 mg/L	Frozen	-20°C	detected	4,11	1,03E+04	-0,2	89,6
17/11/22	Pantano D'Arci	25.047,36 m3/24h	20 mg/L	Refrigerated (fresh)	5±3°C	detected	3,2	8,00E+03	-0,07	73,8
17/11/22	Giarre	6.858,25 m3/24h	10 mg/L	Refrigerated (fresh)	5±3°C	detected	4,56	1,14E+04	-0,45	96,1

17/11/22	Siracusa	36.887 m3/24h	456 mg/L	Refrigerated (fresh)	5±3°C	detected	2,29	5,73E+03	-0,61	22,9
22/11/22	Pantano D'Arci	26.360,64 m3/24h	30 mg/L	Frozen	-20°C	detected	0,92	2,30E+03	-0,32	11
24/11/22	Pantano D'Arci	24.580,80 m3/24h	110 mg/L	Refrigerated (fresh)	5±3°C	detected	0,78	1,95E+03	-0,43	9,5
24/11/22	Giarre	1.642,5 m3/24h	130 mg/L	Refrigerated (fresh)	5±3°C	detected	3,16	7,90E+03	-0,36	29,2
24/11/22	Siracusa	38.509 m3/24h	80 mg/L	Refrigerated (fresh)	5±3°C	detected	7,26	1,82E+04	-0,26	58
29/11/22	Pantano D'Arci	77.941,44 m3/24h	80 mg/L	Frozen	-20°C	detected	6,96	1,74E+04	-0,21	56,1
01/12/22	Pantano D'Arci	26.714,88 m3/24h	110 mg/L	Refrigerated (fresh)	5±3°C	detected	0,48	1,20E+03	-0,27	6,5
01/12/22	Giarre	2.058,75 m3/24h	150 mg/L	Refrigerated (fresh)	5±3°C	detected	1,54	3,85E+03	-0,57	16,6
01/12/22	Siracusa	35.426 m3/24h	364 mg/L	Refrigerated (fresh)	5±3°C	non detected				
06/12/22	Pantano D'Arci	27.622,08 m3/24h	50 mg/L	Refrigerated (fresh)	5±3°C	detected	0,1	2,50E+02	-0,98	26,3
07/12/22	Pantano D'Arci	26.784 m3/24h	80 mg/L	Refrigerated (fresh)	5±3°C	detected	0,11	2,65E+02	-1	23,3
06/12/22	Giarre	2.757,3 m3/24h	40 mg/L	Refrigerated (fresh)	5±3°C	detected	0,06	1,60E+02	-0,81	18,3
07/12/22	Siracusa	38.038 m3/24h	132 mg/L	Refrigerated (fresh)	5±3°C	detected	0,1	2,38E+02	-1,1	24,8
13/12/22	Pantano D'Arci	27.242 m3/24h	30 mg/L	Frozen	-20°C	detected	0,18	4,38E+02	-1,03	39,6
15/12/22	Pantano D'Arci	26.378 m3/24h	90 mg/L	Refrigerated (fresh)	5±3°C	detected	0,12	2,90E+02	-0,81	28,8
14/12/22	Giarre	3.636 m3/24h	110 mg/L	Frozen	-20°C	detected	0,05	1,33E+02	-1,08	12,4
15/12/22	Siracusa	38.378 m3/24h	240 mg/L	Refrigerated (fresh)	5±3°C	detected	0,25	6,35E+02	-1,2	55,4
20/12/22	Pantano D'Arci	23.362,56 m3/24h	40 mg/L	Frozen	-20°C	detected	0,5	1,25E+03	-0,9	90
22/12/22	Pantano D'Arci	24.511,68 m3/24h	30 mg/L	Refrigerated (fresh)	5±3°C	detected	0,37	9,25E+02	-1,01	75,1
20/12/22	Giarre	3.625 m3/24h	60 mg/L	Frozen	-20°C	detected	0,6	1,51E+03	-1,16	87,4
22/12/22	Siracusa	37.659 m3/24h	332 mg/L	Refrigerated (fresh)	5±3°C	detected	0,79	1,97E+03	-0,62	67,9

27/12/22	Pantano D'Arci	21.824,64 m3/24h	30 mg/L	Frozen	-20°C	detected	0,36	8,99E+02	-0,24	37,5
29/12/22	Pantano D'Arci	20.148,48 m3/24h	40 mg/L	Frozen	-20°C	detected	0,1	2,52E+02	-0,61	14,5
27/12/22	Giarre	3.481,67 m3/24h	20 mg/L	Frozen	-20°C	detected	0,47	1,17E+03	-0,33	56
29/12/22	Siracusa	39.000 m3/24h	456 mg/L	Frozen	-20°C	detected	0,29	7,26E+02	-2,23	36,9
03/1/23	Pantano D'Arci	23.708,16 m3/24h	30 mg/L	Frozen	-20°C	detected	0,37	9,36E+02	-0,36	46,1
05/1/23	Pantano D'Arci	22.835,52 m3/24h	40 mg/L	Refrigerated (fresh)	5±3°C	detected	0,42	1,04E+03	-1,35	50,7
04/1/23	Giarre	1.773,33 m3/24h	60 mg/L	Refrigerated (fresh)	5±3°C	detected	0,27	6,70E+02	-0,18	34,4
05/1/23	Siracusa	38.091 m3/24h	248 mg/L	Refrigerated (fresh)	5±3°C	detected	0,35	8,68E+02	-0,22	43,2
10/1/23	Pantano D'Arci	22.584,96 m3/24h	170 mg/L	Frozen	-20°C	detected	0,26	6,54E+02	-0,12	33,6
12/1/23	Pantano D'Arci	21.703,68 m3/24h	60 mg/L	Refrigerated (fresh)	5±3°C	detected	0,32	7,88E+02	-0,9	39,7
11/1/23	Giarre	6.632,75 m3/24h	70 mg/L	Frozen	-20°C	detected	0,23	5,86E+02	-0,15	30,4
12/1/23	Siracusa	38.214 m3/24h	232 mg/L	Refrigerated (fresh)	5±3°C	detected	0,36	9,06E+02	-0,31	44,8
17/1/23	Pantano D'Arci	23.008,32 m3/24h	30 mg/L	Frozen	-20°C	detected	41,26	1,03E+05	0,4	47,1
19/1/23	Pantano D'Arci	24.062,4 m3/24h	160 mg/L	Refrigerated (fresh)	5±3°C	detected	50,92	1,27E+05	0,44	64
18/1/23	Giarre	2.076,66 m3/24h	30 mg/L	Frozen	-20°C	detected	46,57	1,16E+05	0,21	57,2
19/1/23	Siracusa	38.308 m3/24h	256 mg/L	Refrigerated (fresh)	5±3°C	detected	40,82	1,02E+05	0,31	48
24/1/23	Pantano D'Arci	22.032 m3/24h	18 mg/L	Frozen	-20°C	detected	43,92	1,10E+05	0,43	53
26/1/23	Pantano D'Arci	26.748 m3/24h	30 mg/L	Refrigerated (fresh)	5±3°C	detected	84,65	2,12E+05	0,67	72,8
24/1/23	Giarre	1.700 m3/24h	15 mg/L	Frozen	-20°C	detected	52,87	1,32E+05	1,06	64,7
26/1/23	Siracusa	37.486 m3/24h	292 mg/L	Refrigerated (fresh)	5±3°C	detected	43,37	1,08E+05	1,25	59,9
31/1/23	Pantano D'Arci	24.432 m3/24h	100 mg/L	Frozen	-20°C	detected	10,65	2,66E+04	-0,09	89,3

02/2/23	Pantano D'Arci	25.142 m3/24h	200 mg/L	Refrigerated (fresh)	5±3°C	detected	12,26	3,06E+04	-0,37	99,1
01/2/23	Giarre	1.814,5 m3/24h	130 mg/L	Frozen	-20°C	detected	9,25	2,31E+04	-0,11	78,8
02/2/23	Siracusa	38.175 m3/24h	392 mg/L	Refrigerated (fresh)	5±3°C	detected	13,79	3,45E+04	0,02	68,4
07/2/23	Pantano D'Arci	24.235 m3/24h	80 mg/L	Frozen	-20°C	detected	8,53	2,13E+04	0,68	71,9
13/2/23	Pantano D'Arci	30.275 m3/24h	100 mg/L	Refrigerated (fresh)	5±3°C	detected	8,21	2,05E+04	0,24	68,8
13/2/23	Giarre	3.637,66 m3/24h	150 mg/L	Refrigerated (fresh)	5±3°C	detected	8,55	2,14E+04	-0,2	72,1
13/2/23	Siracusa	49.275 m3/24h	1.460 mg/L	Refrigerated (fresh)	5±3°C	detected	9,31	2,33E+04	0,14	78,1
14/2/23	Pantano D'Arci	30.101,76 m3/24h	60 mg/L	Frozen	-20°C	detected	6,831	1,71E+04	0,45	54,6
16/2/23	Pantano D'Arci	26.153,28 m3/24h	20 mg/L	Refrigerated (fresh)	5±3°C	detected	10,451	2,61E+04	0,39	87,9
16/2/23	Siracusa	479,170 m3/24h	96 mg/L	Refrigerated (fresh)	5±3°C	detected	6,08	1,52E+04	0,88	49,1
14/2/23	Giarre	3.517,33 m3/24h	70 mg/L	Frozen	-20°C	detected	7,53	1,88E+04	-0,06	59,8
21/2/23	Pantano D'Arci	19.872 m3/24h	140 mg/L	Frozen	-20°C	detected	9,018	2,25E+04	0,06	73,5
23/2/23	Pantano D'Arci	21.125 m3/24h	30 mg/L	Refrigerated (fresh)	5±3°C	detected	6,533	1,63E+04	-0,38	53,2
20/2/23	Giarre	2.488,66 m3/24h	100 mg/L	Frozen	-20°C	detected	11,632	2,91E+04	0,27	94,7
23/2/23	Siracusa	43.906 m3/24h	316 mg/L	Refrigerated (fresh)	5±3°C	detected	8,875	2,22E+04	0,23	75,1
28/2/23	Pantano D'Arci	21.859,2 m3/24h	20 mg/L	Frozen	-20°C	detected	5,07	1,27E+04	0,97	39,9
02/3/23	Pantano D'Arci	21.479,04 m3/24h	10 mg/L	Refrigerated (fresh)	5±3°C	detected	5,29	1,32E+04	1,81	39
28/2/23	Giarre	1.197,25 m3/24h	40 mg/L	Frozen	-20°C	detected	6,84	1,71E+04	1,93	56
03/3/23	Siracusa	41.263 m3/24h	108 mg/L	Refrigerated (fresh)	5±3°C	detected	7,4	1,85E+04	1,91	61
07/3/23	Pantano D'Arci	22.731,84 m3/24h	100 mg/L	Frozen	-20°C	detected	6,19	1,55E+04	0,35	49,3
09/3/23	Pantano D'Arci	24.797 m3/24h	60 mg/L	Refrigerated (fresh)	5±3°C	detected	4,56	1,14E+04	0,11	32,9

07/3/23	Giarre	1.765,5 m3/24h	80 mg/L	Frozen	-20°C	detected	6,47	1,62E+04	0,68	52,7
09/3/23	Siracusa	41.396 m3/24h	204 mg/L	Refrigerated (fresh)	5±3°C	detected	14,4	3,60E+04	1,05	69,4
14/3/23	Pantano D'Arci	22.766 m3/24h	70 mg/L	Frozen	-20°C	detected	5,45	1,36E+04	1,44	43,3
16/3/23	Pantano D'Arci	21.997,44 m3/24h	50mg/L	Refrigerated (fresh)	5±3°C	detected	16,4	4,10E+04	0,15	88,3
16/3/23	Giarre	1.928,75 m3/24h	120 mg/L	Refrigerated (fresh)	5±3°C	detected	11,09	2,77E+04	0,31	94,8
17/3/23	Siracusa	41.045 m3/24h	224 mg/L	Refrigerated (fresh)	5±3°C	detected	7,93	1,98E+04	0,08	65,4
21/3/23	Pantano D'Arci	23.880,96 m3/24h	20 mg/L	Frozen	-20°C	detected	6,26	1,57E+04	0,1	50,4
23/3/23	Pantano D'Arci	27.241,92 m3/24h	10 mg/L	Refrigerated (fresh)	5±3°C	detected	6,67	1,67E+04	-0,07	52,7
23/3/23	Giarre	2.719,75 m3/24h	30 mg/L	Refrigerated (fresh)	5±3°C	detected	6,91	1,73E+04	-0,31	55,5
24/3/23	Siracusa	39.440 m3/24h	576 mg/L	Refrigerated (fresh)	5±3°C	detected	5,79	1,45E+04	-0,3	44,2
28/3/23	Pantano D'Arci	26.542 m3/24h	470 mg/L	Frozen	-20°C	detected	4,85	1,21E+04	-0,11	35,9
30/3/23	Pantano D'Arci	27.657 m3/24h	500 mg/L	Refrigerated (fresh)	5±3°C	detected	4,1	1,03E+04	-0,03	31,2
29/3/23	Giarre	2.856,25 m3/24h	430 mg/L	Frozen	-20°C	detected	5,09	1,27E+04	0,05	39,6
31/3/23	Siracusa	39.779 m3/24h	244 mg/L	Frozen	-20°C	detected	4,97	1,25E+04	0,65	76,8
04/4/23	Pantano D'Arci	24.401,92 m3/24h	170 mg/L	Refrigerated (fresh)	5±3°C	detected	8,42	2,10E+04	0,28	68,7
06/4/23	Giarre	2.624 m3/24h	70 mg/L	Refrigerated (fresh)	5±3°C	detected	7,21	1,80E+04	0,6	58,8
03/4/23	Siracusa	40.527 m3/24h	604 mg/L	Frozen	-20°C	detected	10,15	2,54E+04	0,44	83,1
09/5/23	Pantano D'Arci	28.641,6 m3/24h	150 mg/L	Refrigerated (fresh)	5±3°C	detected	8,06	2,01E+04	1,09	67,1
08/5/23	Giarre	2.744 m3/24h	90 mg/L	Frozen	-20°C	detected	5,9	1,47E+04	0,97	47,4
10/5/23	Siracusa	40.524 m3/24h	328 mg/L	Refrigerated (fresh)	5±3°C	detected	9,12	2,28E+04	0,62	75,9
08/6/23	Pantano D'Arci	40.504,32 m3/24h	80 mg/L	Frozen	-20°C	detected	4,69	1,17E+04	0,12	34

09/6/23	Giarre	2.248 mg3/24h	400 mg/L	Frozen	-20°C	detected	3,89	9,73E+03	0,91	26,3
07/6/23	Siracusa	39.163 m3/24h	840 mg/L	Frozen	-20°C	detected	4,52	1,13E+04	1,26	30,8
05/7/23	Pantano D'Arci	36.279,36 m3/24h	140 mg/L	Frozen	-20°C	detected	5,75	1,44E+04	1,26	46,1
03/7/23	Giarre	3.523,33 m3/24h	300 mg/L	Frozen	-20°C	detected	5,92	1,48E+04	1,93	46,5
06/7/23	Siracusa	39.562 m3/24h	84 mg/L	Refrigerated (fresh)	5±3°C	detected	5,97	1,49E+04	1,94	48,1
08/8/23	Pantano D'Arci	32.788,8 m3/24h	200 mg/L	Frozen	-20°C	detected	3,64	9,09E+03	0,74	27,5
01/8/23	Giarre	5.530 m3/24h	380 mg/L	Frozen	-20°C	detected	3,13	7,83E+03	1,69	22,9
02/8/23	Siracusa	39.065 m3/24h	1384 mg/L	Frozen	-20°C	detected	3,7	9,25E+03	1,69	25,3
11/9/23	Pantano D'Arci	23.224,32 m3/24h	120 mg/L	Refrigerated (fresh)	5±3°C	detected	5,78	1,45E+04	1,38	46,3
08/9/23	Giarre	2.693,25 m3/24h	200 mg/L	Frozen	-20°C	detected	5,97	1,49E+04	0,91	48,1
11/9/23	Siracusa	36.526 m3/24h	152 mg/L	Refrigerated (fresh)	5±3°C	detected	4	1,00E+04	0,87	30,3
03/10/23	Pantano D'Arci	25.090,56 m3/24h	10 mg/L	Frozen	-20°C	detected	5,95	1,49E+04	1,28	47,7
02/10/23	Giarre	2.491,75 m3/24h	20 mg/L	Frozen	-20°C	detected	5,24	1,31E+04	1,51	40,2
05/10/23	Siracusa	37.985 m3/24h	176 mg/L	Refrigerated (fresh)	5±3°C	detected	5,22	1,30E+04	0,08	36,1
07/11/23	Pantano D'Arci	20.321,28 m3/24h	160 mg/L	Frozen	-20°C	detected	4,36	1,09E+04	1,47	33,3
07/11/23	Giarre	2.717 m3/24h	60 mg/L	Frozen	-20°C	detected	5,65	1,41E+04	0,53	44,9
13/11/23	Siracusa	34.442 m3/24h	140 mg/L	Frozen	-20°C	detected	1,33	3,32E+03	1,44	8,9
05/12/23	Pantano D'Arci	21.893,76 m3/24h	120 mg/L	Frozen	-20°C	detected	5,11	1,28E+04	1,62	36,9
05/12/23	Giarre	2.005,5 m3/24h	230 mg/L	Frozen	-20°C	detected	17,35	4,34E+04	1,44	78
06/12/23	Siracusa	34.598 m3/24h	88 mg/L	Frozen	-20°C	detected	12,72	3,18E+04	0,29	70,7
09/1/24	Pantano D'Arci	21.790,08 m3/24h	200 mg/L	Frozen	-20°C	detected	5,12	1,28E+04	1,95	41,1

08/1/24	Giarre	2.079,5 m3/24h	130 mg/L	Frozen	-20°C	detected	3,45	8,63E+03	1,79	26
15/1/24	Siracusa	39.065 m3/24h	1384 mg/L	Refrigerated (fresh)	5±3°C	detected	5,59	1,40E+04	1,96	42,6
08/2/24	Pantano D'Arci	21.824,64 m3/24h	100 mg/L	Refrigerated (fresh)	5±3°C	detected	5,84	1,46E+04	1,23	45,8
06/2/24	Giarre	12.618 m3/24h	150 mg/L	Frozen	-20°C	detected	1,54	3,86E+03	0,44	10,3
06/2/24	Siracusa	33.829 m3/24h	316 mg/L	Frozen	-20°C	detected	2,24	5,59E+03	0,58	15,7
05/3/24	Pantano D'Arci	18.895,68 m3/24h	190 mg/L	Frozen	-20°C	non detected				
04/3/24	Giarre	3.078,75 m3/24h	140 mg/L	Frozen	-20°C	detected	1,65	4,14E+03	1,35	8,5
01/3/24	Siracusa	1.570 m3/24h	104 mg/L	Frozen	-20°C	detected	0,66	1,66E+03	1,33	4
04/4/24	Pantano D'Arci	29.021,76 m3/24h	110 mg/L	Frozen	-20°C	non detected				
03/4/24	Giarre	3.092,75 m3/24h	60 mg/L	Frozen	-20°C	non detected				
04/4/24	Siracusa	35.695 m3/24h	204 mg/L	Frozen	-20°C	detected	0,39	9,65E+02	1,96	2,2
07/5/24	Pantano D'Arci	32.771,52 m3/24h	200 mg/L	Refrigerated (fresh)	5±3°C	detected	1,91	4,77E+03	0,58	11,4
03/5/24	Giarre	2.730 m3/24h	140 mg/L	Frozen	-20°C	detected	2,02	5,04E+03	0,97	14,2
02/5/24	Siracusa	36.778 m3/24h	300 mg/L	Frozen	-20°C	detected	1,65	4,13E+03	1,25	11,3
04/6/24	Pantano D'Arci	31.847,04 m3/24h	40 mg/L	Frozen	-20°C	detected	0,93	2,33E+03	0,94	5,3
03/6/24	Giarre	2.793 m3/24h	140 mg/L	Frozen	-20°C	detected	0,44	1,10E+03	1,19	2,5
03/6/24	Siracusa	36.971 m3/24h	332 mg/L	Frozen	-20°C	detected	1,15	2,89E+03	1,66	6,1
02/7/24	Pantano D'Arci	43.649,28 m3/24h	80 mg/L	Frozen	-20°C	detected	1,48	3,71E+03	1,9	10
02/7/24	Giarre	2.728,75 m3/24h	170 mg/L	Frozen	-20°C	detected	2,48	6,19E+03	1,99	17
01/7/24	Siracusa	34.477 m3/24h	120 mg/L	Frozen	-20°C	detected	0,61	1,54E+03	1,59	3,7
01/8/24	Pantano D'Arci	39.847,68 m3/24h	90 mg/L	Frozen	-20°C	detected	6,23	1,56E+04	1,23	50

02/8/24	Giarre	2.276,5 m3/24h	70 mg/L	Frozen	-20°C	detected	6,58	1,65E+04	1,64	53,3
02/8/24	Siracusa	37.436 m3/24h	172 mg/L	Frozen	-20°C	detected	9,54	2,39E+04	1,95	79,8
04/9/24	Pantano D'Arci	40.996,8 m3/24h	80 mg/L	Refrigerated (fresh)	5±3°C	detected	1,58	3,95E+03	0,75	8
04/9/24	Giarre	1.457,25 m3/24h	170 mg/L	Refrigerated (fresh)	5±3°C	detected	4,25	1,06E+04	1,96	31,8
02/9/24	Siracusa	37.768 m3/24h	304 mg/L	Frozen	-20°C	detected	3,26	8,14E+03	0,67	23,7

Table 3. Standard thermal-cycling profile

	(*Optional*) UNG incubation[1]	Polymerase activation[2,3]	PCR (40 cycles)	
Real-time PCR instrument	**Hold 50°C**	**Hold 95°C**	**Denature 95°C**	**Anneal/extend 60°C**
QuantStudio™ Dx Real-Time PCR Instrument	2 minutes	20 seconds	15 seconds	1 minute

[1] Heat-labile UNG is active during the reaction setup and does not require a 50°C incubation for UNG activity. Product performance was validated with the optional 50°C UNG incubation step.
[2] The polymerase activation/initial denaturation can be increased up to 10 minutes for templates that are difficult to denature.
[3] Heat-labile UNG is completely inactivated during the first ramp to the 95°C hold step.

Figure 2. Selection of studies included in the scoping review "The Impact of Wastewater on Antimicrobial Resistance: Transmission Pathways and Contributing Factors."

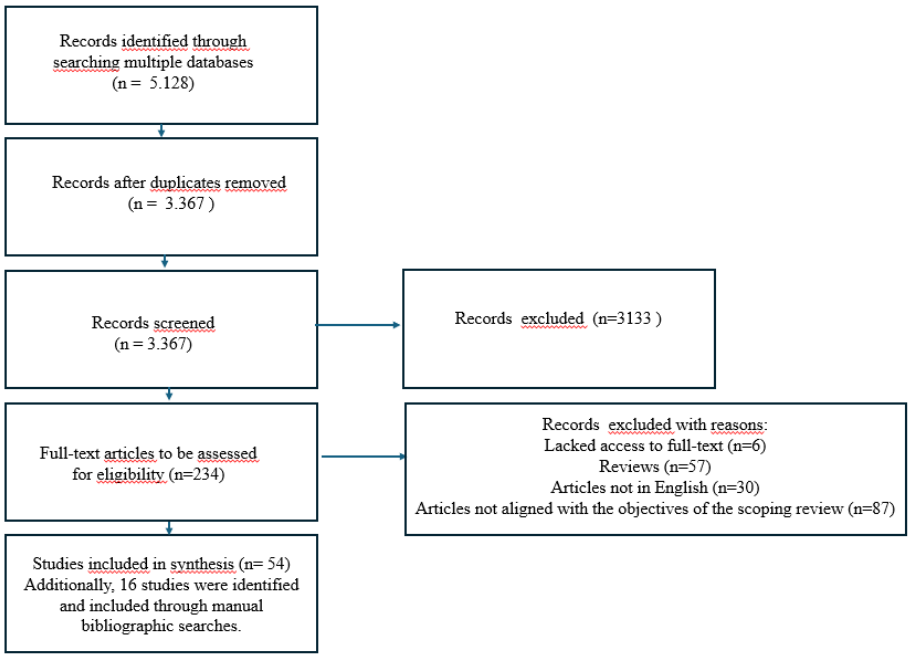


Figure 3. Selection of studies included in the scoping review "Updates on developing and applying biosensors for the detection of microorganisms, antimicrobial resistance genes and antibiotics: a scoping review."

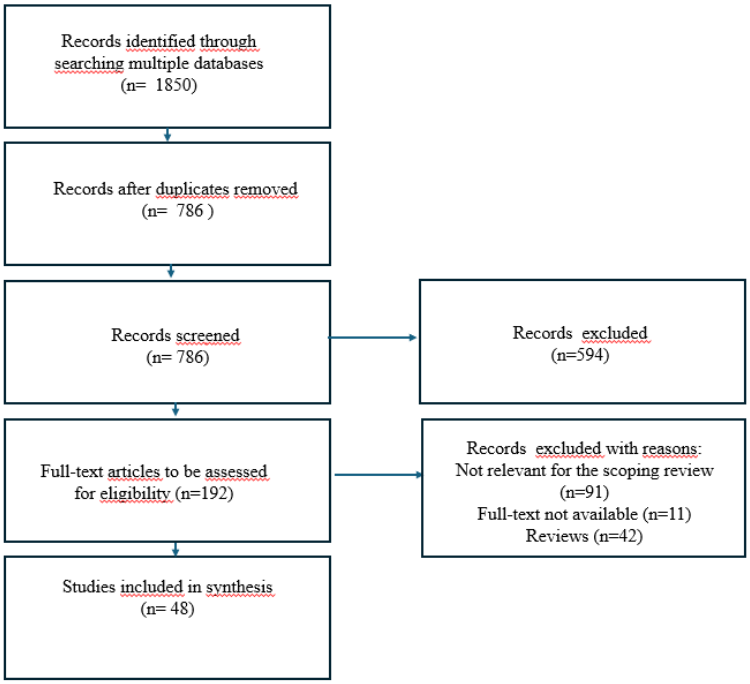


Table 4. Thermocycler conditions for quantification of the different ARGs by qPCR

		aadA	*aadB*	*ampC*	*bla* SHV	*bla* TEM	*dfrA* 1	*ermB*	*mecA*	*qnrS*	*tetA* (A)
Initial denaturalization		95 °C, 10 min	95 °C, 10 min	95 °C, 10 min	95 °C, 10 min	95 °C, 10 min	95° C, 10 min	95 °C, 10 min	95 °C, 10 min	95 °C, 10 min	95 °C, 10 min
Amplification (× 35 cycles)	Denaturalization	95 °C, 30 s	95 °C, 30 s	95 °C, 30 s	95 °C, 15 s	95 °C, 15 s	95 °C, 15 s	95 °C, 15 s	95 °C, 30 s	95 °C, 15 s	95 °C, 30 s
	Annealing	60 °C, 30 s	60 °C, 30 s	60 °C, 30 s	55 °C, 30 s	60 °C, 30 s	55 °C, 30 s	55 °C, 30 s	50 °C, 30 s	55 °C, 30 s	55 °C, 30 s
	Elongation	72 °C, 40 s	72 °C, 40 s	72 °C, 40 s	72 °C, 40 s	72 °C, 40 s	72 °C, 40 s	72 °C, 40 s	72 °C, 40 s	72 °C, 40 s	72 °C, 40 s
Melting curve		60 °C–95 °C + 0.15 °C/s. Fluorescence measured each 15 s									

12. OTHER RESEARCH ACTIVITIES

— Periodic team meetings for planning activities and defining protocols to be adopted within the Research Project;

— I conducted lectures and training sessions on "Preventive Interventions in Primary Care: Optional and Mandatory Vaccinations" for the 1st-year curriculum (2021–2024 cycle) of the School of Specialized Training in General Medicine (Course Code: MMG2022CT1; CdC: 03011101) at Azienda Ospedaliera Cannizzaro, Catania;

— Oral presentation at the 55th National Congress of SItI, “RERUM COGNOSCERE CAUSAS: costruire saperi e competenze in prospettiva One Health” titled "Epidemiological and Molecular Characterization of *Acinetobacter baumannii* during the COVID-19 Pandemic," at the Padua Congress Center, September 28 – October 1, 2022;

— Participation in the educational and seminar activities provided by the PhD course, titled: i) “Liquid Biopsy: An innovative multimodal tool in molecular pathology and clinical oncology,” Dr. Michele Massimino ii) “Understanding clinical trial design in molecular pathology and clinical oncology,” Prof. Stefania Stella iii) “AIRC: the path of research. When DNA is not enough: how matrix stiffness influences tumor progression,” Prof. Sirio Dupont iv) “AIRC: the path of research. Innate lymphoid cells in inflammation and colorectal cancer,” Dr. Giuseppe Sciumè v) Eosinophilic Immune Dysfunction and Respiratory Diseases,” Dr. Raffaele Campisi vi). “Design and Conduct of Clinical Research,” Dr. Raffaele Campisi vii) “Molecular Mechanisms of Cancer Cell Plasticity, Immune Evasion and Organ Metastasis,” Prof.Tony B. D'Assoro vii). “Combination of Stemness-Targeted Therapy with Immune Checkpoint Inhibitors in Metastatic Triple Negative Breast Cancer,” Prof.Tony B. D'Assoro

— Participation, via the Microsoft Teams platform, in a 40-hour English language course organized by the University Language Center for PhD students of the University of Catania, and successful completion of the internal course exam

— Qualified tutoring activities for the Bachelor's Degree in Biological Sciences – Course "Hygiene and Statistics," Notice no. 2051 of 20/06/2022.

— From 13 to 17 April 2024 participation as a learner in the LXI COURSE *"Adopting a One Health strategy to combat HAIs and AMR: the priorities and challenges of Public Health"* held in Erice at the “Ettore Majorana” Foundation Center for Scientific Culture. The course aimed to present the current epidemiological scenario of HAIs and AMR at European and national level, the main factors determining these phenomena and the counteractions identified in the PNCAR 2022-2025. It also described the state of the art on the One Health strategy to combat AMR, reflecting on the challenges and priorities for Public Health

- Junior tutoring activities for the Bachelor's Degree in Biological Sciences – Course titled 'Hygiene and Statistics,' Notice No. 2196 dated 30/05/2023.

- Participation in the Interregional Siculo-Calabro Congress of the Italian Society of Hygiene, Preventive Medicine and Public Health (Società Italiana di Igiene, Medicina Preventiva e Sanità Pubblica, S.It.I.), held in Enna from the 23rd to the 25th of May 2024. To participate in this event, I have prepared a poster titled 'Monitoring of SARS-CoV-2 and Antimicrobial Resistance through Wastewater.

- Participation in the G7 Agriculture and Fisheries Day held at the Syracuse venue.

- September - October 2024: Participation in the REUNICE Certificate Programme-Sustainability Management.

- Participation in the 57th National Congress of SItI, “Public Health for the Future of the Country: Innovation, Alliances, and Institutional Synergies for Prevention,” at the University of Palermo, October 23-26, 2024.

List of scientific publications

Scientific articles from November 2021

- Barchitta M, Maugeri A, **La Rosa MC**, La Mastra C, Murolo G, Corrao G, Agodi A. Burden of Healthcare-Associated Infections in Sicily, Italy: Estimates from the Regional Point Prevalence Surveys 2016-2018. Antibiotics (Basel). 2021 Nov 8;10(11):1360. doi: 10.3390/antibiotics10111360. PMID: 34827298; PMCID: PMC8614974.

-Maugeri A, Barchitta M, Magnano San Lio R, Favara G, La Mastra C, **La Rosa MC**, Agodi A. The Relationship between Body Mass Index, Obesity, and LINE-1 Methylation: A Cross-Sectional Study on Women from Southern Italy. Dis Markers. 2021 Dec 3;2021:9910878. doi: 10.1155/2021/9910878. PMID: 34900031; PMCID: PMC8664509.

- Maugeri A, Magnano San Lio R, **La Rosa MC,** Giunta G, Panella M, Cianci A, Caruso MAT, Agodi A, Barchitta M. The Relationship between Telomere Length and Gestational Weight Gain: Findings from the Mamma & Bambino Cohort. Biomedicines. 2021 Dec 30;10(1):67. doi: 10.3390/biomedicines10010067. PMID: 35052747; PMCID: PMC8773008.

- Ojeda-Granados C, Barchitta M, **La Rosa MC**, La Mastra C, Roman S, Panduro A, Agodi A, Maugeri A. Evaluating Dietary Patterns in Women from Southern Italy and Western Mexico. Nutrients. 2022 Apr 12;14(8):1603. doi: 10.3390/nu14081603. PMID: 35458165; PMCID: PMC9027885.

- Magnano San Lio R, Maugeri A, **La Rosa MC**, Giunta G, Panella M, Cianci A, Caruso MAT, Agodi A, Barchitta M. Nutrient intakes and telomere length of cell-free circulating DNA from amniotic fluid: findings from the Mamma & Bambino cohort. Sci Rep. 2022;12(1):11671. Published 2022 Jul 8. doi:10.1038/s41598-022-15370-9.

- Magnano San Lio R, Barchitta M, Maugeri A, **La Rosa MC**, Giunta G, Panella M, Cianci A, Galvani F, Pappalardo E, Ettore G, Agodi A. The Impact of the COVID-19 Pandemic on Dietary Patterns of Pregnant Women: A Comparison between Two Mother-Child Cohorts in Sicily, Italy. Nutrients. 2022 Aug 17;14(16):3380. doi: 10.3390/nu14163380. PMID: 36014886; PMCID: PMC9416210.

- Maugeri A, Magnano San Lio R, Favara G, **La Rosa MC**, La Mastra C, Riela PM, Guarnera L, Battiato S, Barchitta M, Agodi A. Impact of Eating Context on Dietary Choices of College Students: Evidence from the HEALTHY-UNICT Project. Nutrients. 2022 Oct 21;14(20):4418. doi: 10.3390/nu14204418. PMID: 36297101; PMCID: PMC9609717.

-Maugeri A, Barchitta M, Favara G, La Mastra C, **La Rosa MC**, Magnano San Lio R, Agodi A. The Application of Clustering on Principal Components for Nutritional Epidemiology: A Workflow to Derive Dietary Patterns. Nutrients. 2022 Dec 30;15(1):195. doi: 10.3390/nu15010195. PMID: 36615850; PMCID: PMC9824338.

- Agodi A, Montineri A, Manuele R, Noto P, Carpinteri G, Castiglione G, Grassi P, Lazzara A, Mattaliano AR, Granvillano G, La Mastra C, **La Rosa MC**, Maugeri A, Barchitta M. Molecular Typing and Resistance Profile of Acinetobacter baumannii Isolates during the COVID-19 Pandemic: Findings from the "EPIRADIOCLINF" Project (2023) Antibiotics, DOI: 10.3390/antibiotics12101551.

- Magnano San Lio R, Barchitta M, Maugeri A, **La Rosa MC**, Favara G, Agodi A. Updates on developing and applying biosensors for the detection of microorganisms, antimicrobial resistance genes and antibiotics: a scoping review. Front Public Health. 2023 Sep 7;11:1240584. doi: 10.3389/fpubh.2023.1240584. PMID: 37744478; PMCID: PMC10512422.

- Barchitta M, Maugeri A, Favara G, Lio RMS, **La Rosa MC**, D'Ancona F, Agodi A; SPIN-UTI network. The intertwining of healthcare-associated infections and COVID-19 in Italian intensive care units: an analysis of the SPIN-UTI project from 2006 to 2021. J Hosp Infect. 2023 Oct;140:124-131. doi: 10.1016/j.jhin.2023.07.021. Epub 2023 Aug 9. PMID: 37562591.

- Barchitta M, Magnano San Lio R, **La Rosa MC**, La Mastra C, Favara G, Ferrante G, Galvani F, Pappalardo E, Ettore C, Ettore G, Agodi A, Maugeri A. The Effect of Maternal Dietary Patterns on Birth Weight for Gestational Age: Findings from the MAMI-MED Cohort. Nutrients. 2023 Apr 16;15(8):1922. doi: 10.3390/nu15081922. PMID: 37111140; PMCID: PMC10147093.

- Barchitta M, Maugeri A, Favara G, Lio RMS, **La Rosa MC**, D'Ancona F, Agodi A; SPIN-UTI network. The intertwining of healthcare-associated infections and COVID-19 in Italian intensive care units: an analysis of the SPIN-UTI project from 2006 to 2021. J Hosp Infect. 2023 Oct;140:124-131. doi: 10.1016/j.jhin.2023.07.021. Epub 2023 Aug 9. PMID: 37562591.

- Barchitta M, Maugeri A, La Mastra C, Favara G, **La Rosa MC**, Magnano San Lio R, Gholizade Atani Y, Gallo G, Agodi A. Pre-pregnancy BMI, Gestational Weight Gain, and Telomere Length in Amniotic Fluid: a Causal Graph Analysis. Sci Rep. 2024 Oct 8;14(1):23396. doi: 10.1038/s41598-024-74765-y. PMID: 39379607.

- Favara G, Maugeri A, Barchitta M, Magnano San Lio R, **La Rosa MC**, La Mastra C, Galvani F, Pappalardo E, Ettore C, Ettore G, Agodi A. Social and nutritional profiles of pregnant women: a cluster analysis on the "MAMI-MED" cohort. Submitted to Nutrients on 15 October 2024.

Contributions to national and international conferences and congresses since November 2021

- **La Rosa MC,** Barchitta M, Maugeri A, La Mastra C, Favara G, Magnano San Lio R, Campisi E, Agodi A. "Caratterizzazione epidemiologica e molecolare di *Acinetobacter baumannii* durante la pandemia COVID-19". Accettato al 55° Congresso della Società Italiana di Igiene, Medicina preventiva e Sanità Pubblica (SItI) Padova 28 settembre- 1 ottobre 2022.

- Campisi E, Barchitta M, Maugeri A, La Mastra C, **La Rosa MC**, Favara G, Magnano San Lio R, Magro G, Basile G, Agodi A. "Infezione da *Polyomavirus* in pazienti affetti da carcinoma a cellule di Merkel: un approccio di epidemiologia molecolare". Accettato al 55° Congresso della Società Italiana di Igiene, Medicina preventiva e Sanità Pubblica (SItI) Padova 28 settembre- 1 ottobre 2022.

- Favara G, Barchitta M, Maugeri A, Campisi E, Magnano San Lio R, La Mastra C, **La Rosa MC,** Mura I, Agodi A, S. Network. "Applicazione della *Cluster Analysis* per lo studio dei fattori associati al rischio di Infezione Correlate all'Assistenza nelle Unità di Terapia Intensiva: i risultati del progetto SPIN-UTI". Accettato al 55° Congresso della Società Italiana di Igiene, Medicina preventiva e Sanità Pubblica (SItI) Padova 28 settembre- 1 ottobre 2022.

- Magnano San Lio R, Barchitta M, Maugeri A, **La Rosa MC**, La Mastra C, Campisi E, Favara G, Ferrante G, Galvani F, Pappalardo E, Ettore G, Agodi A. "Relazione tra assunzione materna di folati e lunghezza dei telomeri dei neonati: risultati della coorte MAMI-MED". Accettato al 55° Congresso della Società Italiana di Igiene, Medicina preventiva e Sanità Pubblica (SItI) Padova 28 settembre- 1 ottobre 2022.

- La Mastra C, Barchitta M, Maugeri A, **La Rosa MC**, Magnano San Lio R, Campisi E, Favara G, Ferrante G, Galvani F, Pappalardo E, Ettore G, Agodi. "Valutazione dei profili nutrizionali in una coorte di donne in gravidanza: risultati dello studio MAMI-MED". Accettato al 55° Congresso della Società Italiana di Igiene, Medicina preventiva e Sanità Pubblica (SItI) Padova 28 settembre- 1 ottobre 2022.

- Magnano San Lio R, Barchitta M, Maugeri A, **La Rosa MC**, La Mastra C, Favara G, Ferrante G, Galvani F, Ettore G, Agodi A. Vaccination choices among pregnant women: findings from

the MAMI-MED cohort. The 16th European Public Health Conference, Dublin, Ireland, 9 – 11 November 2023.

- La Mastra C, Gholizade Atani Y, Barchitta M, Maugeri A, Magnano San Lio R, Favara G, **La Rosa MC**, Gallo G, Agodi A. Applicazione di modelli causali per la valutazione della relazione tra aumento di peso gestazionale e lunghezza dei telomeri: risultati della coorte mamma e bambino. Oral presentation at the National Public Health Conference Extraordinary SItI, Cernobbio, Como, Italy, 12- 14 October 2023.

-Maugeri A, Barchitta M, **La Rosa MC**, La Mastra C, Magnano San Lio R, Favara G, Galvani F, Pappalardo E, Ettore C, Ettore G, Agodi A. Effetti dell'incremento di peso gestazionale sugli *outcome* neonatali: risultati della coorte MAMI-MED. The National Public Health Conference Extraordinary SItI, Cernobbio, Como, Italy, 12- 14 October 2023.

- Favara G, Barchitta M, Maugeri A, **La Rosa MC**, La Mastra C, Magnano San Lio R, Galvani F, Pappalardo E, Ettore C, Ettore G, Agodi A. Dieta e profili nutrizionali nelle donne in gravidanza: risultati della coorte MAMI-MED. Oral presentation at the National Public Health Conference Extraordinary SItI, Cernobbio, Como, Italy, 12- 14 October 2023.

-Campisi E, Barchitta M, Maugeri A, Manoli M, La Mastra C, **La Rosa MC**, Favara G, Magnano San Lio R, Ojeda Granados C, Agodi A. Coperture vaccinali tra gli operatori sanitari e i pazienti dei presidi ospedalieri siciliani: risultati della "Sorveglianza europea mediante prevalenza puntuale delle infezioni correlate all'assistenza e sull'uso di antibiotici negli ospedali per acuti". 5° Congresso Società Italiana di Igiene Medicina Preventiva e Sanità Pubblica (SItI), Taormina 2023.

-Magnano San Lio R, Barchitta M, Maugeri A, **La Rosa MC**, La Mastra C, Favara G, Ferrante G, Galvani F, Pappalardo E, Ettore C, Ettore G, Agodi A. Coperture vaccinali nelle donne in gravidanza: risultati della coorte MAMI-MED. 5° Congresso Società Italiana di Igiene Medicina Preventiva e Sanità Pubblica (SItI), Taormina 2023.

-Magnano San Lio R, Barchitta M, Maugeri A, **La Rosa MC**, La Mastra C, Favara G, Ferrante G, Galvani F, Pappalardo E, Ettore C, Ettore G, Agodi A. The effect of gestational weight gain on delivery and neonatal characteristics: exploring sex differences in the "MAMI-MED" cohort. 17th World Congress on Public Health, 2023.

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