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Wastewater valorisation for sustainable reuse of water for mitigating the impact of climate change

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Table of Contents

1. *General introduction*

The topic of climate change is an increasingly relevant issue, as the environmental alterations associated with it have severe consequences for both human health and the environment. For the first time, in 2009, the *Lancet Commission on Managing the Health Effects of Climate Change* defined climate change as "the biggest global health threat of the 21st century," underscoring that, in addition to causing irreparable ecosystem damage, it has a substantial impact on human health worldwide (Watts et al., 2015). The primary causes of climate change are rooted particularly in human activities; indeed, there are multiple correlations between anthropogenic activities and climatic and ecosystem anomalies (IPCC, 2022). These effects can manifest in various forms across different regions of the planet.

The direct effects of climate change on natural or human-modified environments, such as extreme weather events and their consequences (flooding, severe storms, droughts, heatwaves, glacial melting), are among the most widely recognised (Fig. 1). However, there are also indirect effects mediated by direct risk-induced changes at the biosphere level, including water quality degradation, increased air pollution, altered disease vector distribution, reduced food availability, and more (Fig. 1). No region on the planet remains unaffected by these phenomena, as climate change impacts are observed globally (Watts et al., 2015).

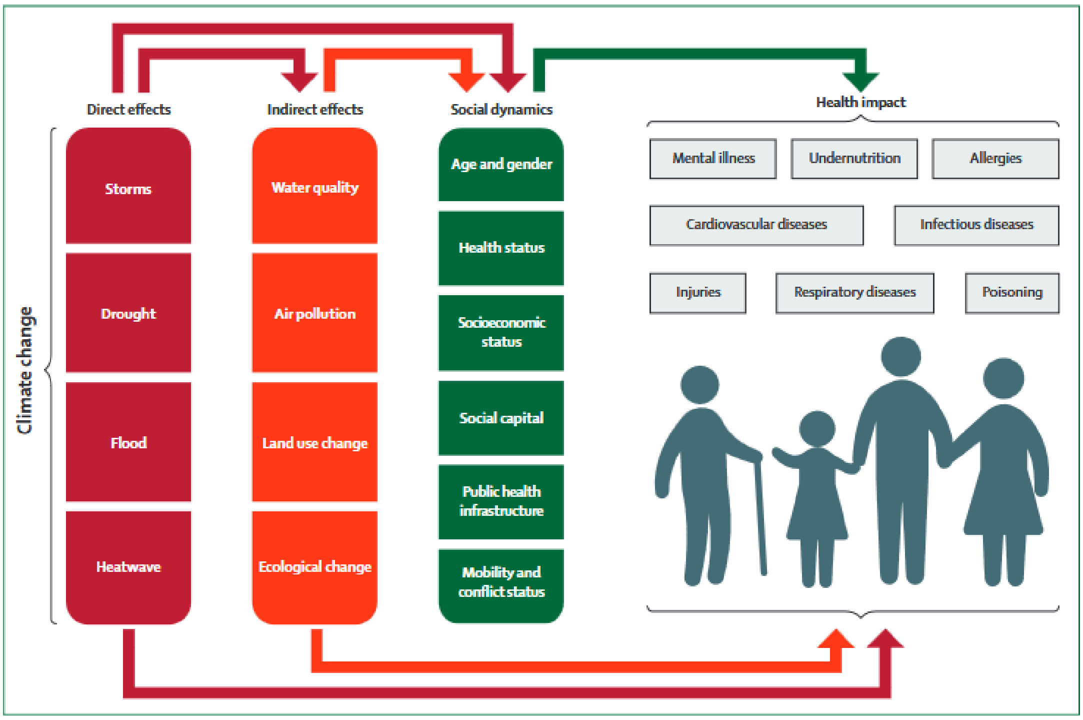

Fig. 1. The direct and indirect effects of climate change on health and wellbeing (from Watts et al., 2015).

While the effects on human health and survival (such as the direct loss of human lives) are more easily linked to extreme events driven by climate change (e.g., floods and inundations), the connection between the indirect effects of climate change and public health is more complex (Hales et al., 2014; Watts et al., 2015). Among the most significant indirect effects is the development of diseases resulting from the degraded quality of environmental matrices. These matrices, subjected to ongoing stress due to human overexploitation, are heavily compromised both quantitatively and qualitatively, making them more vulnerable to additional anthropogenic or natural stresses. This creates a cascade of events that could lead to a reduction in the overall availability of resources, impacting both natural ecosystems and human needs (UN - Water, *The United Nations World Water Development Report 2020*).

Water is one of the environmental matrices most susceptible to climate change effects. As a resource closely linked to climate, any alteration in it can also induce changes in the hydrological cycle. Water is a fundamental element for the survival of biological organisms, including humans; thus, a reduction in its availability — both in quantitative terms and in quality degradation associated with climate change — can have significant economic and health repercussions, as illustrated in Fig. 2 (UN - Water, *The United Nations World Water Development Report 2020*).

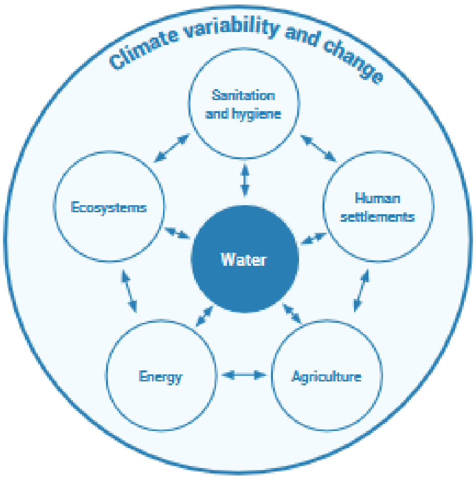

Fig. 2. Interactions between water and other major socio-economic sectors affected by climate variability and change (from UN - Water, *The United Nations World Water Development Report 2020*).

From a quantitative perspective, water has generally been relatively abundant, leading humanity to assume its perpetual availability. On a global scale, significant sources of water are still available, yet at a regional level, demands do not always match actual supply. Analysing the global distribution of water resources reveals that freshwater availability is markedly uneven across regions: just nine countries (Brazil, Russia, China, Canada, Indonesia, the United States, India, Colombia, and the Democratic Republic of the Congo) hold 60% of the world's freshwater resources.

It is clear that climate changes observed over recent decades have impacted the global hydrological cycle, resulting in seasonal changes in river flows and increased severity and frequency of floods and/or droughts in some regions. This trend is expected to lead to a decline in river flow and an overall reduction in water availability in tropical and semi-arid regions, particularly in the Mediterranean Basin, the eastern United States, South Africa, and north-eastern Brazil. Exacerbating the issue of freshwater scarcity—driven by global reductions in polar and alpine glacier reserves and increasing drought events—is the rising demand for water to meet the essential needs of a constantly growing global population (Watts et al., 2015; UN - Water, *The United Nations World Water Development Report 2020*).

As shown in Fig. 3, various regions worldwide experience water stress. Specifically, Italy is classified within the high to extremely high-water stress range across its territory, except for alpine regions. These data point to a potential water supply issue for Italy, which becomes more pronounced as climate change-related phenomena intensify (UN - Water, *The United Nations World Water Development Report 2021*).

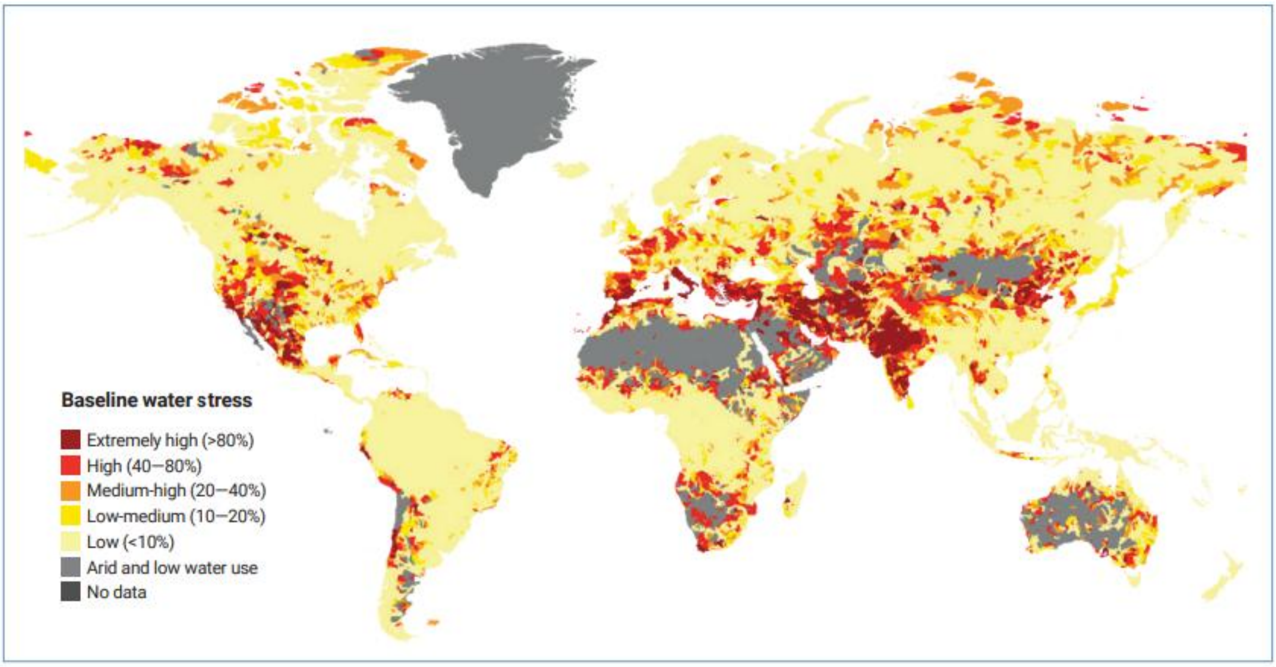

Fig. 3. Annual baseline water stress (from UN - Water, *The United Nations World Water Development Report 2021*).

Climate change and variability can impact not only water availability but also water quality, with diverse consequences for human health. According to the IPCC report (2022), a significant reduction in the quality of both surface and groundwater is expected, with substantial implications for public health. In particular, floods, droughts, storms, changes in the frequency and intensity of rainfall, warming, and sea level rise can all affect water quality, influencing its biological and physicochemical characteristics (IPCC, 2022).

Additionally, the rapid increase in the global population in recent decades, occurring in a political context unprepared for such growth, has led to a demand for water that exceeds natural supplies, resulting in overexploitation and a general decline in water quality (UN - Water, *The United Nations World Water Development Report 2020*).

In the context of the climate crisis, attention to water quality is crucial, especially given the drastic reduction in water resources due to rising temperatures and reduced availability. In industrialised countries with high water usage, health risks may arise from the discharge of wastewater containing potential chemical and microbiological contaminants, an issue of public health concern (Khalid et al., 2018; Bonetta et al., 2022). Managing anthropogenic impacts, such as those associated with wastewater treatment processes, is essential, particularly for surface water resources, as declining water quality could pose health risks related to drinking, recreational, and irrigation uses.

Water scarcity linked to climate change also necessitates the development of new sustainable strategies, both short- and long-term, to address this shortage while safeguarding public health. A frequently proposed approach in this context is the reuse of treated wastewater, which could serve as an alternative to groundwater or surface water withdrawals to meet the irrigation demands of the agricultural and industrial sectors.

1.1. *Treated wastewater reuse*

Among the strategies to reduce withdrawals from surface and groundwater sources and ensure water availability less impacted by climate phenomena and seasonality, there is the reuse of treated wastewater for agricultural and industrial purposes (Tortajada, 2020). The principle of reuse involves using treated wastewater—processed to remove hazardous substances and pathogenic microorganisms—for various applications. Reclaimed water can be used for irrigating agricultural crops, cooling in industrial plants, ornamental purposes (e.g., fountains, artificial ponds, urban landscaping) and street cleaning. Such broad applications could help reduce freshwater consumption, reserving it for potable uses, and favouring recycled water with a lower environmental, economic, and ecological impact (Cui & Liang, 2019; Giannoccaro et al., 2019).

The strategy of reusing treated water could be especially advantageous given the large number of wastewater treatment plants (WWTPs) in Italy equipped with technologies suitable to produce an effluent for reuse.

SNPA (Italian National System for Environmental Protection) presented a preliminary analysis regarding technologies currently installed in WWTPs in Italy, aimed at assessing the potential for reusing urban wastewater for irrigation purposes. Fig. 4 illustrates the distribution of the number of WWTPs, classified according to their technologies suitable for reuse.

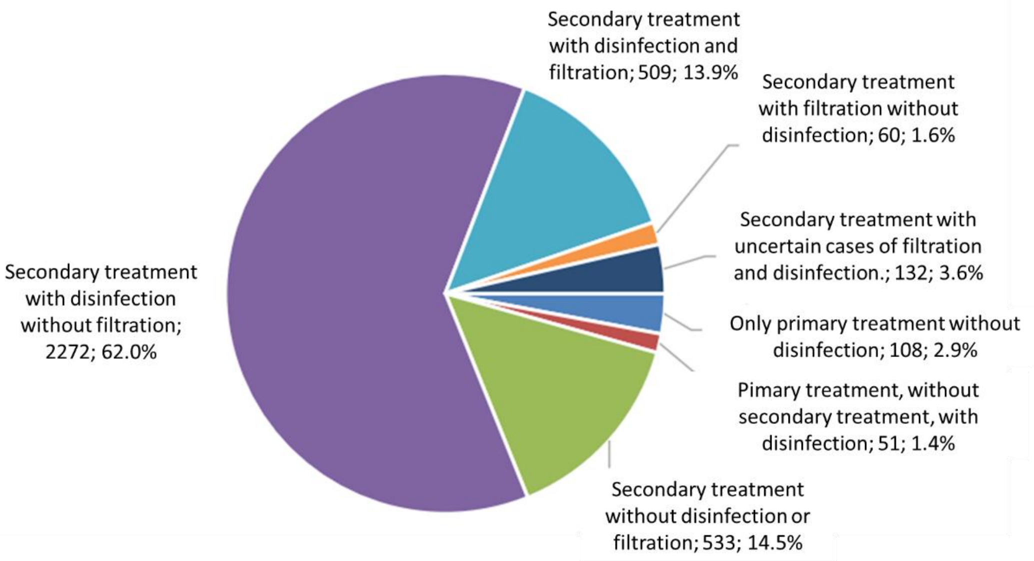

Fig. 4. Distribution of wastewater treatment plants based on the treatment technologies present, suitable for reuse, classified by the number of plants (modified from SNPA Council Resolution No. 254/24 of 23.07.2024).

Today, there are many industrial and agricultural cases employing water-saving techniques or practicing conscious water use. Some authors report that approximately 20 million hectares of land across 50 countries are already irrigated using reclaimed water (Khalid et al., 2018). However, widespread use of this resource is still limited, accounting for about 2.4% of the total water used across Europe (Bonetta et al., 2022).

The reuse of treated wastewater presents several advantages. Key benefits include the reuse of a waste matrix, particularly relevant within a circular economy framework, the ability to irrigate even during periods of water scarcity, and the reduction in infrastructure needed for water extraction and transport from groundwater or surface water bodies, favouring local water sourcing. Conversely, there are challenges to consider in water reuse, such as the need to adapt existing facilities for reuse, since new infrastructure will need to be designed for the collection and distribution of treated wastewater, the importance of educating the public to help them understand the reasons for using treated wastewater, and the overall costs, which can vary depending on the type of treatment and transport infrastructure required.

More scientific research is necessary to maximize the benefits of water reuse practices (Florides et al., 2024). When examining the specific pros and cons of reusing treated wastewater in agriculture, potential health risks emerge, particularly from microorganisms not completely eliminated during treatment that could cause infections in consumers of crops irrigated with this water (Bonetta et al., 2022). Additionally, from an agronomic perspective, there are concerns about the mineral content of reclaimed water. On one hand, these minerals may contribute to fertilization and reduce fertilization costs; on the other, they could affect agricultural product safety, yielding lower-quality crops and potentially harmful products for consumers, while also posing risks of groundwater contamination due to the high mineral content (Khalid et al., 2018; Cui & Liang, 2019).

1.2. *Risks related to wastewater reuse and in the context of urban water cycle*

Wastewater reuse is an increasingly adopted practice aimed at enhancing water resource sustainability. It involves the treatment and recycling of wastewater for various applications, such as irrigation, industrial processes, and even potable use in some cases. While this approach helps mitigate water scarcity and reduce environmental pollution, it also presents several risks that must be carefully managed.

The production of wastewater results from various human activities, which can contribute to both microbiological (bacteria, viruses, protozoa, and helminths) and chemical (metals, pesticides, disinfection by-products, and emerging pollutants) contamination. Among the emerging pollutants, it is mandatory to mention antibiotic resistance. The contaminants referred to antibiotic resistance are not only antibiotics, but also antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs).

For its characteristics, wastewater reuse can introduce both direct and indirect risks through many different pathways.

Direct risks involve immediate threats to human health and the environment. The direct risk could occur, for example, through the inhalation of aerosols (e.g. during irrigation or industrial reuse) and with dermal contact in occupational settings or during recreational activities, such as swimming or bathing in rivers or lakes that receive treated effluents from WWTPs.

Indirect exposure to wastewater can, for example, occur through the consumption of contaminated water and food. In this context, wastewater reuse in agriculture presents potential public health risks since the presence of pathogenic microorganisms and potentially toxic chemical compounds in wastewater can lead to food chain contamination, negatively affecting soil, crops, and agricultural productivity (Fig. 5) (Khalid et al., 2018). The transmission of faecal-oral diseases in such scenarios depends on the survival of pathogens in irrigated crops and soil, which is influenced by factors such as moisture, temperature, pH, sunlight exposure, soil characteristics, and crop type (Sing, 2021).

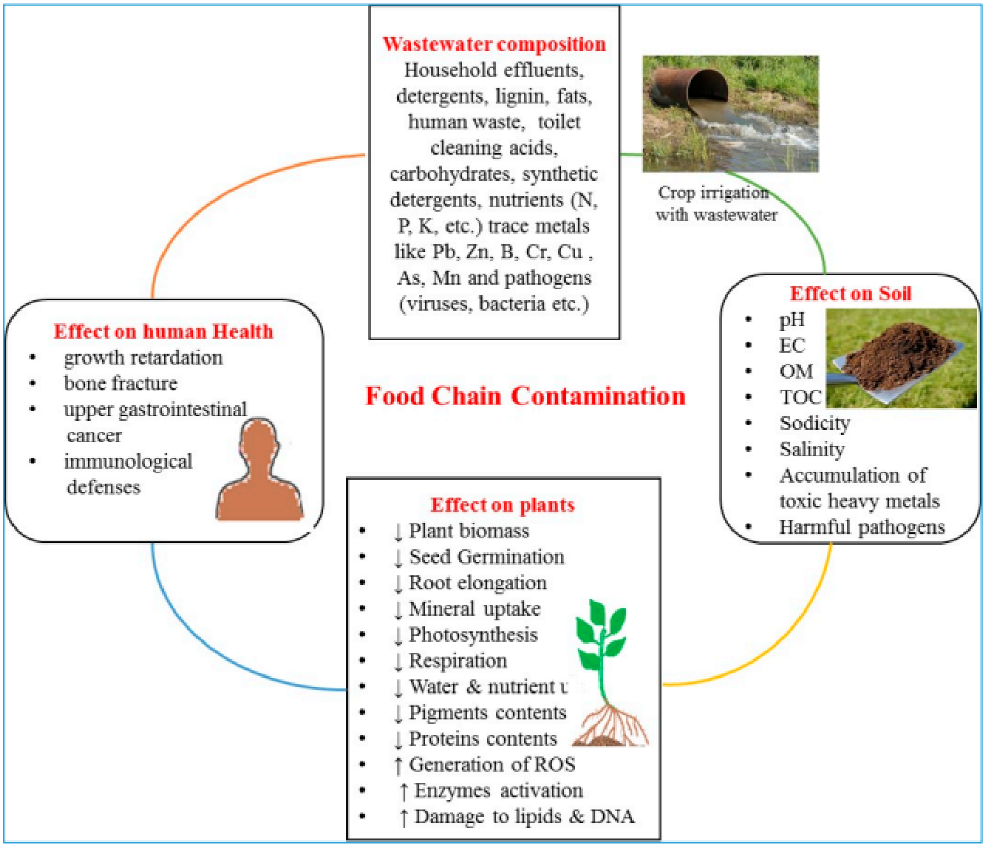

Fig. 5. The possible food chain contamination by wastewater crop irrigation (from Khalid et al., 2018).

Moreover, in the broader context of the urban water cycle, it is important to highlight the close relationship between wastewater treatment and drinking water production, as surface water bodies often serve as key sources for potable water supply. In many cases, rivers receiving treated wastewater are involved in both indirect reuse, when the effluent contributes to the flow used downstream for drinking water abstraction, and direct reuse, when reclaimed water is purposefully diverted from these water bodies for agricultural, industrial, or even potable applications. Consequently, inadequate treatment of wastewater before discharge may severely impact both the microbiological and chemical quality of water intended for human consumption and other uses.

The interconnection between wastewater treatment, surface water quality, and drinking water supply (as shown in Fig. 6) highlights the importance of advanced treatment technologies and continuous monitoring to minimize public health risks.

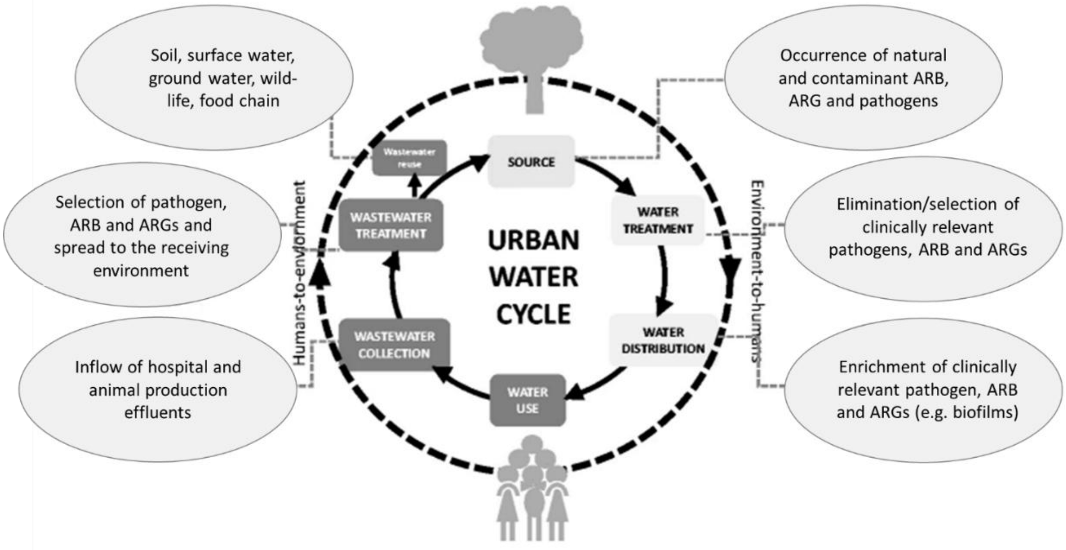

Fig. 6. Schematic representation of the urban water cycle and of sites or processes potentially critical for pathogens, antibiotic resistance selection, spread, and control (modified from Manaia et al., 2016).

For these reasons, the reuse of wastewater especially in the context of the urban water cycle, could presents significant microbiological risks, including the spread of ARB and persistent pathogens that can compromise Public Health and environmental safety. Without stringent treatment processes and continuous monitoring, these hazards may lead to long-term challenges, such as increased antimicrobial resistance and the emergence of new infectious diseases, underscoring the need for sustainable and precautionary wastewater management strategies.

1.3. *Antibiotic resistance*

The discovery of antibiotics, a little less than a century ago, along with the ability to synthesize them, marked a turning point in medical history. Many bacterial infections that were previously often fatal or capable of causing permanent damage have now become mild conditions that can be easily treated. However, in recent years, the effectiveness of these drugs has been increasingly threatened by resistance phenomena, which occur when the microorganism responsible for an infection acquires the ability to survive and proliferate (PNCAR 2022-2025).

The health risks are significant, particularly because, in some cases, bacteria exhibit multidrug resistance, meaning they can withstand multiple classes of antibiotics, including last-generation ones.

The World Health Organization (WHO) has long classified antibiotic resistance as one of the major global health challenges, predicting that by 2050, this phenomenon could cause an additional 10 million direct deaths annually, that is the equivalent to the total number of cancer-related deaths recorded worldwide in 2020 (UNEP, 2023). It is estimated that antibiotic resistance could become the leading cause of death globally and the latest OECD analysis shows that every year, antibiotic resistance costs nearly USD PPP (United States Dollar Purchasing Power Parity) 66 billion across 34 OECD and EU/EEA countries (OECD, 2023).

The expansion of antibiotic resistance is strongly driven by the inappropriate use—both in terms of quantity and quality—of antibiotics in human and veterinary medicine. Specifically, the administration of high or subtherapeutic doses promotes the selection of bacterial strains capable of resisting the selective pressure exerted by antibiotics. Furthermore, horizontal gene transfer mechanisms allow bacteria to acquire ARGs, potentially leading to human exposure to ARB and ARGs through the environment, animals, and food.

In recent years, numerous studies have highlighted the relationship between environmental factors and the presence of antibiotic resistance (Surette et al., 2017).

The European One Health action plan against antimicrobial resistance, launched in 2017, recognizes the environment *as a factor contributing to the development and spread of antimicrobial resistance in humans and animals, particularly in areas at high risk due to human, animal, or industrial waste flows* (European Commission, 2017).

The aforementioned recent report published by the United Nations Environment Programme (UNEP, 2023), reaffirms the key role of the environment in the development, transmission, and dissemination of antimicrobial resistance. It emphasizes that the health of individuals, animals, plants, and ecosystems is closely interconnected and interdependent.

Among various environmental matrices, water is considered a major vehicle for antibiotic resistance, particularly within WWTPs.

Wastewater accumulates ARB and ARGs of human and animal origin, antibiotic residues, and other contaminants such as heavy metals, pharmaceuticals, and parabens. During treatment processes, these compounds interact with the environmental microbiota, undergoing chemical and biological transformations. Specifically, biological treatment processes in wastewater treatment and the use of disinfectants in tertiary treatment could contribute to the spread of antibiotic resistance, through various mechanisms.

For example, to develop resistance, natural mutations can occur in bacterial communities. Consequently, certain bacteria may be resistant to one or more antibiotics due to intrinsic characteristics of the bacterial species, such as membrane impermeability to specific antibiotics. Naturally occurring antibiotic resistance is genetically determined, manifests in all bacterial strains of the same species, and is the result of a long evolutionary process of genetic adaptation.

Antibiotic resistance can also be acquired through genetic exchange. This process, which involves vectors used for the mobilization, acquisition, and encoding of genes derived from other bacteria, confers resistance (Baquero et al., 2008). In susceptible bacterial populations, resistance acquisition depends on modifications to existing DNA through chromosomal mutation (alterations in hereditary DNA sequences due to replication errors) or the acquisition of new genetic material from a resistant donor bacterial cell via horizontal gene transfer (HGT).

HGT occurs through the transfer of DNA fragments from a donor to a susceptible recipient. This exchange can take place across different microbial species and genera. Genetic material is transferred through mobile genetic elements such as plasmids, which, unlike chromosomes that encode essential growth functions, carry genes responsible for functions such as antibiotic resistance. Antibiotic resistance genes migrate within the bacterial genome using vectors such as transposons and integrons, which can move independently of homologous DNA. These mobile genetic elements are directly responsible for the spread of antibiotic resistance genes within bacterial populations through HGT (Nguyen et al., 2021; UNEP, 2023).

The phenomenon of resistance is also biologically associated with the ability of microorganisms to survive in the presence of substances other than antibiotics or pharmaceuticals (e.g., heavy metals, disinfectants). This process is known as co-selection and occurs through three mechanisms: cross-resistance, co-resistance and co-regulation (Murray et al., 2024).

Cross-resistance arises when a single resistance mechanism confers resistance to multiple compounds. This phenomenon has been observed in bacteria through efflux mechanisms that are common to structurally diverse toxic agents, such as metals and antibiotics. For instance, various drug efflux pumps reduce susceptibility to both antibiotics and disinfectants by expelling these compounds from the bacterial cell.

Co-resistance, on the other hand, occurs when two or more distinct resistance genes are located on the same mobile genetic element (e.g., plasmids, integrons, transposons) in bacteria, simultaneously conferring resistance to multiple toxic compounds (Seiler et al., 2012).

Co-regulation takes place when translational and transcriptional mechanisms are interconnected, resulting in a synchronized response, such as the activation of multiple distinct efflux pumps in reaction to a single agent. Co-selection has been recognized as a crucial process driving the selection of ARGs across various microbial communities (Murray et al., 2024).

Despite the role of WWTPs in antibiotic resistance spreading is well established in literature (Cacace et al., 2019; Nguyen et al., 2021; Kang et al., 2022; Bonetta et al., 2023), less is known about the quality of the effluent intended for reuse that usually is obtained with more advanced wastewater treatment technologies.

1.4. *Pathogens*

The wastewater environment serves as an optimal habitat for both harmful and harmLess microorganisms. Hazardous pathogens include intestinal bacteria, viruses, protozoa, parasitic worms, and their eggs. Faecal material is a primary constituent of household sewage and the origin of most human disease-causing agents in wastewater. Industrial discharge from food manufacturing, especially from livestock processing, can also contribute to the presence of pathogenic microbes. This intrinsic wastewater characteristic is crucial when the effluent is designated for reuse purposes, but also in the case of surface water discharge, since this water could be withdrawn for direct or indirect use. In fact, the contamination of surface waters by pathogenic microorganisms, as a consequence of inadequate wastewater disinfection, may promote waterborne disease spread, including those caused by Shiga toxin (Stx)-producing *Escherichia coli* (STEC) and *Salmonella* (Bonetta et al., 2021).

One of the most frequent bacteria, found in wastewaters and in waters impacted by wastewater discharge is *Salmonella* spp. *Salmonella* belongs to the *Enterobacteriaceae* family and includes Gram-negative, rod-shaped, facultative anaerobic microorganisms, typically motile due to peritrichous flagella. Based on clinical manifestations in humans, *Salmonella* strains are classified into two groups: typhoidal and non-typhoidal (NTS). The former includes *S. typhi* and *S. paratyphi*, etiological agents of typhoid (enteric) fever, while the latter comprises strains such as *S. enteritidis* and *S. typhimurium*, primarily causing less severe enteric salmonellosis.

Typhoidal *Salmonella* exclusively infects humans, with transmission occurring via ingestion of food or water contaminated with faeces from infected individuals or asymptomatic carriers. In contrast, NTS strains are widespread in domestic and wild animals, particularly poultry, cattle, and swine, and transmission can occur through direct contact with infected animals (Crump, 2019).

Enteric fever is rare in the EU, mostly linked to travel in regions with poor sanitation and inadequate food and water safety infrastructure. In 2021, 19 EU/EEA countries reported 304 confirmed cases and the notification rate was 0.10 cases per 100,000 inhabitants (ECDC, 2024).

In industrialised nations, NTS is more prevalent, with foodborne transmission as the primary route, but water can also be a source of human exposure. Salmonellosis is the second most common gastrointestinal infection in Europe. Salmonellosis remained the second most prevalent zoonotic infection in humans in the EU in 2023, following campylobacteriosis. The notification rate rose in 2023 compared to 2022 (18.0 *vs* 15.4, respectively). Both the number of cases linked to outbreaks and the incidence of salmonellosis outbreaks increased compared to 2022 (EFSA & ECDC, 2024). Growing global concern surrounds treatment options due to widespread antibiotic resistance in both typhoidal and NTS strains (Dyson et al., 2019; Kumar et al., 2025). The recent literature suggests that irrigation water represent a possible source of *Salmonella* contamination in produce, highlighting its possible role as a transmission vehicle (Bonetta et al., 2021).

Another relevant bacterium is *Escherichia coli*. *E. coli* is a Gram-negative, rod-shaped, facultative anaerobe of the *Enterobacteriaceae* family. Most strains colonize the gastrointestinal tract of humans and animals harmLessly, but some are pathogenic, classified by virulence factors and clinical symptoms. Among these, enterohemorrhagic *E. coli* (*EHEC*), also known as verocytotoxin-producing (*VTEC*) or Shiga toxin-producing (*STEC*), cause haemorrhagic colitis and haemolytic uremic syndrome (*HUS*). The most prevalent serotype, *E. coli* O157:H7, expresses the O157 somatic and H7 flagellar antigens.

E. coli O157:H7 persists in soil, water, wastewater, and food for extended periods—contaminated water can harbour the bacterium for over eight months, posing an infection risk (Ormsby et al., 2023).

A key virulence factor is Shiga toxin (*Stx*), classified into *Stx1* and *Stx2*. *Stx1* is highly conserved and similar to *Shigella dysenteriae* type 1 toxin, while *Stx2* is more toxic and strongly linked to *HUS*. The acidic pH of the stomach (1.5–3) is a primary defence against enteric pathogens, but *E. coli* O157:H7 tolerates pH < 3, enhancing intestinal colonization and infection potential. This acid resistance correlates with a low infectious dose, making the strain highly virulent (Newell et al., 2017).

STEC infections are a global health issue. Although case numbers are lower than *Salmonella* and *Campylobacter*, hospitalization and mortality rates are significantly higher. In 2023, Europe reported 10,217 cases with 66 foodborne outbreaks and, among the totality of waterborne outbreaks (n=10), 40% were caused by STEC (n=4) (EFSA and ECDC, 2024).

Cattle and sheep are primary reservoirs, often asymptomatic carriers. Transmission occurs mainly via contaminated food and water but also through direct human-to-human or animal-to-human contact.

Common sources include raw meat, unpasteurized milk, and improperly washed vegetables (Ramos et al., 2020).

Regarding wastewater, non-pathogenic *E. coli* in WWTPs is a significant concern due to its potential to indicate faecal contamination and the presence of pathogenic strains. Wastewater treatment processes, including primary, secondary, and tertiary treatments, aim to reduce *E. coli* concentrations before discharge or reuse (Ofori et al., 2021). However, studies have shown that while conventional treatment methods significantly lower bacterial loads, some *E. coli*, including antibiotic resistant strains, can persist in treated effluent and sludge.

Among the antibiotic resistant strains, extended-spectrum β-lactamase-producing *E. coli* (ESBL-Ec) is a significant Public Health concern. These strains hydrolyse a broad range of β-lactam antibiotics, including penicillins, cephalosporins, and monobactams, rendering treatment challenging. ESBL genes are often plasmid-encoded, facilitating their horizontal transfer among bacterial populations (Pitout & Laupland, 2008). Infections caused by ESBL-Ec are associated with increased morbidity, mortality, and prolonged hospital stays. These resistant strains are commonly found in healthcare settings but are also emerging in community-acquired infections, often linked to contaminated food, water, and environmental sources (Liu et al., 2024).

In 2021, 29 EU/ EEA countries reported 144,260 isolates of *E. coli*. Among these, 108,730 (75.4%) isolates reported antibiotic susceptibility test results for aminopenicillins, 143,180 (99.3%) for third-generation cephalosporins (β-lactam antibiotics), 143,253 (99.3%) for fluoroquinolones, 139,435 (96.7%) for aminoglycosides and 137,526 (95.3%) for carbapenems (ECDC, 2023).

At EU/ EEA level, more than half (52.3%) of the *E. coli* isolates were resistant to at least one of the antimicrobial groups under surveillance (aminopenicillins, fluoroquinolones, third-generation cephalosporins, aminoglycosides and carbapenems) (ECDC, 2023).

Another bacterium that is necessary to mention, especially in relation to the risk of aerosolization, is *Legionella*. The aerosolization can occur both during biological treatment within WWTPs but also during the agricultural spray irrigation with treated wastewater (Bonetta et al., 2022). *Legionella* is the sole genus of the *Legionellaceae* family, comprising weakly Gram-negative, motile, cocco-bacillary, obligate aerobic bacteria that thrive at 20–42°C. It was named in 1976 following an outbreak at an American Legion convention in Philadelphia, where 221 individuals were infected, resulting in 34 deaths. Currently, 50 *Legionella* species are identified, with *L. pneumophila* being the primary cause of legionellosis. This species includes at least 16 serogroups, with serogroup 1 responsible for 90% of cases.

Legionellosis are manifested mainly in two forms: Legionnaires' disease and Pontiac fever. The former is a pneumonia with or without extrapulmonary involvement, lacking specific clinical or radiological features, making it indistinguishable from other bacterial pneumonias like pneumococcal pneumonia. Pontiac fever is a milder, self-limiting influenza-like illness without pulmonary involvement. Transmission occurs via inhalation of aerosolized water droplets containing *Legionella*.

These bacteria are nutritionally demanding, requiring L-cysteine, iron, and carbon for growth. Despite this, they are ubiquitous, found in natural aquatic environments and moist soils, where they survive long periods as intracellular parasites of protozoa. *Legionella* withstands wide temperature (0–68°C) and pH (5–8.5) ranges and exhibits resistance to chlorine disinfection. As a result, it can persist in treated wastewater, potable water distribution systems, and biofilms on pipe surfaces (Caicedo et al., 2019; Ofori et al., 2021).

Warm, humid environments favour *Legionella* proliferation. Cooling towers, air conditioning systems, and aerobic wastewater treatment plants provide optimal conditions due to their carbon, nitrogen, and phosphorus content, along with controlled oxygen levels and temperatures (Caicedo et al., 2019). Additionally, water reuse practices pose risks; spray or mist irrigation and recycled water in sanitary systems and cooling networks generate aerosols that can spread *Legionella* over kilometres (Hamilton et al., 2017). This led to the inclusion of *Legionella* as a monitored parameter in reclaimed water under EU Regulation 2020/741 when airborne transmission is possible.

Legionnaires' disease in the EU is primarily sporadic, peaking between June and October, likely due to higher temperatures promoting bacterial growth in water systems. As stated in the most recent ECDC report on this subject, in 2021, the EU/EEA recorded the highest yearly incidence rate of Legionnaires' disease to date, reaching 2.4 cases per 100,000 inhabitants. Four nations (Italy, France, Spain, and Germany) were responsible for 75% of all documented cases (ECDC, 2023).

The reasons for this trend remain unclear but may include aging populations, increased travel, water system maintenance, and climate change, which influence *Legionella* presence and aerosol exposure.

The pathogens mentioned are not the only ones that can be present in water and the detection of all potential pathogens is impractical. It is not feasible to investigate all microorganisms that may pose a risk to human health due to their large numbers.

Furthermore, the absence of detection does not guarantee the absence of pathogens, as these can intermittently appear in effluents and receiving water bodies. Additionally, the presence of abundant contaminating flora may interfere with the ability to detect pathogens, even when present.

A method to overcome this issue is using faecal contamination indicator microorganisms.

Therefore, indicator organisms are used, as their presence suggests the potential presence of pathogens. However, it is important to note that the use of indicators provides probability, not certainty, regarding pathogen presence. Historically, enteric bacteria have been proposed as indicators of faecal contamination, as they inhabit the gastrointestinal tract of humans and warm-blooded animals and are transmitted via the oro-faecal route.

According to Italian regulations (Decree-Law 39/2023), the effluent must be tested for a single indicator microorganism, *E. coli*. However, total coliforms, faecal coliforms, enterococci, and clostridia are commonly tested as well. Coliforms, part of the *Enterobacteriaceae* family, are Gram-negative, rod-shaped, facultative anaerobes, and non-spore-forming. These are the most used indicators, with concentrations in raw wastewater reaching up to 10^7 CFU/100 mL. *E. coli* is the most abundant coliform in mammalian faeces, making it the most specific indicator of faecal contamination. Italian legislation sets a maximum concentration limit of 5,000 CFU/100 mL for *E. coli* in treated effluents.

Enterococci, part of the faecal streptococci group, are Gram-positive, spherical, facultative anaerobes, and non-spore-forming. Clostridia, often used as surrogates for more resistant microorganisms and protozoa, are Gram-positive, obligate anaerobic, spore-forming bacilli. *Clostridium perfringens* spores are heat-resistant and resistant to disinfection, allowing them to survive longer in the environment. Their presence can indicate previous faecal contamination (Holcomb et al., 2020; Bonetta et al., 2022).

1.5. *Legislation*

The practice of wastewater reuse is a circular economy measure that has been implemented in Italy for some time and is regulated by the decree of the Minister of the Environment and the Protection of the Territory No. 185 of June 12, 2003 ("Regulation containing technical standards for the reuse of wastewater").

The Ministerial Decree aims to establish technical standards for the reuse of domestic, urban, and industrial wastewater, regulating their intended uses and the water quality requirements. The goal of this regulation is to reduce the extraction of surface and groundwater, minimizing the impact of discharges on receiving water bodies, thus promoting water conservation through its multiple uses. Regarding the reuse of treated wastewater for irrigation purposes, Article 10 of the Decree defines the need for its use through methods that ensure water savings.

In relation to microbiological parameters, in the subsequent Ministerial Decree of May 2, 2006, it was specified few indications for treated effluent intended for reuse regarding *E. coli* (max 100 CFU/100 mL) and *Salmonella* (absent).

With the European Parliament and Council Regulation No. 2020/741 of May 25, 2020 (that is in force since June 2023), establishing minimum requirements for water reuse, the first-ever European-level standards were set for the use of so-called reclaimed water, i.e., treated wastewater, for agricultural purposes.

In this regulation are reported 4 “minimum reclaimed water quality class” reported in Table 1 with different requirements for each class, reported in Table 2.

Minimum reclaimed water quality class	**Crop category**	**Irrigation method**
A	All food crops consumed raw where the edible part is in direct contact with reclaimed water and root crops consumed raw	All irrigation methods
B	Food crops consumed raw where the edible part is produced above ground and is not in direct contact with reclaimed water, processed food crops and non-food crops including crops used to feed milk- or meat-producing animals	All irrigation methods
C	Food crops consumed raw where the edible part is produced above ground and is not in direct contact with reclaimed water, processed food crops and non-food crops including crops used to feed milk- or meat-producing animals	Drip irrigation or other irrigation method that avoids direct contact with the edible part of the crop
D	Industrial, energy and seeded crops	All irrigation methods

Table 1. Classes of reclaimed water quality and permitted agricultural use and irrigation method.

<table>
<tr><th rowspan="2">Reclaimed water quality class</th><th rowspan="2">Indicative technology target</th><th colspan="2">Quality requirements</th></tr>
<tr><th>E. coli (number/100 mL)</th><th>Other</th></tr>
<tr><td>A</td><td>Secondary treatment, filtration, and disinfection</td><td>≤ 10</td><td rowspan="4">Legionella spp.: < 1 000 CFU/l where there is a risk of aerosolization.

Intestinal nematodes (helminth eggs): ≤ 1 egg/l for irrigation of pastures or forage</td></tr>
<tr><td>B</td><td>Secondary treatment, and disinfection</td><td>≤ 100</td></tr>
<tr><td>C</td><td>Secondary treatment, and disinfection</td><td>≤ 1 000</td></tr>
<tr><td>D</td><td>Secondary treatment, and disinfection</td><td>≤ 10 000</td></tr>
</table>

Table 2. Reclaimed water microbiological quality requirements for agricultural irrigation. CFU: colony-forming unit.

On April 14, 2023, Decree-Law No. 39 "Urgent Provisions for Combating Water Scarcity and Enhancing and Upgrading Water Infrastructure" was published in the Italian Official Journal (converted into Law No. 68 on June 13, 2023). Article 7, paragraph 1 of this decree states that the reuse of treated wastewater for agricultural irrigation purposes from plants already in operation is authorised, in accordance with Regulation (EU) 2020/741, until December 31, 2023.

Currently, the Italian Ministry of the Environment and Energy Security (MASE) is working on updating the decree within the regulatory framework to produce a new regulation that aligns with the implementation of European regulations on the reuse of treated urban wastewater, as established by Regulation (EU) 2020/741. This decree extends beyond the scope of irrigation reuse, also encompassing other purposes such as civil, environmental, and industrial uses.

Specifically, the decree will define the operational and regulatory framework for the management of treated wastewater intended for reuse, ensuring compliance with quality and safety standards. A key aspect is risk management, with the Risk Management Plan serving as a fundamental tool to address risks associated with the production, distribution, storage, and use of reclaimed water.

In January 2024, the European Parliament adopted an agreement with the Council on revising EU regulations on water management and urban wastewater treatment for better public health and environmental protection published on December 12, 2024 (procedure 2022/0345/COD). The new directive mandates that by 2035, urban wastewater in all agglomerations with at least 1,000 equivalent inhabitants must undergo secondary treatment (removal of biodegradable organic matter) before being discharged into the environment. By 2039, tertiary treatment (removal of nitrogen and phosphorus) will be required in wastewater treatment plants serving 150,000 equivalent inhabitants and beyond, and by 2045 for those serving 10,000 equivalent inhabitants or more. Additionally, a "quaternary treatment" for removing a wide range of micro-pollutants will be mandatory for plants above 150,000 equivalent inhabitants (and those over 10,000 based on a risk assessment) by 2045.

The law will also require strict monitoring of public health parameters (such as known viruses and emerging pathogens), chemical pollutants (including PFAS, "forever chemicals"), microplastics, and antimicrobial resistance.

Furthermore, extended producer responsibility (EPR) will be introduced for human pharmaceuticals and cosmetics, covering the costs of quaternary treatment to remove micro-pollutants from urban wastewater. At least 80% of these costs will be covered by producers, with additional national funding.

EU member states will be required to promote the reuse of treated wastewater from all urban WWTPs, especially in water-stressed areas.

1.6. *Sustainable Development Goals (SDGs)*

The present Ph.D. project aligns with different Sustainable Development Goals (SDGs), in particular:

- SDG 3 (Good Health and Well-being), by ensuring the safe reuse of treated wastewater for agricultural purposes, it helps reduce the risk of waterborne diseases and supports public health by promoting access to clean and safe water sources.
- SDG 6 (Clean Water and Sanitation), which aims to ensure the availability and sustainable management of water and sanitation for all. Wastewater reuse helps reduce pressure on water resources, promoting water conservation and responsible management, which is crucial in areas facing water scarcity.
- SDG 11 (Sustainable Cities and Communities), by promoting water conservation and enhancing the resilience of urban areas to water scarcity. By effectively reusing treated wastewater, urban communities can reduce their dependence on freshwater sources, thereby contributing to more sustainable and resilient urban water management practices.
- SDG 12 (Responsible Consumption and Production), which focuses on responsible consumption and production, fostering circular economy practices and minimising waste.
- SDG 13 (Climate Action), by contributing to climate adaptation strategies. The reuse of wastewater helps mitigate the effects of droughts and water shortages, which are increasingly exacerbated by climate change. This sustainable water management approach reduces the strain on natural water resources, supports climate resilience, and aids in the mitigation of climate-related risks in both urban and agricultural sectors.

1.7. *Aim*

Climate change has become an increasingly relevant issue due to its severe environmental and public health consequences. In 2015, the *Lancet Commission on Managing the Health Effects of Climate Change* identified climate change as "the biggest global health threat of the 21st century" (Watts et al., 2015).

Water is one of the environmental matrices most vulnerable to climate change. Over recent decades, alterations in the global hydrological cycle have led to reduced river flows and declining water availability in various regions, including the Mediterranean Basin. This issue is exacerbated by the growing global population and the increasing demand for freshwater to meet essential needs (Watts et al., 2015; UN-Water, 2020).

In this context, ensuring water quality is crucial, especially as rising temperatures and declining availability place additional stress on freshwater resources. Anthropogenic impacts, such as wastewater treatment processes, must be carefully managed to prevent degradation of surface water quality, which could pose hygiene and public health risks related to drinking water supply, recreational activities, and irrigation (Sapkota, 2019).

The increasing water scarcity caused by climate change highlights the urgent need for sustainable short- and long-term strategies to mitigate its effects while safeguarding public health. One widely proposed solution is the reuse of treated wastewater as an alternative to groundwater or surface water withdrawals, particularly for agricultural and industrial applications. While wastewater reuse presents a promising strategy for addressing water shortages, inadequate treatment can introduce microbiological and chemical risks, potentially threatening human and environmental health.

The objective of this Ph.D. project is to assess the microbiological risks associated with wastewater reuse, with a specific focus on the presence of pathogens and antibiotic resistance. Through an interdisciplinary approach, this research aims to quantify and characterise the potential health and environmental risks linked to water reuse, providing tools for the sustainable management of water resources.

The study investigates the efficiency of wastewater treatment processes in removing pathogenic microorganisms and antibiotic resistance determinants, evaluating the impact of different reuse strategies in agricultural, industrial, and urban contexts but also in the surrounding environment (e.g. air, rivers) as well as the entire urban water cycle. The results will be helpful to develop risk assessment models and mitigation strategies to ensure the safe reuse of treated wastewater, thereby protecting human health and aquatic ecosystems reducing pressure on natural water resources.

In particular, the aspect investigated can be summarised as follow:

- Different wastewater treatment can have different impact on antibiotic resistance elements (antibiotics, bacteria and genes). In the present Ph.D. project, WWTPs with different technologies were considered in Italy (North and North-East) and in Spain (South). The approach used was multi-disciplinary, with a combination of cultural and advanced molecular microbiological techniques.
- In a broader context of urban water cycle, also drinking water deserve great attention. For this reason, also a literature review on antibiotic resistance in drinking tap water was performed.
- Finally, recent growing interest is also given to the presence of antibiotic resistance genes in the air. In this context, wastewater treatment plant emission in air should be carefully monitored beside other relevant sites, such as farm, hospital and urban area.
- Beside antibiotic resistance elements, in wastewater-related environments, it is also possible to detect pathogens, such as *Legionella* spp. A part of the project was also dedicated to cultural and molecular evaluation of this bacteria within a WWTP that produce an effluent for reuse.
- WWTP effluent, charged with high load of antibiotic resistance determinants, faecal indicator bacteria and pathogens can be, beside reuse, also discharged in the environment especially in rivers that, lacking dilution capacity due to water shortage, are heavily threatened with a direct impact on human health. For this reason, the environmental impact of wastewater discharge in rivers impacted by water shortage was evaluated in terms of faecal indicator bacteria and pathogens.

Finally, this research aligns with global efforts to mitigate climate change by promoting circular economy solutions that enhance water resilience and reduce the overall water footprint, in accordance with international water management policies and sustainable development goals.

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2. *Antibiotic resistance*

2.1. *First published manuscript: Antibiotic resistance and pathogen spreading in a wastewater treatment plant designed for wastewater reuse*

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Antibiotic resistance and pathogen spreading in a wastewater treatment plant designed for wastewater reuse☆

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2.1.1. Abstract

Climate change significantly contributes to water scarcity in various regions worldwide. While wastewater reuse is a crucial strategy for mitigating water scarcity, it also carries potential risks for human health due to the presence of pathogenic and antibiotic resistant bacteria (ARB). Antibiotic resistance represents a Public Health concern and, according to the global action plan on antimicrobial resistance, wastewater role in selecting and spreading ARB must be monitored.

Our aim was to assess the occurrence of ARB, antibiotic resistance genes (ARGs), and potential pathogenic bacteria throughout a wastewater treatment plant (WWTP) designed for water reuse. Furthermore, we aimed to evaluate potential association between ARB and ARGs with antibiotics and heavy metals.

The results obtained revealed the presence of ARB, ARGs and pathogenic bacteria at every stage of the WWTP. Notably, the most prevalent ARB and ARG were sulfamethoxazole-resistant bacteria (up to 7.20 log CFU mL^{-1}) and *sul*II gene (up to 5.91 log gene copies mL^{-1}), respectively. The dominant pathogenic bacteria included *Arcobacter*, *Flavobacterium* and *Aeromonas*. Although the abundance of these elements significantly decreased during treatment (influent *vs* effluent, $p < 0.05$), they were still present in the effluent designated for reuse.

Additionally, significant correlations were observed between heavy metal concentrations (copper, nickel and selenium) and antibiotic resistance elements (ampicillin-resistant bacteria, tetracycline-resistant bacteria, ARB total abundance and *sul*II) ($p < 0.05$).

These results underscore the importance of monitoring the role of WWTP in spreading antibiotic resistance, in line with the One Health approach. Additionally, our findings suggest the need of

interventions to reduce human health risks associated with the reuse of wastewater for agricultural purposes.

2.1.2. Introduction

Climate change significantly amplifies the water scarcity worldwide by exacerbating the existing water stress and poses new challenges for the availability, distribution, and quality of freshwater resources. Water scarcity is a multifaceted and interconnected challenge that requires coordinated efforts at the local, national and international levels to ensure the sustainable management of water resources (IPCC, 2022). Wastewater reuse represents an important strategy for tackling water shortage and promoting environmental sustainability. However, despite this, treated wastewater remains a minor source for the water supply, consisting of only 0.59% worldwide and 2.4% in Europe (EU, 2016; Bonetta et al., 2022). Thus, wastewater reuse could be a promising solution to water scarcity, but it comes with risks and challenges due to the release of harmful microorganisms, such as human pathogenic bacteria, viruses and parasites. If not properly treated, wastewater can pose significant risks to Public Health when the reclaimed wastewater is used particularly for irrigation procedures. A recent European regulation (2020/741) (EU, 2020) established harmonised parameters to ensure the safety of wastewater reuse in agricultural irrigation, with the aim of encouraging the practice and helping to address drought and water scarcity. The EU regulation proposes minimum requirements for reference indicator microorganisms or pathogens and the risk management when wastewater is reused for irrigation purposes. Additional indications within this regulation address heavy metals, pesticides, disinfection by-products, pharmaceuticals (e.g., antibiotics), and antibiotic resistance. The presence of antibiotics in wastewater could influence the selection and spreading of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs). Low levels of antibiotics, i.e., sub-lethal concentrations, may persist in treated wastewater (Manaia et al., 2018), exerting selective pressure on ARB (Mutuku et al., 2022), giving them a survival advantage. Furthermore, heavy metals in wastewater can contribute to antibiotic resistance through mechanisms like co-selection and cross-resistance (Samreen et al., 2021; Engin et al., 2023). Thus, effective wastewater treatment is crucial to remove or inactivate antibiotic resistant and/or pathogenic bacteria. Typically, wastewater treatment plants (WWTPs) are equipped with primary, secondary, and tertiary treatments aiming at reducing nutrients, such as carbon (C), nitrogen (N), and phosphorus (P), as well as faecal bacteria. Moreover, they are not designed to efficiently remove ARB (Bonetta et al., 2023). On the contrary, they can be considered as hotspots for the dissemination of ARGs (Pazda et al., 2019), since it was demonstrated that, in some cases, the tertiary treatments could even select for antibiotic resistance (Di Cesare et al., 2016a). In this context it is important to build a knowledge base on how effective water reuse treatments are in further reducing antibiotic resistance. A limited number of studies evaluating antibiotic resistance dynamics in real-scale water reuse facilities have been published (Rosenberg Goldstein et al., 2014; Turolla et al., 2018; Oliveira et al., 2020; Oliveira et al., 2022; Keenum et al., 2024). In these previous studies, the authors investigated different ARB or ARGs (Rosenberg Goldstein et al., 2014; Turolla et al., 2018; Oliveira et al., 2020; Oliveira et al., 2022; Keenum et al., 2024) or different tertiary treatments (Rosenberg Goldstein et al., 2014; Oliveira et al., 2020; Oliveira et al., 2022). More comprehensive studies are required to explore the potential of water reuse, considering different facility sizes, influent characteristics, and treatment methods applied according to the intended use of the reclaimed water.

Thus, this study aimed to: I) assess the impact of the various treatment steps within a WWTP, designed for effluent reuse, on the dynamics of ARB, ARGs, and pathogens; and II) investigate the correlation between the abundances of ARB and ARGs and the concentrations of antibiotics and heavy metals to explore potential co-selection phenomena. These parameters were monitored in a full-scale WWTP producing effluent intended for agricultural reuse. The reuse of this reclaimed water in agriculture could have a direct impact on human health since antibiotic resistance could spread also in the food chain (Nguyen et al., 2021).

2.1.3. Materials and methods

2.1.3.1. WWTP description and sampling

The investigated WWTP, located in North-East of Italy, has 280,000 population equivalent (p.e.) and treats 19 million m^3 y− 1 of which 5.5 million m^3 y− 1 are reused in agriculture during the irrigation period. The effluent is used for the irrigation of permanent pasture, alfalfa, corn, beet, sorghum, tomato, other vegetables such as melon and watermelon, vineyard. The influent is composed of mixed wastewater, with industrial wastewater accounting for 10% of the total.

After entering, the inlet wastewater is divided into three treatment lines (the third line is the largest one, accounting for an average of 40%) until the secondary treatment (Supplementary material, Fig. S1). After the secondary treatment, the three lines are re-combined into a single treatment line and their product constitutes the WWTP effluent, which, after undergoing additional treatments (i.e., sand filtration, hydrogen peroxide (H_2O_2) and UV treatment) becomes suitable for reuse.

In this study, we collected six different samples (as 24 h integrated samples) at the following steps: A (WWTP influent); B (primary treatment inlet); C (biological treatment inlet); D (secondary treatment effluent); E (WWTP effluent); F (WWTP effluent for agricultural reuse). The B, C and D samples refer to the third WWTP line. The WWTP configuration and the sampling points are shown in Supplementary material, Fig. S1. The six samples were collected during six monthly samplings (January–June 2022) (total number of samples = 36). A 2 L volume of sample was taken from each sampling point, transported refrigerated to the laboratory, and processed within 24 h.

2.1.3.2. ARB quantification

ARB were selected based on their common use in the literature as indicators of antibiotic resistance dynamics in aquatic environments (Munir et al., 2011; Berendonk et al., 2015; Ben et al., 2017; Bonetta et al., 2023; Gholipour et al., 2024) and were evaluated using a previously published method (Bonetta et al., 2023). Briefly, wastewater samples were serially diluted and plated in duplicate on media with and without antibiotic supplementation. ARB were enumerated on R2Agar (VWR International) supplemented with ampicillin, tetracycline and sulfamethoxazole (concentration of 32 mg L^{-1}, 16 mg L^{-1} and 50.4 mg L^{-1}, respectively). Medium without antibiotics was used to determine the total heterotrophic count (HPC). After 30 ◦C incubation for 7 days the colonies were counted and the results were expressed as log CFU mL^{-1}. The antibiotic resistance rate for each antibiotic was calculated as the ratio between the CFU mL^{-1} of each ARB and the CFU mL^{-1} of HPC.

2.1.3.3. DNA extraction

A volume of samples (ranging from 20 to 200 mL) was filtered onto 0.22-μm pore size polycarbonate filter membrane (Millipore). The filters were stored at − 20 ◦C until DNA extraction. The filters were processed using the DNeasy PowerWater kit (Qiagen) according to the manufacturer's instructions. Concentration of the extracted DNA of each sample was quantified by spectrophotometry using a NanoDrop® ND-1000 (NanoDrop Technologies). Extracted DNA was divided in two aliquots: one aliquot was used for ARG quantification and the other one for 16S rRNA gene amplicon sequencing.

2.1.3.4. ARG detection and quantification

ARGs were selected based on some of the most commonly used ones in the literature (Munir et al., 2011; Berendonk et al., 2015; Ben et al., 2017; Sabri et al., 2020; Nguyen et al., 2021; Gholipour et al., 2024; Keenum et al., 2024; Su et al., 2024). The selected genes were *bla*$_{\mathrm{TEM}}$, *tet*A and *sul*II (representative ARGs encoding for the resistance against β-lactams, tetracycline and sulphonamides, respectively) and 16S rRNA gene (used to normalise the ARG abundances).

The gene quantification was performed by droplet digital PCR (ddPCR). Extracted DNA samples were diluted according to the type of sample and to the detected gene target (Supplementary material, Table S1). For the ddPCR, 22 µL of reaction mix were prepared, containing QX200 ddPCR EvaGreen Supermix with primer at concentration of 100 nM and 5 µL of diluted DNA (or 5 µL of nuclease-free water for the no template control). 20 µL of the reaction mix and 70 µL of QX200 Droplet Generation Oil for EvaGreen were transferred to the DG8 Cartridge that was finally placed in the QX200 Droplet Generator (BioRad). Generated droplets were then transferred to a 96-well PCR plate for the DNA amplification with T100 Thermal Cycler (BioRad). The amplification program was 95 ◦C for 30 s, annealing temperature specific for each primer for 1 min at ramp rate of 2 ◦C sec^{-1}, 4 ◦C for 5 min and 90 ◦C for 5 min (Supplementary material, Table S1). After the amplification, the plates were transferred to a QX200 Droplet reader (BioRad) for the DNA quantification. Only reactions with >10,000 droplets were considered. Data obtained with QuantaSoft Analysis Pro Software (BioRad) were expressed as gene copy µL^{-1} of sample. Finally, relative ARG abundances were obtained by dividing the number of each ARG copies by the number of 16S rRNA gene copies.

2.1.3.5. Pathobiome characterization

DNA samples were sent to IGA Technology Services Srl (Udine, Italy) for the 16S rRNA gene amplicon sequencing of the V3-V4 hypervariable region (Herlemann et al., 2011). Raw reads were quality checked, filtered and trimmed, and merged following the DADA2 pipeline, as proposed in the online tutorial (https://benjjneb.github.io/dada2/tutorial_1_8.htmL), in the R environment v4.3.0 (R Core Team, 2019). The obtained amplicon sequence variants (ASVs) were annotated against the Silva database v138.1 to assign taxonomy. Starting from the whole bacterial community, a subset of data (hereafter referred to as pathobiome) was prepared containing only the bacterial genera listed as established human pathogens, as reported by Bartlett and colleagues (Bartlett et al., 2022).

2.1.3.6. Antibiotic quantification

High-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) was used to quantify antibiotics, i.e. ampicillin, tetracycline and sulfamethoxazole as reported in the literature (Lindberg et al., 2004). Prior to treatment, each sample was spiked with a known amount of internal standard (Cortisol D3, Toronto Research Chemicals) for the assessment of extraction recovery and the correction of instrumental response. The sample was filtered through a Büchner filtration apparatus onto filter paper and concentrated (800x) using 47 mm ENVI-18DSK extraction disks (Sigma-Merck). The eluate was dried using a centrifugal evaporator and reconstituted in running solvent. Subsequently, the quantitative analysis was performed using the HPLC-MS/MS, in MRM mode (chromatograph: Nexera XR, Shimadzu; mass analyser: LCMS8045 triple quadrupole, Shimadzu, Kyoto, Japan; chromatographic column: Restek Raptor C18 150 × 3 mm, 2.7 µm). The eluents were 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) (Sigma-Merck, Milan, Italy) in the following gradient conditions: from 20 to 100% of solvent A in 7 min, with final re-equilibration of 5 min. The injection volume was 10 µL, the flow rate was 200 L min^{-1} and the column was maintained at a temperature of 40 °C. A calibration curve was prepared using standard solutions of the analytes at a final concentration of 0.5–200 µg L^{-1}. The instrumental limit of quantification (LOQ) of ampicillin, tetracycline and sulfamethoxazole was 0.01 µg L^{-1} corresponding to 0.013 ng L^{-1} in real samples before preconcentration.

2.1.3.7. Heavy metal quantification

Water samples were filtered and treated with 70% extra pure nitric acid (Thermo Scientific Chemicals) (final acid concentration 0.14%) and directly injected in the ICP source. The inductively coupled plasma-mass spectrometry (ICP- MS) was used to perform the quantification of 28 heavy metal, i.e., silver (Ag), arsenic (As), barium (Ba), bismuth (Bi), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), mercury (Hg), manganese (Mn), molybdenum (Mo), nickel (Ni), lead (Pb), palladium (Pd), platinum (Pt), rubidium (Rb), rhodium (Rh), ruthenium (Ru), antimony (Sb), selenium (Se), tin (Sn), strontium (Sr), titanium (Ti), thallium (Tl), uranium (U), vanadium (V), zinc (Zn), and zirconium (Zr). The analysis was performed with a Thermo iCAP Q instrument (Thermo Scientific) with a quadrupolar analyser. Argon was used for plasma at 15 mL min^{-1} and as atomizing gas at 1 L min^{-1}. RF power was 1.55 kW. Calibration was performed using a multielement standard solution for ICP (Sigma-Merck, Milan, Italy) in the range 0.3–100 µg L^{-1}. LOQ values were in the range 0.003–1.49 µg L^{-1}. LOQ values are calculated as a 10-fold the standard deviation of blank signal.

2.1.3.8. Statistical analysis

The statistical analyses were performed in the R environment with the aim to assess the dynamics of ARB, ARGs and pathobiome along the different treatment steps of the WWTP. To achieve this, the ARB and ARG absolute concentrations were converted to log10 (log CFU mL^{-1} and log gene copies mL^{-1}, respectively); while, the ARB and ARG relative abundances were converted to the arcsin of the square root (arcsin of square root of ARB/HPC and arcsin of square root of gene copies/16s rRNA gene copies, respectively). Differences in absolute and relative concentration between the treatment steps and sampling months were analysed, first, by MANOVA, to evaluate the general trend, and then by ANOVA followed by Tukey's post-hoc test for HPC, single ARB or ARGs. Potential correlations between ARB, ARGs, pathogens, antibiotic and heavy metal concentrations were investigated by Pearson's correlation. The correlation was considered strong and significant when $p < 0.05$ and the correlation coefficient (r) ≥ 0.6. Moreover, for the pathobiome, alfa (as richness, the number of the different genera), and beta diversity (as abundance-based Bray-Curtis dissimilarity index) were determined using the vegan package. Total abundance per sample of pathogenic genera was calculated too. Richness and total abundance were analysed by ANOVA followed by Tukey's post hoc test. Beta-diversity was used to test differences in sample composition via PERMANOVA. In both cases, treatment step and sampling month were used as explanatory variables in the model.

2.1.4. Results and discussion

2.1.4.1. Antibiotic resistant bacteria

Total HPCs, ampicillin-resistant bacteria (AmRB), tetracycline- resistant bacteria (TRB), and sulfamethoxazole-resistant bacteria (SRB) at all steps of the WWTP are shown in Fig. 1. The ARB resistance rate is displayed in Fig. 2. In the influent and in samples B and C, the concentrations ranged from 5.27 to 7.03 log CFU mL^{-1} for AmRB, from 4.37 to 5.93 log CFU mL^{-1} for TRB, and from 5.71 to 7.20 log CFU mL^{-1} for SRB. The highest mean concentration found was 6.73 log CFU mL^{-1} for SRB, and the lowest was 4.88 log CFU mL^{-1} for TRB. The mean resistance rates of AmRB, TRB, and SRB across all three samples were 7.44%, 0.64%, and 24.21%, respectively. The highest ratios observed were 25.00%, 1.81%, and 53.13% for AmRB, TRB, and SRB, respectively, and were consistently found in the C samples. In the effluents (E and F) and the D sample, the concentrations of AmRB ranged from 1.64 to 4.45 log CFU mL^{-1}, TRB from 0.89 to 3.30 log CFU mL^{-1}, and SRB from 1.70 to 4.77 log CFU mL^{-1}. The highest mean concentration observed was 4.21 log CFU mL^{-1} for SRB, while the lowest was 1.70 log CFU mL^{-1} for TRB. The mean resistance rates of AmRB, TRB, and SRB across all three samples were 8.83%, 1.15%, and 18.66%, respectively, with the highest rates recorded in the D samples reaching 18.91%, 3.84%, and 28.67%, respectively. For all the samples, the trend of mean resistance rate was SRB >AmRB > TRB. The concentration of ARB measured in the influent of the WWTP was comparable to that reported in other studies (Munir et al., 2011; Gao et al., 2012; Bonetta et al., 2023). Also the ARB levels monitored in the WWTP effluent were similar (Gao et al., 2012; Bonetta et al., 2023) or lower (Munir et al., 2011) compared to the concentrations reported in the literature.

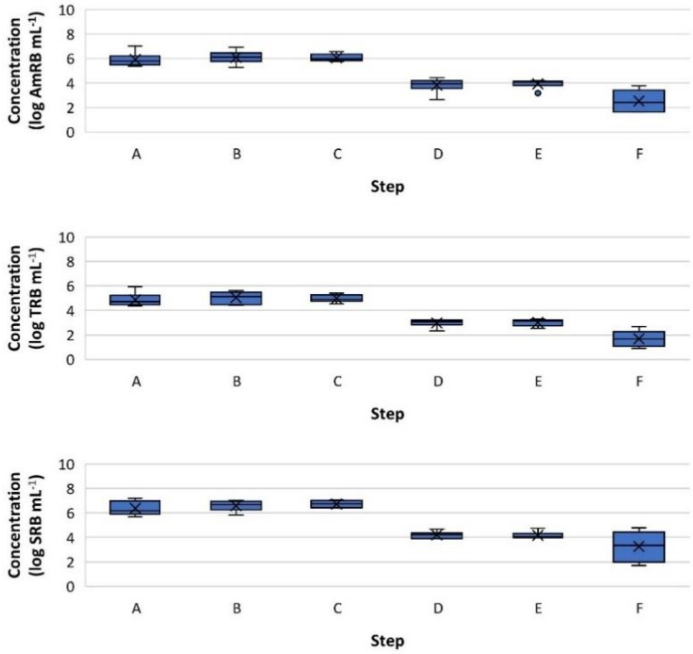

Fig. 1. Log-transformed concentrations of ampicillin-, tetracycline-, sulfamethoxazole-resistant bacteria in the different steps of the WWTP investigated. Box plots represent median and range values. WWTP: wastewater treatment plant; AmRB: ampicillin-resistant bacteria; TRB: tetracycline-resistant bacteria; SRB: sulfamethoxazole-resistant bacteria. Different WWTP steps: A (WWTP influent); B (primary treatment inlet); C (biological treatment inlet); D (secondary treatment effluent); E (WWTP effluent); F (WWTP effluent for reuse).

The statistical analysis revealed significant differences in the treatment steps for HPC, AmRB, TRB and SRB counts (Supplementary material, Table S2): specifically, the concentrations of HPC and all ARB monitored in samples A, B, and C were significantly higher compared to those in samples D, E, and F ($p < 0.05$). Furthermore, the HPC concentration was lower in F sample (effluent for reuse) compared to E sample (effluent) ($p < 0.05$), the SRB concentration was lower in F sample (effluent for reuse) compared to D sample ($p < 0.05$) and the concentrations of AmRB and TRB in F sample were lower compared to samples D and E ($p < 0.01$). Regarding the total abundance of ARB, the F sample showed a statistically significant lower value compared to all the other samples ($p < 0.05$). This demonstrates a substantial reduction in bacterial load achieved through both biological and tertiary treatments.

The comparison between the sampling periods did not reveal any significant differences, except for SRB, which had a lower concentration in the first sampling (January) compared to the last one (June) ($p < 0.05$). A decrease in ARB between the initial wastewater treatments (A, B and C) and the final ones (D, E and F) was also observed in a previous study investigating the dynamics of ARB in a full-scale WWTP (Dias et al., 2022). Moreover, other studies (Rosenberg Goldstein et al., 2014; Turolla et al., 2018; Keenum et al., 2024) found a reduction in ARB abundance following treatment in WWTPs intended for reuse, though they investigated different bacterial species compared to those examined in the present study. Overall, these findings underscore the effectiveness of the wastewater treatment in reducing both heterotrophic flora and ARB. No statistically significant difference was observed in the antibiotic resistance rate for AmRB and TRB among the different treatment steps, except for SRB, where the C step showed higher resistance than the A and E steps ($p < 0.05$)

(Supplementary material, Table S2). These results highlight that both ARB and HPCs generally decreased in a similar manner and, for this reason, the reduction trend observed between influent and effluent, and among the different steps, for ARB absolute quantification (log CFU mL^{-1}), was not observed for ARB relative abundance. This result indicates that the wastewater treatments did not appear to promote the selection of ARB, which is consistent with previous findings (Le et al., 2018; Bonetta et al., 2023). Although some studies have identified the biological treatment as a possible "critical" step, where a potential selection of ARB could occur (Vaz-Moreira et al., 2014; Kurasam et al., 2022), the present study did not observe a statistically significant increase in the antibiotic resistance ratio following the biological treatment. Nevertheless, it is crucial to emphasise that ARB were still detected in the effluent intended for reuse after all the treatments performed, as evidenced by other studies (Munir et al., 2011; Gao et al., 2012; Turolla et al., 2018; Rosenberg Goldstein et al., 2014; Keenum et al., 2024). The performance of specific reuse treatments (i.e., sand filtration, H2O2 and UV radiation) in reducing antibiotic resistance varies widely in the literature. Sand filtration is commonly used in tertiary treatment of wastewater (Burch et al., 2019) and it is effective in removing indicator bacteria from the effluents. However, in another study (Turolla et al., 2018) ARB were still detectable in the effluents after biological treatment and sand filtration. While H_2O_2 combined with UV radiation is a relatively low-cost process, its effectiveness in removing a broader range of pollutants, including antibiotics, is still under investigation (Anjali and Shanthakumar, 2019; Gajdos et al., 2023). One of the main advantages of the UV radiation treatment is that it does not produce disinfection by-products (Hazra et al., 2024). However, the overall UV treatment efficiency is highly dependent on the UV doses applied. Consequently, the performance of this treatment can vary significantly, as reported in the literature (Rizzo et al., 2020).

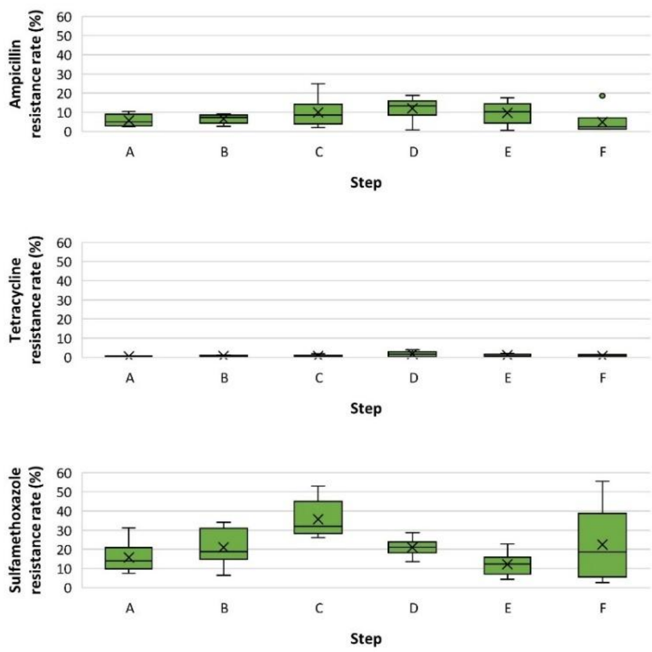

Fig. 2. Antibiotic resistance rate of ampicillin-, tetracycline-, and sulfamethoxazole-resistant bacteria in the different steps of the WWTP investigated. All values are normalised to HPC abundances. Box plots represent median and range values. WWTP: wastewater treatment plant; HPC: total heterotrophic count; different WWTP steps: A (WWTP influent); B (primary treatment inlet); C (biological treatment inlet); D (secondary treatment effluent); E (WWTP effluent); F (WWTP effluent for reuse).

Moreover, it is important to highlight that the ARB may harbour resistance genes and transfer antibiotic resistance determinants to the resident microbial community through horizontal gene transfer (Mosaka et al., 2023). In the context of wastewater reuse in agriculture, this phenomenon represents a significant pathway for the spread of antibiotic resistance, raising concerns for human health. Considering the sampling period, the antibiotic resistance rate differed for AmRB and TRB (Supplementary material, Table S2): specifically, in February and March samplings, higher rates were observed compared to June for AmRB ($p < 0.05$); for TRB, higher rates were measured in February compared to May and June samplings ($p < 0.05$), and in March compared to May and June samplings ($p < 0.01$). The rate observed seems to follow an opposite trend compared to the water flow at the WWTP inlet (higher ARB rate loads, lower water flow) and temperature. Factors such as temperature, precipitation, and water flow rates can affect the performance of treatment plants, but the evidence in the literature is inconsistent. Moreover, seasonal variations in human activities, such as agricultural practices and antibiotic usage, can also influence the composition of wastewater. Concerning the antibiotic use, it is plausible to assume that they are prescribed for the treatment of bacterial infections, which might be higher when outdoor temperatures are low (Harnisz et al., 2020).

2.1.4.2. ARG abundances

The absolute and relative abundance observed for *bla*$_{\mathrm{TEM}}$, *tet*A and *sul*II genes are reported in Figs. 3 and 4, respectively. In the influent and in samples B and C the absolute abundance of ARGs ranged from 3.65 to 5.12 log gene copies mL^{-1} for *bla*$_{\mathrm{TEM}}$, from 4.03 to 5.57 log gene copies mL^{-1} for *tet*A, and from 4.41 to 5.91 log gene copies mL^{-1} for *sul*II. On the contrary, in sample D and in the effluents (E and F), the absolute abundance ranged from 1.47 to 3.09 log gene copies mL^{-1} for *bla*$_{\mathrm{TEM}}$, from 1.88 to 3.69 log gene copies mL^{-1} for *tet*A, and from 2.57 to 4.34 log gene copies mL^{-1} for *sul*II. Across all samples, the lowest and highest mean absolute ARG abundance was measured for the *bla*$_{\mathrm{TEM}}$ and *sul*II genes, respectively. The *sul*II gene followed the same trend of SRB, as they were the most abundant ARG and ARB, respectively. This is not surprising, as this trend is well established in the literature (Munir et al., 2011; Ferro et al., 2016; Ben et al., 2017). The spreading of ARGs in WWTP is well documented in the literature. The genes investigated in the present study were detected in all the wastewater samples, as also observed in the study of Bonanno Ferraro et al. (2024), in which they analysed, among others, also *tet*A and *bla*$_{\mathrm{TEM}}$ genes. In the study by Wang and collaborators (Wang et al., 2021), the absolute abundances of ARGs (*tet*A and *sul*II) were similar to those obtained in this study. Consistently, our data indicated that *sul*II gene showed the highest detection frequencies. This pattern has been previously observed in other studies (Munir et al., 2011; Ferro et al., 2016; Ben et al., 2017).

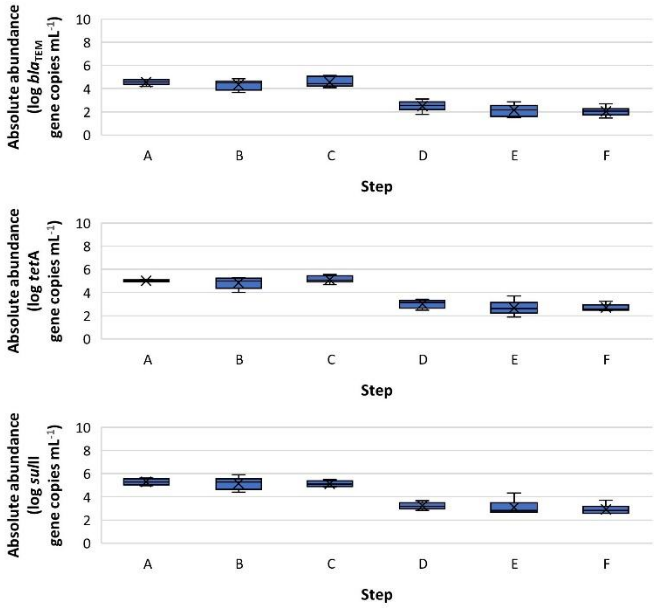

Fig. 3. Log-transformed absolute abundances of *bla*$_{\mathrm{TEM}}$, *tet*A, *sul*II genes in the different steps of the WWTP investigated. Box plots represent median and range values. Different WWTP steps: A (WWTP influent); B (primary treatment inlet); C (biological treatment inlet); D (secondary treatment effluent); E (WWTP effluent); F (WWTP effluent for reuse).

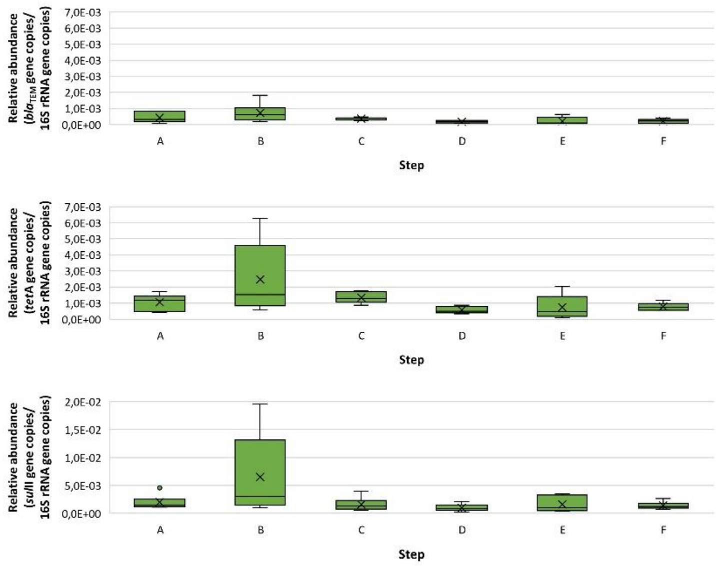

Fig. 4. Relative abundances of *bla*$_{\mathrm{TEM}}$, *tet*A, *sul*II genes in the different steps of the WWTP investigated. All values are normalised to 16S rRNA gene copies. Box plots represent median and range values. Different WWTP steps: A (WWTP influent); B (primary treatment inlet); C (biological treatment inlet); D (secondary treatment effluent); E (WWTP effluent); F (WWTP effluent for reuse).

Also Bonetta and colleagues (Bonetta et al., 2023) reported higher absolute abundance of sulII gene in their study. The variability of ARB and ARG abundance in WWTPs worldwide is well known and may be attributed to the influence of local environmental and anthropogenic factors, such as antimicrobial residue levels, bacterial taxonomic composition, and local antimicrobial use

(Hendriksen et al., 2019). Statistical analysis revealed differences in the ARG abundance among the treatment steps within the WWTP (Supplementary material, Table S3). Specifically, for all ARGs the influent and samples B and C showed higher levels compared to sample D and the effluents E and F ($p < 0.001$). This demonstrates a substantial reduction in bacterial load, particularly through biological treatment. This trend reflects the measured concentration pattern of ARB, confirming that the treatments were effective in reducing absolute ARG abundances along the wastewater production chain as shown in other studies assessing ARG removal by different treatments (Liang et al., 2021), including UV disinfection (Kucukunsal et al., 2020). As previously noted for ARB, ARGs were still present in the effluent samples. This finding is consistent with various studies that investigate antibiotic resistance in WWTPs intended for effluent reuse (Oliveira et al., 2020; Oliveira et al., 2022). Moreover, differences were observed across sampling periods for *tet*A (January > March, April, May and June samplings) ($p < 0.05$) and *bla*TEM (January > April, May and June samplings) ($p < 0.05$) genes. As expected, the total abundance of ARGs (gene copies mL^{-1}) showed a similar trend, being the A, B and C samples > D, E and F samples ($p < 0.001$) and January sampling > March, April and May samplings ($p < 0.05$). Similar to ARB, the influence of the seasonality on ARG abundance within WWTPs remains unclear (Sims et al., 2023). This could be attributed to the different types of WWTPs or to the genes selected for quantification. Sui and collaborators (Sui et al., 2017) found that the abundance of all the quantified genes (*tet*G, *tet*M, *tet*X, *erm*B, *erm*F, *ere*A, and *mef*A, *int*I1) were higher in winter than in summer, indicating a potential seasonal effect on ARG abundance. This is partially in accordance with our results, as certain ARGs (bla_{TEM} and *tet*A) showed significantly higher absolute abundance in January compared to warmer months (such as May and June). The relative ARG abundance in the influent and in samples B and C ranged from 7.70×10^{-5} to 1.83×10^{-3} copies/16S rRNA gene copies for bla_{TEM}, from 4.24×10^{-4} to 6.28×10^{-3} copies/16S rRNA gene copies for *tet*A, and from 5.55×10^{-4} to 1.96×10^{-2} copies/16S rRNA gene copies for *sul*II. Conversely, in sample D and in the effluents (E and F), the relative abundance ranged from 3.51×10^{-5} to 6.37×10^{-4} copies/16S rRNA gene copies for bla_{TEM}, from 8.51×10^{-5} to 2.04×10^{-3} copies/16S rRNA gene copies for *tet*A, and from 2.80×10^{-4} to $3.50 \times 10- 3$ copies/16S rRNA gene copies for *sul*II.

The values of the ARG relative abundances were similar to those reported in other studies performed in municipal WWTPs (Wang et al., 2021; Bonetta et al., 2023) indicating the same proportion between ARG-carrying bacteria and heterotrophic flora. The relative abundance compared to the absolute abundance of ARGs, revealed a different trend (Supplementary material, Table S3). In particular, the bla_{TEM} and *tet*A relative abundances in the different treatment steps was significantly higher in sample B compared to D and E ($p < 0.05$). Additionally, the relative abundance of *sul*II was higher only in sample B compared to D ($p < 0.05$). The total relative abundance of ARGs (gene copies/16S rRNA gene copies) measured in sample B was higher than in samples D and E ($p < 0.05$). The relative abundances of ARGs were significantly different only between the intermediate steps, showing that the inlet of the WWTP had similar relative abundances of ARGs to those measured in the effluent for reuse. This suggests that there are no steps within WWTP that select for ARGs compared to heterotrophic flora as reported in previous studies (Mao et al., 2015; McConnell et al., 2018). In contrast, Bonanno Ferraro et al. (2024) reported an increase in the relative abundance of ARGs in the effluents compared to the influents. Additionally, Keenum et al. (2024) observed occasional increases in the relative abundance of ARGs following chlorination treatment. Furthermore, there was no difference among ARG relative abundances across different sampling periods, indicating that seasonality did not affect them, even though some differences in absolute quantification were detected.

2.1.4.3. Pathobiome

A total of 4189 ASVs were retrieved, representing 553 bacterial genera. Of these ones, 90 genera comprised potential pathogens for humans. Overall, within the pathobiome, the most abundant bacterial genus was Arcobacter, followed by Flavobacterium and Aeromonas (Fig. 5). Looking at the single treatment step, Arcobacter remained the most abundant bacterium from A to D; while, Flavobacterium had the highest abundance in E and F (Fig. 5). In literature, the presence of Arcobacter is reported in WWTPs by several studies in different countries (Al-Jassim et al., 2015; Webb et al., 2016; Wu et al., 2019) and bacteria from this taxon are associated to gastroenteritis. Among others, Arcobacter is consistently identified in WWTP influent and is considered a resident of sewer infrastructure (Yasir et al., 2020; Dias et al., 2022). Recent studies have also highlighted Arcobacter as one of the most prevalent genera in the influent of various WWTPs (Oliveira et al., 2020; Cuetero-Martínez et al., 2023; Bonanno Ferraro et al., 2024). The presence of Aeromonas, again, is not surprising, since it was already detected in other WWTPs (Ye et al., 2011; Yasir et al., 2020). These bacteria are known for their versatility in degrading a variety of organic pollutants and their ability to survive in different environmental conditions (Pessoa et al., 2022). However, Aeromonas species can also include opportunistic pathogens, raising concerns about their presence in effluents, as they might pose a risk to public health if discharged into natural water bodies (Pessoa et al., 2022). Moreover, they are known to harbour ARGs (Baron et al., 2017; Grilo et al., 2020) and, for this reason, along with their potential pathogenicity, they necessitate careful monitoring and control measures to ensure the safety of the treated water released into the environment and reused. The increased presence of Flavobacterium in effluent is reported also in another study (Yasir et al., 2020). Conversely, in the study of Bonanno Ferraro (2024), a slight decrease in Flavobacterium prevalence respect to the influents was observed. These bacteria are commonly found in WWTPs and they are part of the microbial community involved in the decomposition of organic matter. In the effluent of WWTPs, Flavobacterium can be prevalent due to their role in breaking down complex organic compounds (Waskiewicz and Irzykowska, 2014). Their presence in effluent indicates effective biological treatment processes and microbial activity. Usually, human infections are rare but some members can be human opportunistic pathogens (Zurbuchen et al., 2023). Moreover, in this study we used some diversity indexes to analyse the dynamics of the pathobiome along the WWTP. Richness of pathobiome significantly varied throughout the treatments (Fig. 6 and Supplementary material, Table S4). In particular, after the biological treatment (D and E samples), richness significantly decreased in respect with C samples ($p < 0.05$). Our result is in accordance with other studies (Yanget al., 2022; Ríos-Castro et al., 2023), which showed the efficacy of the treatments applied in the WWTPs in reducing pathogen diversity. A possible explanation is that, in the extremely heterogeneous environment present in the activated sludge, some pathogenic bacteria could be unable to grow or could die because of the presence of the inhibitory flora.

Also the total abundance of potential pathogenic bacterial genera followed a similar trend (Fig. 7 and Supplementary material, Table S4). Indeed, the biological treatment determined an abatement of bacterial load, with a significant reduction of pathogen abundances of A and C samples in respect with D, E, and F ones ($p < 0.05$, Supplementary material, Table S4).

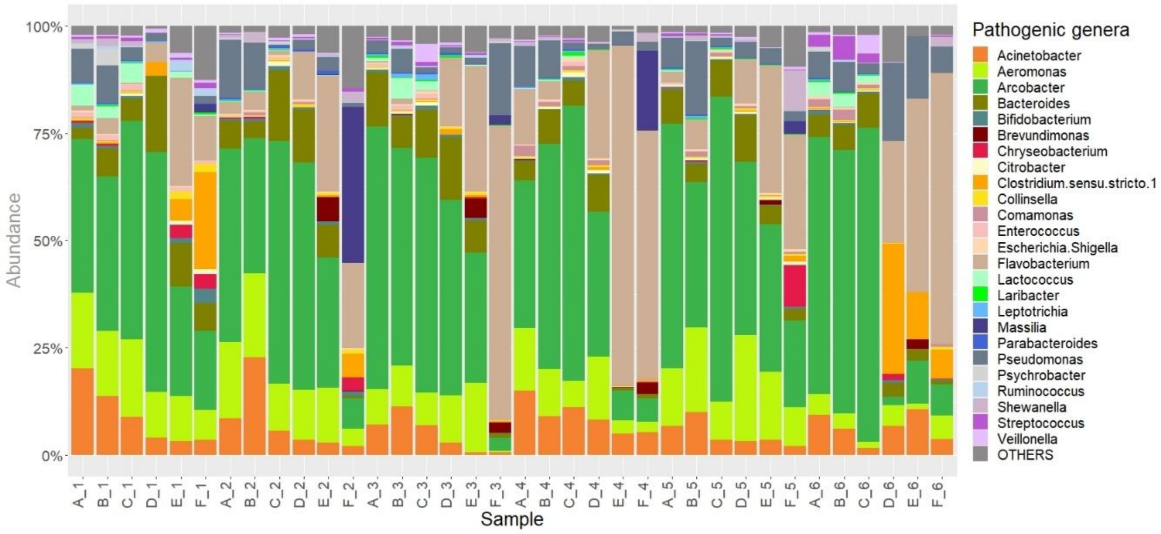

Fig. 5. Abundance of pathogenic bacteria. Different WWTP steps: A (WWTP influent); B (primary treatment inlet); C (biological treatment inlet); D (secondary treatment effluent); E (WWTP effluent); F (WWTP effluent for reuse). Numbers from 1 to 6 indicate the different samplings.

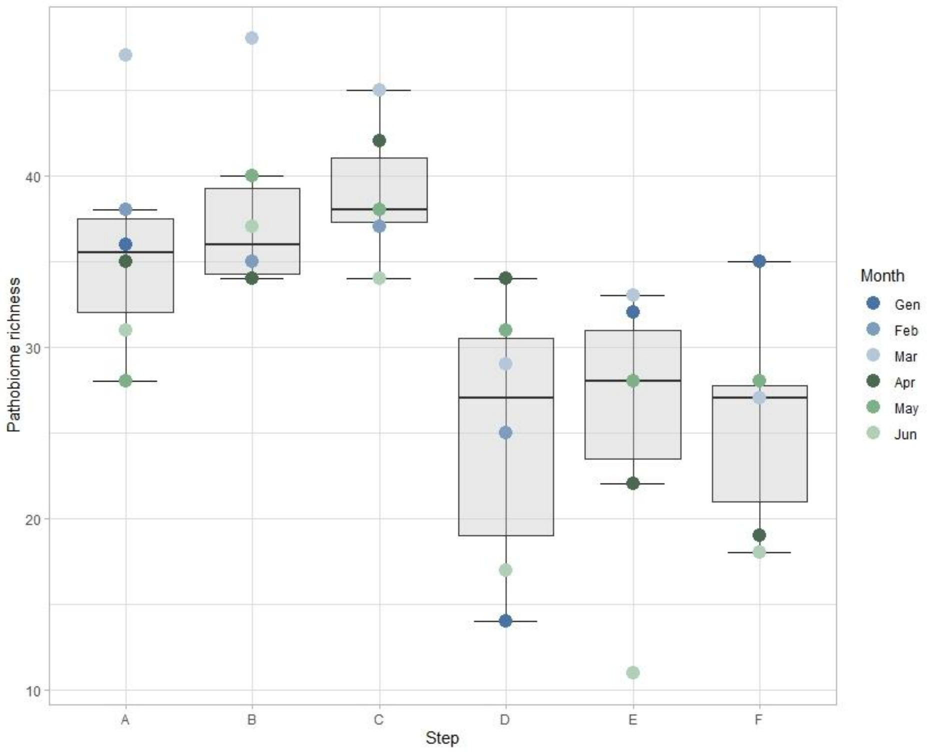

Fig. 6. Pathogen richness (the number of the different genera associated with pathogens) in the different steps of the WWTP investigated. Box plots represent median and range values. Different WWTP steps: A (WWTP influent); B (primary treatment inlet); C (biological treatment inlet); D (secondary treatment effluent); E (WWTP effluent); F (WWTP effluent for reuse).

Regarding the beta diversity, the treatment step is the main factor driving the pathobiome composition and explains 59.2% of sample variance ($p < 0.05$, Supplementary material, Table S4). In general, it is possible to observe a significant dissimilarity between the samples before the biological treatment and the ones after it ($p < 0.05$, Supplementary material, Table S4), as shown in the compositional tree, based on Bray-Curtis dissimilarity index, which clustered A, B and C samples in same branch (Fig. 8). This diversity in the pathobiome composition observed in the different steps of wastewater treatment is in accordance with other studies (Schneeberger et al., 2019; Zhang et al., 2020; Verburg et al., 2021) and could be due to different factors. The variation of nutrients, pH, oxygen and chemical substance presence could influence the growth of different microbial groups, affecting the bacterial diversity and the pathobiome composition (Tong et al., 2019).

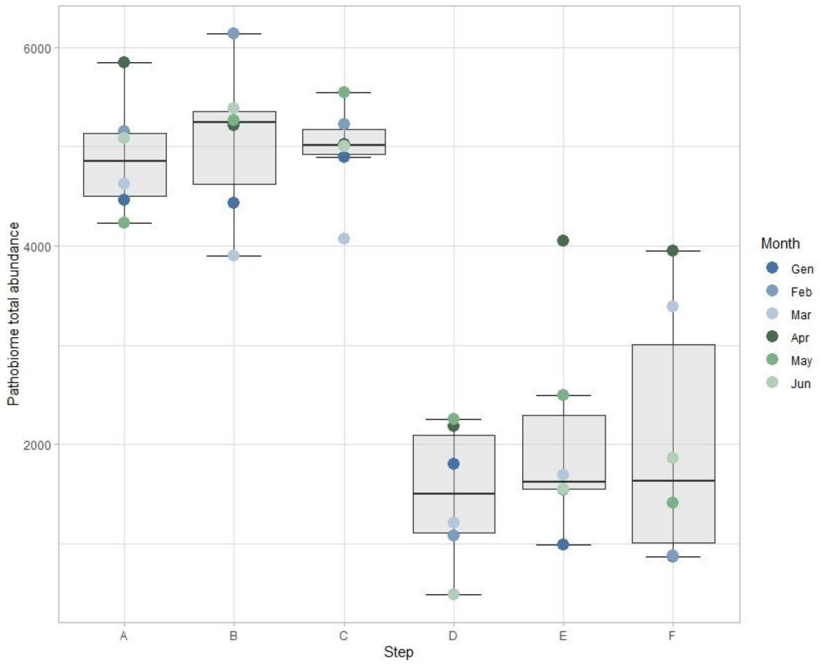

Fig. 7. Pathobiome total abundance in the different steps of the WWTP investigated. Box plots represent median and range values. Different WWTP steps: A (WWTP influent); B (primary treatment inlet); C (biological treatment inlet); D (secondary treatment effluent); E (WWTP effluent); F (WWTP effluent for reuse).

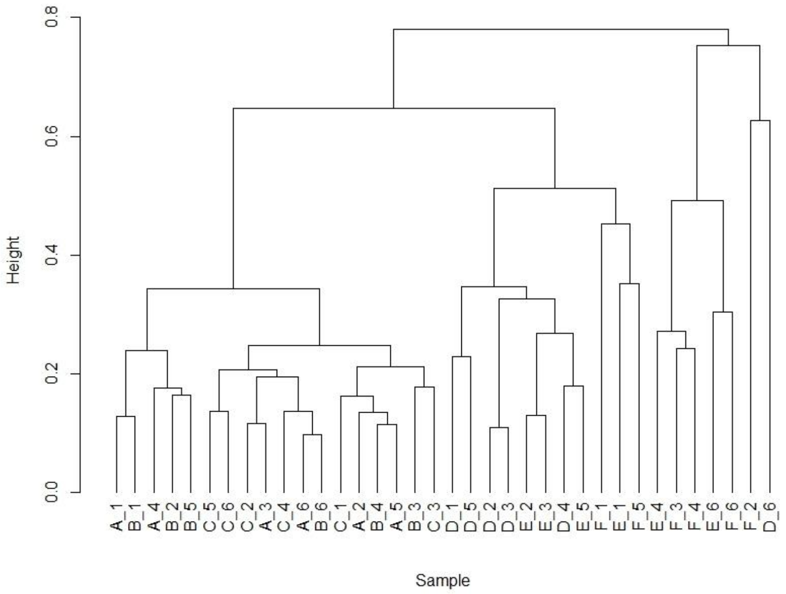

Fig. 8. Beta diversity (as abundance-based Bray-Curtis dissimilarity index) of pathogen community within the different treatment steps. Different WWTP steps: A (WWTP influent); B (primary treatment inlet); C (biological treatment inlet); D (secondary treatment effluent); E (WWTP effluent); F (WWTP effluent for reuse). Numbers from 1 to 6 indicate the different samplings.

Moreover, wastewater contains organic and inorganic components that, during the treatment are degraded or transformed by various microorganisms, leading to the selection of different species (Wang et al., 2017). The effluent can also contain bacteria that are introduced by the treatment itself, e.g., biofilm or active sludge, and they could not be present in the original influent. In addition, also the environmental selective pressure present within wastewaters could exert an activity favouring some microbial groups to the detriment of others (such as pathogenic genera) (Marutescu et al., 2023). However, it is important to highlight that, even if pathobiome richness and abundance decreased along the WWTP, the pathogenic bacteria still present could be released into the environment and reach humans in the case of water reuse.

2.1.4.4. Antibiotics and heavy metals

All the monitored antibiotics were detected at very low levels across the samples within WWTP (Supplementary materials, Table S5), with ampicillin being the most detected in the WWTP both in the influent (mean of 0.122 ng L^{-1}) and effluent for reuse (mean of 0.056 ng L^{-1}) and tetracycline the lowest in the influent and effluent for reuse (<LOQ). The intermediate steps B and C, instead, reported a different trend because tetracycline showed the highest values (0.053 and 0.038 ng L^{-1}, respectively). For D and E samples, the ampicillin again showed the highest values (0.129 ng L^{-1} and 0.073 ng L^{-1}, respectively). Regarding heavy metals, 7 were present in quantities < LOQ in every WWTP step (Table 1) but in general, all the concentrations detected were very low. For all the WWTP steps, the most present metal was Sr with the highest concentration detected in the influent at 939 µg L^{-1}. The lowest concentration, instead, was detected for Pd in the C sample (0.045 µg L^{-1}). These data were used to study the correlation with ARB and ARGs.

	WWTP steps						
	A	**B**	**C**	**D**	**E**	**F**	
	Mean concentration (µg L^{-1})						**Mean LOQ (µg L^{-1})**
Ag	0.487	0.482	1.02 ± 1.15	< LOQ	< LOQ	< LOQ	0.200
As	0.460 ± 0.167	0.402 ± 0.164	0.298 ± 0.099	0.295 ± 0.063	0.311 ± 0.200	0.293 ± 0.094	0.043
Ba	73.9 ± 6.26	58.2 ± 17.2	46.0 ± 5.72	31.5 ± 8.19	29.5 ± 11.1	29.4 ± 8.30	0.249
Bi	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.023
Cd	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.018
Co	0.462 ± 0.130	0.549 ± 0.330	0.279 ± 0.124	0.473 ± 0.086	0.351 ± 0.121	0.397 ± 0.109	0.030
Cr	1.23 ± 0.521	0.879 ± 0.383	0.682 ± 0.401	0.454 ± 0.203	0.629 ± 0.488	0.394 ± 0.227	0.116
Cu	1.33 ± 1.80	0.597 ± 0.376	0.235 ± 0.109	0.483 ± 0.246	1.08 ± 0.961	0.502 ± 0.197	0.040
Hg	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.792

Mn	67.2 ± 11.8	69.9 ± 30.2	55.2 ± 16.6	56.5 ± 27.8	47.7 ± 32.1	57.1 ± 24.5	0.069
Mo	2.12 ± 1.16	2.18 ± 1.69	0.975 ± 0.394	3.17 ± 1.27	2.30 ± 1.42	2.29 ± 1.33	0.304
Ni	9.71 ± 3.52	13.0 ± 10.8	3.09 ± 0.819	7.31 ± 1.32	4.96 ± 1.79	4.88 ± 0.942	0.101
Pb	0.232 ± 0.113	0.100 ± 0.073	0.492 ± 0.387	0.083 ± 0.038	0.082 ± 0.060	0.208 ± 0.136	0.043
Pd	< LOQ	0.061 ± 0.025	0.045	< LOQ	< LOQ	< LOQ	0.041
Pt	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.003
Rb	10.5 ± 1.88	8.26 ± 3.54	7.53 ± 2.23	10.1 ± 2.34	8.51 ± 2.97	8.86 ± 2.09	0.104
Rh	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.007
Ru	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.028
Sb	0.300 ± 0.045	0.105 ± 0.011	0.125 ± 0.039	0.388 ± 0.095	0.160 ± 0.065	0.156 ± 0.041	0.048
Se	1.13 ± 0.570	1.03 ± 0.737	0.496 ± 0.099	0.502 ± 0.129	0.447 ± 0.228	0.426 ± 0.112	0.165
Sn	0.198	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.139
Sr	939 ± 117	796 ± 223	620 ± 103	878 ± 180	813 ± 306	775 ± 181	0.305
Ti	< LOQ	0.473	< LOQ	< LOQ	< LOQ	< LOQ	0.356
Tl	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.007
U	0.645 ± 0.092	0.534 ± 0.163	0.372 ± 0.107	0.495 ± 0.133	0.606 ± 0.465	0.465 ± 0.140	0.010
V	0.328 ± 0.194	0.301 ± 0.129	0.242 ± 0.072	0.306 ± 0.125	0.732 ± 1.15	0.263 ± 0.127	0.101
Zn	14.0 ± 9.73	8.47 ± 5.14	10.5 ± 7.56	19.4 ± 6.67	15.8 ± 3.96	15.4 ± 5.15	1.46
Zr	0.066 ± 0.025	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.051

Table 1. Overall mean heavy metal concentration (μg L^{-1}) ± standard deviation (SD). LOQ: limit of quantification; A: WWTP influent; B: primary treatment inlet; C: biological treatment inlet; D: secondary treatment effluent; E: WWTP effluent; F: WWTP effluent for reuse.

2.1.4.5. Association of ARB and ARGs with antibiotics, heavy metals and pathogens

The Pearson's correlation test did not indicate any correlation between antibiotic concentration and related ARB and ARGs (neither absolute nor relative abundance). The correlation between the levels of ARG and ARB abundances in WWTPs with antibiotic concentrations remains unclear, as the results in the literature are discordant. In other studies (Gao et al., 2012; Li et al., 2015), a correlation between TRB and tetracycline was found, albeit at higher antibiotic concentrations than those measured in our study. Conversely, they did not observe any correlation between *tet* genes (*tet*O and *tet*W) and tetracycline but a correlation among *sul*I gene and SRB and sulphonamide was observed. Moreover, according to our work, in the study of Hubeny and collaborators (Hubeny et al., 2021), no correlations between sulphonamide and tetracycline concentrations (comparable to our study) and the most prevalent genes encoding resistance to these antibiotics were observed. The inconsistent results from literature support the idea that the selective pressure exerted by the antibiotics, often present at very low levels, as in the present study, is not the only mechanism selecting for antibiotic resistance.

In addition, heavy metals, which persist longer in wastewater, can contribute to the selection and spread of antibiotic resistance (Di Cesare et al., 2016b; Hubeny et al., 2021). The presence of heavy metals in wastewater could, in presence of ARGs, contribute to the increasing amount of ARB in the receiving environment (Hazra et al., 2024). In the present study, the statistical analysis showed some correlations between ARB (AmRB, TRB and ARB total abundance), *sul*II (both absolute and relative concentration) and heavy metals (Cu, Ni and Se) (Supplementary materials, Table S6). According to our results, other studies have also observed a positive correlation between *sul*II and Ni (Hubeny et al., 2021; Zhang et al., 2016). This could be due to the fact that *sul* genes are among the most widespread genes in wastewater environments and, in a similar way, Ni is one of the most distributed heavy metals in the same setting.

Compared to the association between ARGs and heavy metals, the correlation between ARB and heavy metals has been less thoroughly explored. Interestingly, we found an association between TRB, AmRB and Cu and between TRB, ARB total abundance and Se ($r > 0.6$, $p < 0.001$, Supplementary materials, Table S6). To the best of our knowledge there are no studies deepening this aspect in WWTPs. However, in the study by Icgen and collaborators (Icgen et al., 2014), multiple co-resistance to heavy metals, including Cu, and to antibiotics in strains isolated from a river was observed. The association between Se and ARB, remains unclear since this finding has not been previously obtained. These relationships are complex and multifactorial. Furthermore, they can be influenced by heavy metal concentrations, different bacterial species, and environmental conditions that vary depending on the specific wastewater treatment step being considered (Engin et al., 2023). Also, the correlation between the ARG total abundance and pathobiome total abundance was statistically significant ($r = 0.6912$, $p = 0.0000$), indicating that, probably, there is a consistent part of resistance genes carried by pathogenic bacteria. The association of ARGs with pathogens is supported by other studies (Fan et al., 2018; Narciso-da-Rocha et al., 2018; Leroy-Freitas et al., 2022).

2.1.5. Conclusions

Although the concentration of various micropollutants, including potentially pathogenic bacteria and ARGs, decreased significantly from the inlet to the final effluent in WWTP, these contaminants were still detectable in treated wastewater intended for reuse. This once again indicates that wastewater is a source of antibiotic resistant and potentially pathogenic bacteria. Consequently, these micropollutants might spread into the environment through irrigation, potentially reaching humans and posing a public health risk. Understanding the dynamics of antibiotic resistant pathogens within WWTPs is critical for developing effective monitoring and management strategies. However, regulations are needed because, among all the parameters monitored in this study, only heavy metals are subject to control, with their levels consistently below Italian legislative limits (Legislative Decree 152/2006). Considering this aspect, it seems relevant that the upcoming future EU directive on urban wastewater treatment specifies the need to monitor the phenomenon of antibiotic resistance (EU, 2022). The importance of monitoring antimicrobial resistance in the environment to assess the potential risks it poses to human health has also been emphasised by the Council of the European Union (2023). Given this, further research into antibiotic resistance is essential, as our current knowledge in this area remains limited. This is particularly crucial when considering wastewater reuse, which is an important strategy to address water scarcity.

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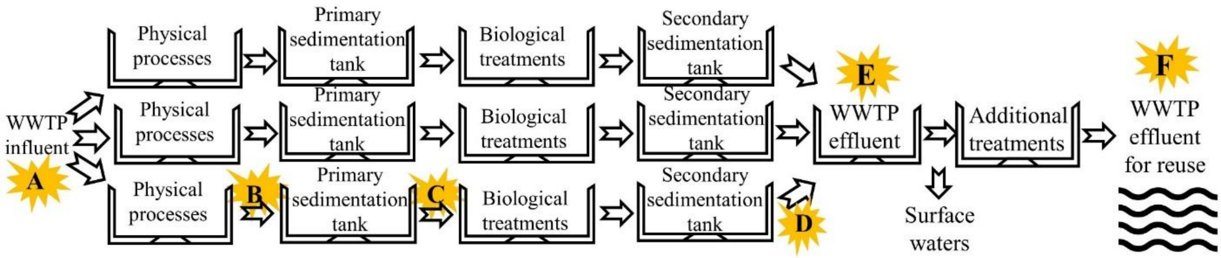
2.1.7. Supplementary materials

Supplementary Fig. S1. Schematic representation of the studied WWTP with the sampling points analysed. The plant is composed of three different lines with the third line representing the biggest one. The additional treatments applied to the effluent for reuse are: sand filtration, hydrogen peroxide (optional) and UV disinfection. A: WWTP influent; B: primary treatment inlet; C: biological treatment inlet; D: secondary treatment effluent; E: WWTP effluent; F: WWTP effluent for reuse.



Supplementary Table S1. Primer pairs and annealing temperatures used to quantify 16S rRNA gene and antibiotic resistance genes with droplet digital PCR (ddPCR).

Target Name	Primer name	Primer sequence (5'- 3')	Amplicon size (bp)	Annealing temperature (°C)	Optimal sample dilution	Reference
16S rRNA	Bact1369F	CGGTGAATACGTTCYCGG	142	55	10^{-5}-10^{-6}	Modified from Suzuki et al., 2000
	Prok1492R	GGHTACCTTGTTACGACTT				
***tet*A**	tet(A)F	GCTACATCCTGCTTGCCTTC	210	59.6	10^{-1}- 10^{-2}	Ng et al., 2001
	tet(A)R	CATAGATCGCCGTGAAGAGG				
***bla*TEM**	blaTEMF	TTCCTGTTTTTGCTCACCCAG	112	56.3	10^{-1}- 10^{-2}	Bibbal et al., 2007
	blaTEMR	CTCAAGGATCTTACCGCTGTTG				
***sul*II**	sulIIF	TCCGGTGGAGGCCGGTATCTGG	191	58.5	10^{-1}- 10^{-2}	Pei et al., 2006
	sulIIR	CGGGAATGCCATCTGCCTTGAG				

Supplementary Table S2. Results of Manova test and ANOVA test (followed by Tukey's post-hoc test) considering the heterotrophic plate count (HPC) and antibiotic resistant bacteria (ARB) (AmRB: ampicillin-resistant bacteria; TRB: tetracycline-resistant bacteria; SRB: sulfamethoxazole-resistant bacteria). Considering treatment steps, A: WWTP influent; B: primary treatment inlet; C: biological treatment inlet; D: secondary treatment effluent; E: WWTP effluent; F: WWTP effluent for reuse. Considering sampling months: Jan: January; Feb: February; Mar: March; Apr: April; Jun: June; n.s.: not significant.

	Differences between treatment steps		Differences between sampling month	
	Absolute concentration	**Relative concentration**	**Absolute concentration**	**Relative concentration**
Manova	p = 0.0000	p = 0.0094	p = 0.0000	p = 0.0001
HPC	Anova: p = 0.0000 A, B, C > D, E, F: p = 0.0000 E > F: p = 0.011	-	n.s.	-
AmRB	Anova: p = 0.0000 A, B, C > D, E, F: p = 0.0000 D > F: p = 0.0082 E > F: p = 0.0035	n.s.	n.s.	Anova: p = 0.0036 Feb > Jun: p = 0.0108 Mar > Jun: p = 0.0012
TRB	Anova: p = 0.0000 A, B, C > D, E, F: p = 0.0000 D > F: p = 0.0064 E > F: p = 0.0048	n.s.	n.s.	Anova: p = 0.0011 Feb > May: p = 0.0458 Feb > Jun: p = 0.0108 Mar > May: p = 0.0083 Mar > Jun: p = 0.0012
SRB	Anova: p = 0.0000 A, B, C > D, E, F: p = 0.0000 D > F: p = 0.0376	Anova: p = 0.0060 A < C: p = 0.0171 C > E: p = 0.0026	Anova: p = 0.0007 Jan < Jun: p = 0.0347	n.s.
ARB total abundance	Anova: p = 0.0000 A, B, C > D, E, F: p = 0.0000 D > F: p = 0.0298 E > F: p = 0.0273	-	n.s.	-

Supplementary Table S3. Results of Manova test and ANOVA test (followed by Tukey's post-hoc test) considering the antibiotic resistance genes (ARGs). Considering treatment steps, A: WWTP influent; B: primary treatment inlet; C: biological treatment inlet; D: secondary treatment effluent; E: WWTP effluent; F: WWTP effluent for reuse. Considering sampling months, Jan: January; Feb: February; Mar: March; Apr: April; Jun: June. N.s.: not significant.

	Differences between treatment steps		Differences between sampling month	
	Absolute concentration	**Relative concentration**	**Absolute concentration**	**Relative concentration**
Manova	p = 0.0000	p = 0.0498	p = 0.0018	n.s.
bla$_{TEM}$	Anova: p = 0.0000 A, B, C > D, E, F: p = 0.0000	Anova: p = 0.0204 B > D: p = 0.0377 B > E: p = 0.0361	Anova: p = 0.0000 Jan > Apr: p = 0.0059 Jan > May: p = 0.0210 Jan > Jun: p = 0.0023	n.s.
*tet*A	Anova: p = 0.0000 A, B, C > D, E, F: p = 0.0000	Anova: p = 0.0172. B > D: p = 0.0214 B > E: p = 0.0301	Anova: p = 0.0010 Jan > Mar: p = 0.0351 Jan > Apr: p = 0.0127 Jan > May: p = 0.0414 Jan > Jun: p = 0.0150	n.s.
*sul*II	Anova: p = 0.0000 A, B, C > D, E, F: p = 0.0000	Anova: p = 0.0278 B > D: p = 0.0197	n.s.	n.s.
ARG total abundance	Anova: p = 0.0000 A, B, C > D, E, F: p = 0.0000	Anova: p = 0.0175 B > D: p = 0.0128 B > E: p = 0.0372	Anova: p = 0.0082 Jan > Mar: p = 0.0277 Jan > Apr: p = 0.0220 Jan > May: p = 0.0115	n.s.

Supplementary Table S4. Results of Anova followed by Tukey's post hoc (for richness and total abundance of pathogen) and of PERMANOVA (used for beta diversity). A: WWTP influent; B: primary treatment inlet; C: biological treatment inlet; D: secondary treatment effluent; E: WWTP effluent; F: WWTP effluent for reuse. N.s.: not significant.

	Differences between treatment steps	Differences between sampling month
Pathobiome richness	Anova: p = 0.0017 A > D: p = 0.0133 A > E: p = 0.0137 A > F: p = 0.0380 C > D: p = 0.0327 C > E: p = 0.0337	n.s.
Pathobiome total abundance	Anova: p = 0.0000 A > D: p = 0.0133 A > E: p = 0.0137 A > F: p = 0.0380 C > D: p = 0.0327 C > E: p = 0.0337	n.s.
Beta diversity	Permanova: p = 0.001 A < D: p = 0.0075 A < E: p = 0.0075 A < F: p = 0.0075 B < C: p = 0.0245 B < D: p = 0.0075 B < E: p = 0.0075 B < F: p = 0.0075	n.s.

	C < D: p = 0.0075 C < E: p = 0.0083 C < F: p = 0.0075 D < F: p = 0.0120	

Supplementary Table S5. Overall mean antibiotic concentration (ng L^{-1}) ± standard deviation (SD). LOQ: limit of quantification; A: WWTP influent; B: primary treatment inlet; C: biological treatment inlet; D: secondary treatment effluent; E: WWTP effluent; F: WWTP effluent for reuse.

	WWTP steps					
	A	**B**	**C**	**D**	**E**	**F**
	Mean concentration (ng L^{-1})					
Ampicillin	0.122 ± 0.069	0.014 ± 0.023	< LOQ	0.129 ± 0.066	0.073 ± 0.004	0.056 ± 0.027
Tetracycline	< LOQ	0.053 ± 0.073	0.038 ± 0.057	< LOQ	< LOQ	< LOQ
Sulfamethoxazole	0.073 ± 0.113	0.028 ± 0.027	0.037 ± 0.018	0.080 ± 0.100	< LOQ	< LOQ

Supplementary Table S6. Results of Pearson's correlation between antibiotic resistant bacteria and antibiotic resistance genes (absolute and relative concentration and their total abundances) with heavy metals. The correlation was considered strong and significant when the correlation coefficient (r) ≥ 0.6 and p < 0.05. Bold font indicates statistically significant results. HPC: heterotrophic plate count; AmRB, ampicillin-resistant bacteria; TRB, tetracycline-resistant bacteria; SRB: sulfamethoxazole-resistant bacteria.

	Antibiotic resistant bacteria (ARB)							**Antibiotic resistance genes (ARGs)**								
	Absolute concentration				**Relative concentration**			**Absolute concentration**				**Relative concentration**				
	AmRB	**TRB**	**SRB**	**ARB total abundance**	**AmRB /HPC**	**TRB /HPC**	**SRB /HPC**	***bla***$_{TEM}$	***tet*A**	***sul*II**	**ARG total abundance**	***bla***$_{TEM}$ **/16S rRNA**	***tet*A /16S rRNA**	***sul*II /16S rRNA**	**ARG total abundance**
Heavy metals																
Ag	r = -0.0366 p = 0.8323	r = 0.0253 p = 0.8834	r = 0.3252 p = 0.0530	r = 0.2136 p = 0.2115	r = -0.1473 p = 0.3914	r = - 0.1175 p = 0.4949	r = 0.2641 p = 0.1195	r = 0.0476 p = 0.7826	r = 0.1164 p = 0.4991	r = 0.1599 p = 0.3515	r = 0.1496 p = 0.3838	r = 0.1321 p = 0.4426	r = 0.1308 p = 0.447	r = 0.0898 p = 0.6026	r = 0.1077 p = 0.5320
As	r = 0.4628 p = 0.0045	r = 0.5575 p = 0.0004	r = 0.4694 p = 0.0039	r = 0.5072 p = 0.0016	r = -0.3054 p = 0.0701	r = -0.1968 p = 0.2499	r = 0.0727 p = 0.6735	r = 0.1699 p = 0.3218	r = 0.1360 p = 0.4291	r = 0.2735 p = 0.1065	r = 0.2460 p = 0.148	r = -0.0653 p = 0.7051	r = -0.1555 p = 0.3652	r = -0.0314 p = 0.8557	r = -0.0653 p = 0.7050
Ba	r = 0.4213 p = 0.0105	r = 0.5243 p = 0.0010	r = 0.5270 p = 0.0010	r = 0.5314 p = 0.0009	r = -0.2358 p = 0.1663	r = -0.2873 p = 0.0894	r = 0.1085 p = 0.5289	r = 0.4407 p = 0.0071	r = 0.4445 p = 0.0066	r = 0.5624 p = 0.0004	r = 0.5723 p = 0.0003	r = 0.2191 p = 0.1991	r = 0.0912 p = 0.5967	r = 0.1667 p = 0.3312	r = 0.1617 p = 0.3461
Co	r = 0.1064 p = 0.5369	r = 0.2151 p = 0.2076	r = 0.0354 p = 0.8374	r = 0.0686 p = 0.6910	r = 0.0863 p = 0.6166	r = 0.2921 p = 0.0838	r = -0.1274 p = 0.4592	r = 0.0767 p = 0.6565	r = 0.0287 p = 0.8682	r = 0.3860 p = 0.0202	r = 0.2722 p = 0.1083	r = 0.0487 p = 0.7778	r = -0.0107 p = 0.9508	r = 0.3636 p = 0.0293	r = 0.2776 p = 0.1012
Cr	r = 0.2679 p = 0.1142	r = 0.3059 p = 0.0696	r = 0.1445 p = 0.4005	r = 0.2048 p = 0.2308	r = -0.0268 p = 0.8765	r = -0.0267 p = 0.8770	r = -0.2613 p = 0.1238	r = 0.5544 p = 0.0005	r = 0.5525 p = 0.0005	r = 0.5130 p = 0.0014	r = 0.5901 p = 0.0002	r = 0.1006 p = 0.5596	r = -0.0107 p = 0.9508	r = 0.0698 p = 0.6859	r = 0.0566 p = 0.743

Cu	**r = 0.6559 p = 0.0000**	**r = 0.6690 p = 0.0000**	r = 0.3395 p = 0.0428	r = 0.4895 p = 0.0024	r = -0.0694 p = 0.6876	r = - 0.0467 p = 0.7868	r = -0.2662 p = 0.1166	r = 0.0837 p = 0.6274	r = -0.0716 p = 0.6782	r = 0.1145 p = 0.5059	r = 0.0619 p = 0.7200	r = 0.1538 p = 0.3704	r = -0.0430 p = 0.8034	r = 0.0335 p = 0.846	r = 0.0257 p = 0.8818
Mn	r = 0.1383 p = 0.4210	r = 0.2069 p = 0.2261	r = 0.0764 p = 0.658	r = 0.1083 p = 0.5296	r = -0.0584 p = 0.7351	r = -0.0228 p = 0.895	r = 0.1329 p = 0.4399	r = 0.1426 p = 0.4067	r = 0.0815 p = 0.6366	r = 0.0290 p = 0.8669	r = 0.0641 p = 0.7105	r = -0.1063 p = 0.5371	r = -0.2183 p = 0.2009	r = -0.1775 p = 0.3005	r = -0.1921 p = 0.2617
Mo	r = 0.0264 p = 0.8784	r = 0.0732 p = 0.6714	r = -0.0733 p = 0.671	r = -0.0395 p = 0.8193	r = -0.2871 p = 0.0895	r = -0.0141 p = 0.935	r = -0.1568 p = 0.361	r = -0.3983 p = 0.1613	r = -0.4514 p = 0.0057	r = -0.1963 p = 0.2512	r = -0.3291 p = 0.0500	r = -0.3813 p = 0.0218	r = -0.3666 p = 0.0278	r = -0.1652 p = 0.3355	r = -0.2382 p = 0.1618
Ni	r = 0.1576 p = 0.3587	r = 0.2880 p = 0.0885	r = 0.3123 p = 0.0636	r = 0.2812 p = 0.0966	r = -0.1510 p = 0.3793	r = 0.0245 p = 0.887	r = -0.0121 p = 0.9441	r = 0.0664 p = 0.7003	r = 0.1125 p = 0.5134	**r = 0.6482 p = 0.0000**	r = 0.4709 p = 0.0037	r = 0.2000 p = 0.2422	r = 0.2337 p = 0.1707	**r = 0.6412 p = 0.0000**	r = 0.5478 p = 0.0005
Pb	r = 0.3501 p = 0.0363	r = 0.3049 p = 0.0706	r = 0.0743 p = 0.6667	r = 0.1856 p = 0.2784	r = 0.2942 p = 0.0816	r = 0.1121 p = 0.5152	r = -0.0931 p = 0.5892	r = 0.4698 p = 0.0038	r = 0.2213 p = 0.1945	r = -0.0320 p = 0.8529	r = 0.1118 p = 0.5161	r = 0.1297 p = 0.451	r = -0.0523 p = 0.762	r = -0.1194 p = 0.4878	r = -0.0922 p = 0.5929
Pd	r = -0.1130 p = 0.5117	r = -0.0255 p = 0.8824	r = 0.1544 p = 0.3683	r = 0.0660 p = 0.7023	r = -0.2255 p = 0.1860	r = -0.1389 p = 0.4191	r = 0.0283 p = 0.8697	r = -0.0577 p = 0.7384	r = 0.0315 p = 0.8554	r = 0.3619 p = 0.0301	r = 0.2406 p = 0.1575	r = 0.1347 p = 0.4336	r = 0.2268 p = 0.1835	r = 0.5082 p = 0.0016	r = 0.4425 p = 0.0069
Rb	r = -0.0897 p = 0.6029	r = -0.0659 p = 0.7026	r = -0.3713 p = 0.0258	r = -0.2919 p = 0.0841	r = 0.2696 p = 0.1118	r = 0.4419 p = 0.0070	r = -0.2941 p = 0.0816	r = 0.1688 p = 0.3251	r = 0.0525 p = 0.7612	r = -0.0856 p = 0.6197	r = -0.0174 p = 0.9197	r = -0.2848 p = 0.0923	r = -0.4323 p = 0.0085	r = -0.3702 p = 0.0262	r = -0.3997 p = 0.0157
Sb	r = 0.0542 p = 0.7537	r = 0.0565 p = 0.7434	r = -0.2017 p = 0.2382	r = -0.1190 p = 0.4892	r = 0.1404 p = 0.4141	r = 0.3048 p = 0.0707	r = -0.1813 p = 0.2898	r = -0.1246 p = 0.4691	r = -0.2467 p = 0.1468	r = -0.1832 p = 0.2848	r = -0.2180 p = 0.2016	r = -0.2449 p = 0.1500	r = -0.3808 p = 0.0219	r = -0.3194 p = 0.0575	r = -0.3467 p = 0.0383

Se	r = 0.5333 p = 0.0008	**r = 0.6702 p = 0.0000**	r = 0.5734 p = 0.0003	**r = 0.6079 p = 0.0000**	r = -0.1521 p = 0.3755	r = -0.0722 p = 0.6756	r = -0.0045 p = 0.9793	r = 0.2985 p = 0.0770	r = 0.2611 p = 0.1241	r = 0.5254 p = 0.0010	r = 0.4691 p = 0.0039	r = 0.1108 p = 0.5199	r = 0.0105 p = 0.9515	r = 0.2311 p = 0.1751	r = 0.1825 p = 0.2866
Sr	r = 0.0881 p = 0.6093	r = 0.1569 p = 0.3607	r = -0.0930 p = 0.5900	r = -0.0286 p = 0.8683	r = 0.0492 p = 0.7756	r = 0.3281 p = 0.0507	r = -0.1454 p = 0.3976	r = -0.0574 p = 0.7393	r = 0.5082 p = 0.0016	r = -0.0101 p = 0.9535	r = -0.0603 p = 0.7270	r = -0.2890 p = 0.0885	r = -0.4081 p = 0.0135	r = -0.2242 p = 0.1887	r = -0.2854 p = 0.0916
Ti	r = 0.2018 p = 0.2378	r = 0.2788 p = 0.0997	r = 0.1389 p = 0.4190	r = 0.1765 p = 0.3032	r = -0.0901 p = 0.6011	r = -0.0951 p = 0.5812	r = -0.1902 p = 0.2666	r = 0.1994 p = 0.2436	r = 0.1359 p = 0.4294	r = 0.2782 p = 0.1005	r = 0.2528 p = 0.1369	r = 0.1938 p = 0.2575	r = -0.0022 p = 0.9897	r = 0.0156 p = 0.9279	r = 0.0250 p = 0.8851
U	r = 0.1485 p = 0.3873	r = 0.1992 p = 0.2442	r = 0.0665 p = 0.7000	r = 0.1049 p = 0.5426	r = -0.1625 p = 0.3435	r = 0.0583 p = 0.7356	r = -0.1009 p = 0.5581	r = -0.0185 p = 0.9147	r = -0.0668 p = 0.6989	r = 0.1102 p = 0.5222	r = 0.0477 p = 0.7821	r = -0.2247 p = 0.1877	r = -0.2654 p = 0.1178	r = -0.0572 p = 0.7406	r = -0.1222 p = 0.4775
V	r = 0.0431 p = 0.8030	r = 0.0590 p = 0.7324	r = 0.0157 p = 0.9278	r = 0.0279 p = 0.8715	r = -0.1807 p = 0.2916	r = -0.0533 p = 0.7573	r = 0.0432 p = 0.8024	r = -0.1182 p = 0.4924	r = -0.1380 p = 0.4221	r = -0.0289 p = 0.8673	r = -0.0797 p = 0.6439	r = -0.1474 p = 0.3911	r = -0.1530 p = 0.3731	r = -0.0388 p = 0.8223	r = -0.0761 p = 0.6591
Zn	r = 0.1204 p = 0.4841	r = 0.0822 p = 0.6337	r = -0.2645 p = 0.1190	r = -0.1382 p = 0.4215	r = 0.3396 p = 0.0428	r = 0.4420 p = 0.0072	r = -0.3226 p = 0.0550	r = -0.2377 p = 0.1628	r = -0.4021 p = 0.0150	r = -0.3414 p = 0.0416	r = -0.3876 p = 0.0195	r = -0.3057 p = 0.0698	r = -0.3695 p = 0.0266	r = -0.2913 p = 0.0847	r = -0.3274 p = 0.0513
Zr	r = 0.1823 p = 0.2857	r = 0.2100 p = 0.2189	r = 0.0553 p = 0.7485	r = 0.1097 p = 0.5239	r = -0.1430 p = 0.4053	r = -0.1174 p = 0.4951	r = -0.2501 p = 0.1413	r = 0.1819 p = 0.2885	r = 0.0890 p = 0.6057	r = 0.2341 p = 0.1695	r = 0.2060 p = 0.2280	r = 0.0304 p = 0.8604	r = -0.1330 p = 0.4394	r = -0.0816 p = 0.6363	r = -0.0904 p = 0.5999
Heavy metal total concentration	-	-	-	r = 0.0660 p = 0.9020	-	-	-	-	-	-	r = 0.0010 p = 0.9990	-	-	-	r = 0.0520 p = 0.9220

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2.2. *Period in the company (IREN SpA): Evaluation of the Antibiotic Resistance Spread in Different Wastewater Treatment Processes for Reuse*

2.2.1. Introduction

Climate change and variability can impact not only water availability but also its quality, with various consequences for human health. Water scarcity associated with climate change necessitates the development of sustainable short- and long-term strategies to address this issue, particularly in agriculture and energy production, while ensuring Public Health protection. A widely proposed strategy involves the reuse of treated wastewater as an alternative to groundwater or surface water withdrawals for agricultural and industrial irrigation.

Wastewater reuse relies on using effluent from wastewater treatment plants (WWTPs), where raw sewage undergoes processing to remove hazardous substances and pathogenic microorganisms. Treated effluent can then be reused for agricultural irrigation, industrial cooling systems, ornamental uses (e.g., fountains, artificial lakes, urban irrigation) and street cleaning. This broad range of applications could reduce freshwater consumption for potable use, promoting water recycling with lower environmental, economic, and ecological impact (Cui & Liang, 2019; Giannoccaro et al., 2019).

The reuse of treated wastewater presents several advantages. Key benefits include the valorisation of waste streams within a circular economy framework, irrigation feasibility during droughts, and reduced infrastructure requirements for water extraction and transport. However, potential risks must be considered, particularly in agricultural applications. The safety of wastewater reuse depends on several factors: (1) the presence of infectious pathogens, (2) their concentration being high enough to cause disease, and (3) exposure of a susceptible individual to an infective dose, influenced by factors such as pathogen survival in irrigated soils and the agricultural environment (Partyka & Bond, 2022).

The assessment of the treated wastewater quality is crucial, particularly regarding the effectiveness of different treatment processes and emerging contamination risks. The recent European regulation (EU, 2020) mandates a risk management plan for agricultural wastewater reuse, similar to those for drinking water safety. Among additional considerations, antibiotic resistance is of growing concern.

WWTPs are recognized as key hotspots for antibiotic resistance spreading, acting as both reservoirs and sources of antibiotic resistant bacteria (ARB) and resistance genes (ARGs) released into the environment (Rizzo et al., 2013; Wang et al., 2020). WWTPs receive high loads of ARB, ARGs from human and animal sources, and contaminants such as heavy metals and pharmaceuticals (Nguyen et al., 2021). Moreover, treatment technologies can influence the spread of antibiotic resistance. Biological processes and tertiary disinfection treatments have been shown to promote resistance selection and gene transfer (Pazda et al., 2019; Nguyen et al., 2021).

In order to deepen the knowledges initially acquired with the work presented in section 2.1 (first published manuscript: Antibiotic resistance and pathogen spreading in a wastewater treatment plant designed for wastewater reuse), in the present Ph.D. project, during the period spent in the company IREN SpA, the analysis was extended at two different WWTPs with advanced tertiary treatments

involved in the reuse of their effluent since the impact of different technologies on antibiotic resistant determinant spreading is still underexplored (Lopez et al., 2022; Drane et al., 2024).
Primary and secondary treatments in general are not sufficient in removing this kind of contaminants and this pose the risk of ARG reintroduction and horizontal gene transfer (Pei et al., 2019). For this reason, a proper tertiary process is necessary for greater elimination of ARB and ARGs. The effectiveness of tertiary treatment stages can differ significantly across various processes and technologies.

Among the most commonly used disinfection method, it is possible to mention hydrogen peroxide and UV disinfection. Both methods present relatively low-cost applications and present distinctive significant advantages: in the first case, a simple application and high inactivation capability and, in the second case, little by-product formation. These two common disinfection techniques are sometimes combined in the same WWTPs obtaining an advantageous combination of efficiencies (Gomes et al., 2025).

Another promising and more advanced disinfection technique is the membrane bioreactor (MBR) that can no longer be recognized as an emerging technology as it has been widely applied (Pei et al., 2019). In literature, it is well described the ability of this technology to achieve high removal or ARB, with higher extent, and ARGs. The removal of ARB seems more efficient probably because of extracellular ARG leakage from membrane pores during the filtration process, while most bacteria were entrapped at the membrane (Pei et al., 2019). Despite these promising results, further investigation is deemed required to gain a more complete understanding, because different factors can influence the MBR performance (e.g. membrane integrity, development of a gel/cake layer on membrane surface, the adsorption of pathogens to suspended solids) (Bonetta et al., 2022).

In literature, the distribution of specific antibiotic classes is well-established, such as β-lactams, sulphonamides and tetracycline. Beside sulfamethoxazole-resistant bacteria (SRB) and tetracycline-resistant bacteria (TRB), a greater attention was focused on Extended-Spectrum β-Lactamase-Producing *E. coli* (ESBL-Ec), considered a possible a key sentinel or indicator organism for antibiotic resistance phenomenon in wastewater (WHO, 2021). ESBL-Ec was chosen as target organism because ESBL producing *Enterobacteriaceae* are considered as one of the greatest threats in terms of antibiotic resistance since it is one of the species most often isolated from invasive infections in humans (Milenkov et al. 2024). WHO listed carbapenem-resistant and ESBL-producing *Enterobacteriaceae* among the critical priority pathogens for the discovery of new antibiotics (Silva et al., 2018).

Considering the ARG contamination in wastewater, the existing relevant literature (Pei et al., 2019) indicate that sulphonamide- and tetracycline-resistance genes exhibit the most widespread distribution. This could be attributed to the extensive use of the associated antibiotics, their relatively broad host range, high persistence capacity, and predominant resistance mechanisms. Moreover, ESBLs are encoded by genes belonging mostly to three groups called $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} (Zaatout et al., 2021).

For these reasons, the objectives of this project are to evaluate the spread of ARB and ARGs in different WWTPs that practice reuse, with the aim of identifying the most promising technologies among those currently available to reduce and/or select ARB and ARGs.

The specific objectives of the study were to assess the spread of antibiotic resistance in terms of ARB and ARGs in wastewater treatment plants designed for water reuse, evaluating the effectiveness of specific treatments in reducing and/or promoting antibiotic resistance.

2.2.2. Materials and methods

2.2.2.1. Wastewater treatment plants (WWTPs)

To evaluate the efficacy of different treatments, in the present study were included two WWTPs with different technologies. The samples from these WWTPs were collected 6 times each, accounting for a total of 12 samplings performed from June to October 2024, according to effective period of effluent reuse. In the other part of the year, the reuse is not operated. WWTP1 was already described in the first published manuscript (section 2.1) and the treatment steps are reported in the following Fig 1.

The sampling was performed at: influent (MI), effluent (MIR), effluent after filtration (MTH), effluent after hydrogen peroxide treatment (MTU) (3 ppm with 7 minutes for contact time) and effluent for reuse after the UV treatment (MU) (26 mJ/cm^2). The MTU sample is referred only to the first three samplings due to technical issues occurred at hydrogen peroxide treatment stage.

WWTP2 is a plant equipped with newer MBR technology with a capacity of 90,000 population equivalent (p.e.). It treats about 4.4 million m^3 per year of which 1.8 million m^3 per year are reused for irrigation of green areas, such as parks, and for recreational and sports activities. This WWTP has a significant seasonal load in relation to the population served, since it is in a highly tourist area. The sampling points included for WWTP2 were influent (RI) and effluent for reuse (RU).

Both WWTP steps, with the sampling points are shown in Fig. 1.

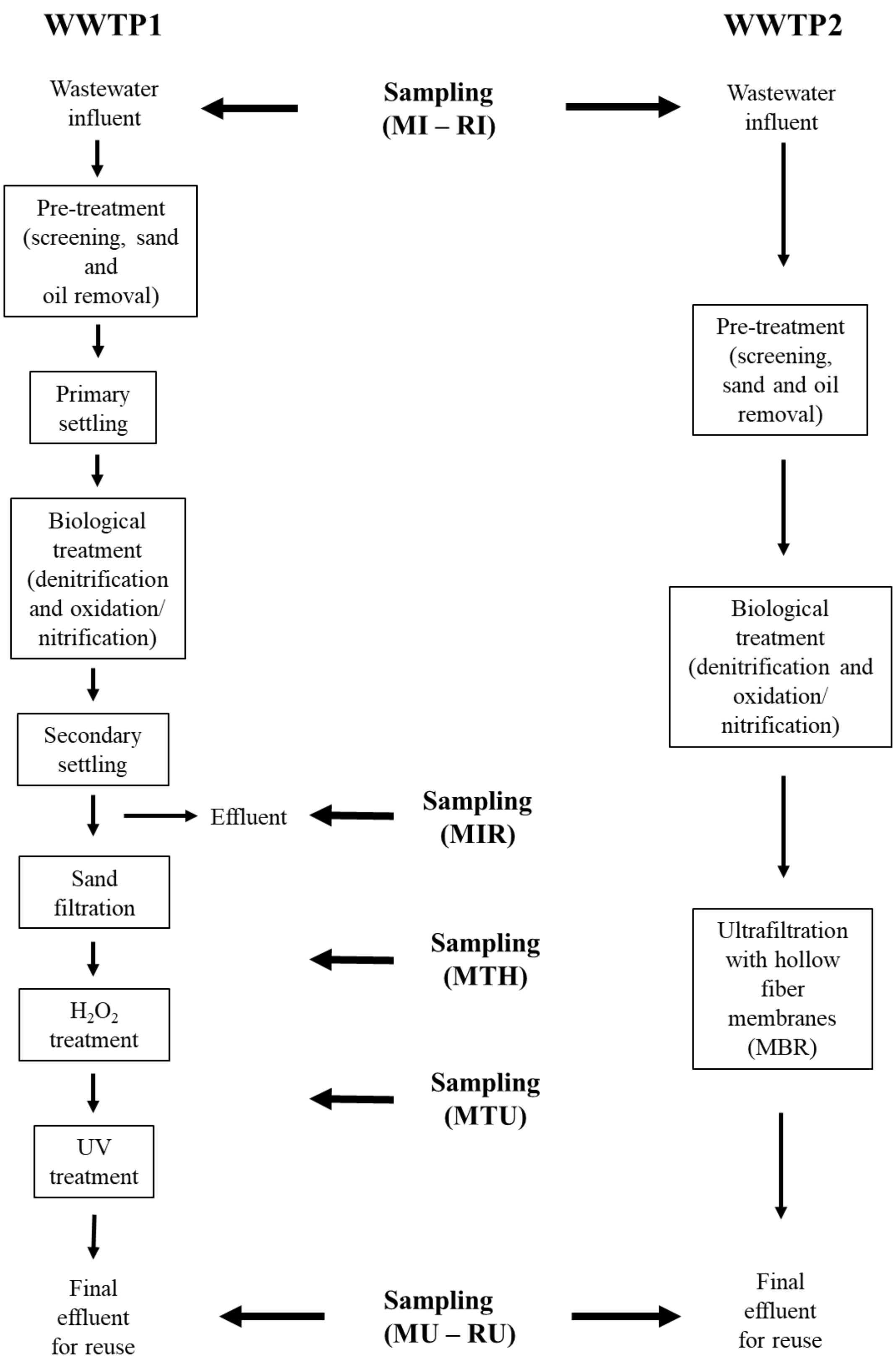


Fig. 1. Diagram of the wastewater treatment plants showing sampling points.

2.2.2.2. Antibiotic resistant bacteria (ARB) quantification

ARB were selected based on their common use in the literature as indicators of antibiotic resistance dynamics in aquatic environments (Munir et al., 2011; Berendonk et al., 2015; Ben et al., 2017; Bonetta et al., 2023; Gholipour et al., 2024; Macrì et al., 2024) and were evaluated using previously published method (WHO, 2021; Bonetta et al., 2023; Macrì et al., 2024).

Briefly, wastewater samples were serially diluted and plated in duplicate on media with and without antibiotic supplementation. ARB were enumerated on R_2Agar (VWR International) supplemented with tetracycline (for tetracycline-resistant bacteria – TRB) and sulfamethoxazole (sulfamethoxazole-resistant bacteria – SRB) (concentration of 16 mg/L and 50.4 mg/L, respectively). Medium without antibiotics was used to determine the total heterotrophic count (HPC). After 30° C incubation for 7 days the colonies were counted, and the results were expressed as log CFU/mL for the absolute abundance. The antibiotic resistance rate for each antibiotic was calculated as the ratio between the CFU mL/of each ARB and the CFU/mL of HPC.

ESBL-Ec were quantified according to the Tricycle method (WHO, 2021). Briefly, wastewater samples were serially diluted and appropriate dilution was filtered onto 0.45 µm nitrocellulose filter. The filters were placed on both tryptone bile X-glucuronide (TBX) agar (Chromocult®) to detect *E. coli* and TBX bile X-glucuronide agar with 4 µg/mL cefotaxime (CTX) (TBX agar + CTX) to detect ESBL-Ec. Presumptive *E. coli* and ESBL-Ec colonies (dark blue) were counted and purified on TBX+CFX and Tryptic Soy Agar (TSA) media, and identification was confirmed by the rapid biochemical identification test, RapID™ ONE System (ThermoFisher Scientific), and subsequently with spectrometry MALDI-TOF (Bruker Daltonics). The confirm of the phenotypical resistance was performed with disk-diffusion assay against cefotaxime (30 µg), ceftazidime (30 µg), cefotaxime and clavulanic acid (30 µg + 10 µg) and ceftazidime and clavulanic acid (30 µg + 10 µg), according to Clinical and Laboratory Standards Institute (CLSI). Results are expressed as concentration (log CFU/100 mL, absolute abundance) and as percentage of ESBL-Ec on total *E. coli* (antibiotic resistance rate).

2.2.2.3. Antibiotic resistance genes (ARGs) abundances

A volume of samples (ranging from 20 to 200 mL) was filtered onto 0.22-µm pore size polycarbonate filter membrane (Millipore). The filters were stored at – 20 °C until DNA extraction. The filters were processed using the DNeasy PowerWater kit (Qiagen) according to the manufacturer's instructions. Extracted DNA of each sample was quantified by spectrophotometry using QuantiFluor® dsDNA protocol (Promega Corporation) and used to perform gene quantification with droplet digital PCR (ddPCR).

ARGs included were: *tet*W, *tet*A (encoding resistance to tetracycline), $bla_{\text{CTX-M}}$, $bla_{\text{OXA-48}}$ (encoding resistance to β-lactams and representative of ESBL-Ec features), *sul*I and *sul*II (encoding resistance to sulphonamides), *intI*1 (gene encoding an integrase, responsible of ARG integration in the bacterial DNA and intended as antibiotic resistance indicator) and 16S rRNA (used to determine the relative ARG abundances). Primer sequences and annealing temperature are reported in Table 1.

Gene	Primer sequence	Annealing Temperature (°C) ddPCR	Reference
16S rRNA	Fw ACTCCTACGGGAGGCAGCAG Rv ATTACCGCGGCTGCTGG	60	Guo et al., 2008
*intI*1	Fw CTGGATTTCGATCACGGCACG Rv ACATGCGTGTAAATCATCGTCG	60	Wang et al., 2014
bla_{OXA-48}	Fw AAGATTTTACTTTGGGCGAAGC Rv CAACTTCCGTGCCTATTTGC	60	Subirats et al. 2017
bla_{CTX-M}	Fw CTATGGCACCACCAACGATA Rv ACGGCTTTCTGCCTTAGGTT	60	Di Cesare et al., 2015
*sul*I	Fw CGCACCGGAAACATCGCTGCAC Rv TGAAGTTCCGCCGCAAGGCTCG	62	Pei et al., 2006
*sul*II	Fw TCCGGTGGAGGCCGGTATCTGG Rv CGGGAATGCCATCTGCCTTGAG	58.5	Di Cesare et al., 2015
*tet*A	Fw GCTACATCCTGCTTGCCTTC Rv CATAGATCGCCGTGAAGAGG	59.6	Petrin et al., 2019
*tet*W	Fw GAGAGCCTGCTATATGCCAGC Rv GGGCGTATCCACAATGTTAAC	60	Aminov et al., 2001

Table 1. Primer pairs and annealing temperatures used to quantify 16S rRNA, *intI*1 and antibiotic resistance genes with droplet digital PCR (ddPCR).

For the ddPCR, 22 µL of reaction mix were prepared, containing QX200 ddPCR EvaGreen Supermix with primer at concentration of 100 nM and 5 µL of diluted DNA (or 5 µL of nuclease-free water for the no template control). 20 µL of the reaction mix and 70 µL of QX200 Droplet Generation Oil for EvaGreen were moved to the DG8 Cartridge that was placed in the QX200 Droplet Generator (BioRad). Generated droplets were then transferred to a 96-well PCR plate for the DNA amplification with T100 Thermal Cycler (BioRad). After the amplification, the plates were transferred to a QX200 Droplet reader (BioRad) for the DNA quantification. Only reactions with >10,000 droplets were considered. Data obtained with QuantaSoft Analysis Pro Software (BioRad) were expressed as gene copy (g.c.) / µL of sample. Finally, relative ARG abundances were obtained by dividing the number of each ARG copies by the number of 16S rRNA gene copies.

2.2.2.4. Chemical parameters

The chemical analyses were performed with certified and standardises methods entirely in IREN SpA laboratories by the company staff.

The wastewater samples collected were analysed for total suspended solids (TSS) (according to APAT CNR IRSA 2090B Man 29 2003), biological oxygen demand (BOD) (according to APHA methods for water Ed 23rd 2017, 5210 D), chemical oxygen demand (COD) (according to ISO 15705:2002), total phosphorus (according to APAT CNR IRSA 4110 A2 Man 29 2003), ammoniacal nitrogen (according to APAT CNR IRSA 4030A1 Man 29 2003), total nitrogen (according to UNI EN ISO 11905-1:2001) and total surfactants. The water flow at the influent and pH values were also collected.

Moreover, also a panel of metals were monitored according to the methods UNI EN ISO 15587-2:2002 and UNI EN ISO 17294-2:2023. In particular: aluminium (Al), arsenic (As), barium (Ba), beryllium (Be), boron (B), cadmium (Cd), calcium (Ca), cobalt (Co), chrome (Cr), iron (Fe), magnesium (Mg), manganese (Mn), mercury (Hg), nickel (Ni), lead (Pb), potassium (K), copper (Cu), selenium (Se), sodio (Na), tin (Sn), thallium (Tl), vanadium (V), zinc (Zn).

2.2.2.5. Statistical analysis

The statistical analyses were performed with SPSS software (version 30.0.0) with the aim to assess the dynamics of ARB and ARGs in the different treatment steps of the two WWTPs. To achieve this, the ARB and ARG absolute concentrations were converted to log10 (log CFU/mL and log gene copies (g.c.) / mL, respectively); while the ARB and ARG relative abundances were converted to the arcsin of the square root (arcsin of square root of ARB/HPC and arcsin of square root of gene copies/16s rRNA gene copies, respectively). Differences between the two WWTPs were studied with T-test. Differences in absolute and relative concentration between the treatment steps, sampling months and different WWTPs were analysed by ANOVA followed by Tukey's post-hoc test for HPC, single ARB or ARGs. Potential correlations between ARB, ARGs and chemical parameters were investigated by Pearson's correlation. Significant differences and correlation were considered significant when $p < 0.05$ and the correlation coefficient $(r) \geq 0.6$.

2.2.3. Results and discussion

2.2.3.1. Antibiotic resistant bacteria (ARB)

ARB (TRB, SRB and ESBL-Ec) detected in the different steps of the WWTP1 are reported in Fig. 2; whereas ARB detected in the influent and effluent of the WWTP2 are reported in Fig. 3.

The average concentrations of SRB, TRB, and ESBL-Ec in the influents of both WWTPs were similar ($p > 0.05$), with values of approximately ~5–6 log CFU/mL and ~4.75 log CFU/100 mL, respectively, according to the values reported in other studies on WWTP influents (Haberecht et al., 2019; Bonetta et al., 2023; Oliveira et al., 2023; Macrì et al., 2024). In general, SRB exhibited the highest concentrations among ARB in the influents of both WWTPs, consistent with previous findings (Li et al., 2015; Bonetta et al., 2022; Macrì et al., 2024).

Unlike the influents, in the treated effluent intended for reuse, the WWTP2 exhibited significantly higher SRB and TRB loads (4.72 and 4.32 CFU/mL, respectively) compared to the WWTP1 (3.22 and 2.25 CFU/mL, respectively) ($p < 0.01$), while ESBL-Ec concentrations are similar (~1 log CFU/100 mL). Comparable SRB and TRB concentrations have been reported in previous studies for both the WWTP1 (Bonetta et al., 2023; Macrì et al., 2024) and the WWTP2 (Li et al., 2015; Li et al., 2016), confirming that WWTPs can contribute to the environmental release of various ARB.

In both WWTPs, ARB concentrations in the effluents intended for reuse (MU and RU samples) decreased significantly compared to influents (MI and RI samples) for all monitored parameters ($p < 0.01$), except for TRB in the WWTP2, highlighting an overall good removal efficiency of the treatment processes.

The best TRB and SRB reduction performances were observed in the WWTP1, with a reduction of approximately ~3 log CFU/mL. Conversely, ESBL-Ec was more effectively removed by the WWTP2 (MBR treatment), achieving a total reduction of ~4 log CFU/100 mL, with three different samples below the detection limit (1 CFU/100 mL). The high removal efficiency observed for resistant *E. coli* in the WWTP2 was observed also in previous studies indicating MBR technologies as among the most effective systems for ARB and related ARGs abatement (Nnadozie et al., 2017; Wang et al., 2020; Lin et al., 2021; Zhu et al., 2021; Drane et al., 2024).

Despite the reduction observed, antibiotic resistant bacteria (SRB and TRB) were still detected in all effluent samples. More notably, resistant *E. coli* strains were found in half of the effluent samples from the WWTP2 and all effluent samples from the WWTP1.

Regarding the WWTP1, the tertiary treatments applied in the effluent intended for reuse production did not significantly improve effluent quality, as no statistically significant differences were observed between MIR samples (reuse treatment influent) and MU samples (effluent for reuse after UV treatment) ($p > 0.05$), although a slight reduction in ARB was noted in the effluent intended for reuse. Moreover, in intermediate tertiary treatment steps, represented by MTH (post-filtration) and MTU (post-hydrogen peroxide) samples, a statistically significant increase in SRB and TRB was observed compared to the reuse effluent ($p < 0.01$), indicating the ineffectiveness of these treatment steps in reducing the microbial load.

Several studies have investigated the efficacy of tertiary treatments in improving the quality of effluents intended for reuse, with often conflicting results. For instance, some studies have reported that UV and hydrogen peroxide treatments can significantly reduce ARB (Ferro et al., 2016), whereas the UV treatment alone at lower UV dosage respect to the present study (5 mJ/cm^2 *vs* 26 mJ/cm^2) exhibited ARB decrease (Guo et al., 2013). Moreover, other studies have indicated limited reduction or even an increase in certain ARB populations after these treatments (Munir et al., 2011; Zheng et al., 2017; Macrì et al., 2024). This suggests that tertiary treatment effectiveness depends on multiple factors, including operational conditions and the characteristics of the treated effluent.

The sampling period did not show significant differences for any of the monitored parameters or between the two WWTPs, which may be attributed to the short sampling duration, limited to the actual effluent reuse period, which occurs almost exclusively during the summer season.

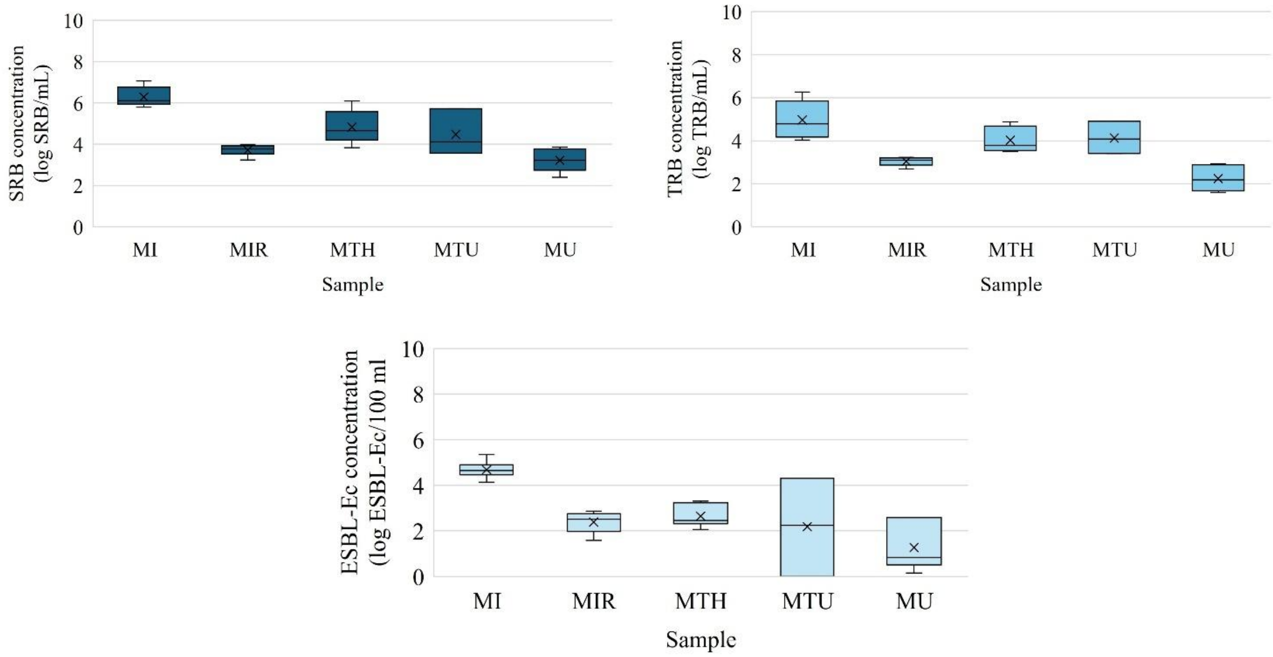


Fig. 2. Log-transformed concentrations of tetracycline-, sulfamethoxazole-resistant bacteria and Extended-spectrum β-lactamase *E. coli* in the different steps of the WWTP1. Box plots represent median and range values. WWTP: wastewater treatment plant; TRB: tetracycline-resistant bacteria; SRB: sulfamethoxazole-resistant bacteria; ESBL-Ec: extended-spectrum β-lactamase *E. coli*. Different WWTP steps: influent (MI), effluent (MIR), effluent after filtration (MTH), effluent after hydrogen peroxide treatment (MTU) and effluent for reuse after the UV treatment (MU).

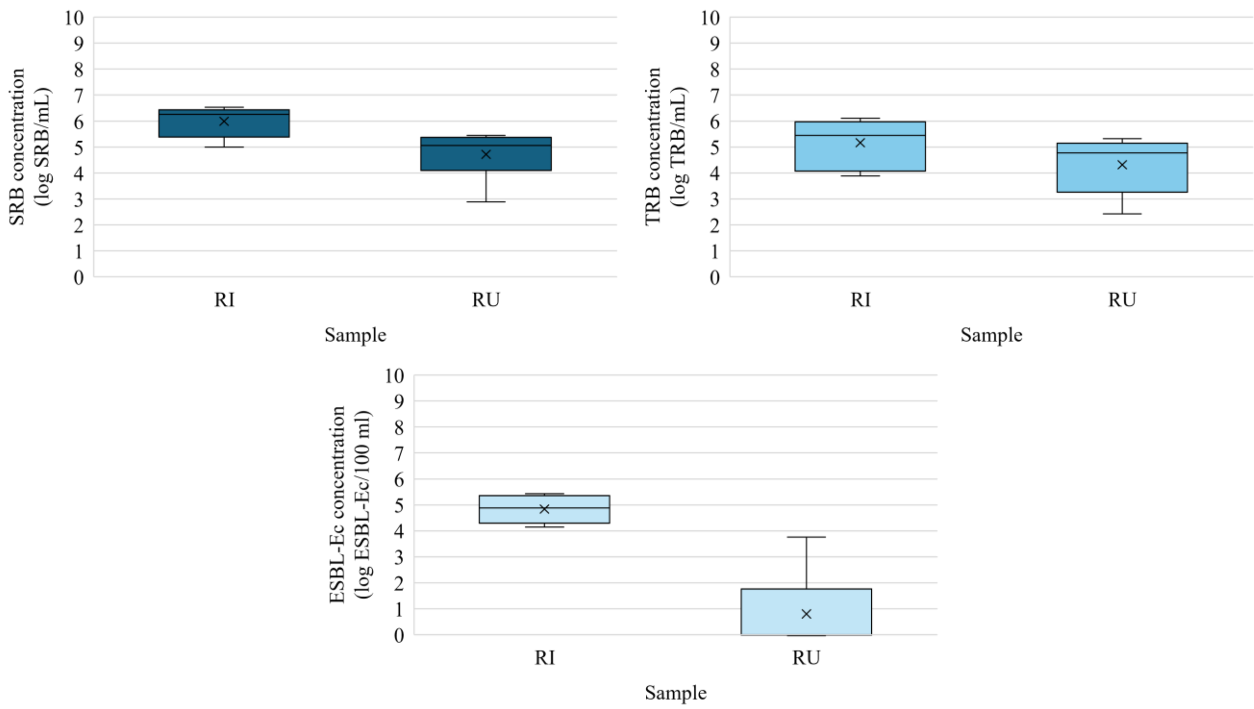


Fig. 3. Log-transformed concentrations of tetracycline-, sulfamethoxazole-resistant bacteria and Extended-spectrum β-lactamase *E. coli* in the different steps of the WWTP2. Box plots represent median and range values. WWTP: wastewater treatment plant; TRB: tetracycline-resistant bacteria; SRB: sulfamethoxazole-resistant bacteria; ESBL-Ec: extended-spectrum β-lactamase *E. coli*. Different WWTP steps: influent (RI) and effluent for reuse (RU).

Antibiotic resistance rate, i.e. ARB relative abundance (SRB/HPC, TRB/HPC and ESBL-Ec/*E. coli*), in the different steps of the WWTP1 and WWTP2 are reported in Fig. 4 and in Fig. 5, respectively.

On the contrary of the absolute ARB abundance, the relative abundance showed significant differences between the influents of the two WWTPs. Specifically, the sulfamethoxazole resistance rate was higher in the WWTP2 than in WWTP1 (37.4% *vs* 9.9%, $p < 0.05$). A similar trend was observed for TRB (10.80% *vs* 0.79%, $p < 0.05$), whereas, the relative abundance of ESBL-Ec was similar between the two WWTPs (~2.4%).

In the effluent samples, the WWTP2 maintained a higher tetracycline resistance rate than the WWTP1 (15.78% *vs* 3%, $p < 0.05$), while the relative abundance of SRB and ESBL-Ec showed no statistically significant differences between the two plants.

When analysing the two WWTPs separately, a notable accumulation of SRB and TRB was observed in the MTU samples (hydrogen peroxide-treated effluent) from WWTP1 compared to all other samples. This indicates that the hydrogen peroxide treatment alone applied in this WWTP was ineffective in ARB removal, as highlighted by absolute abundance data, and may have favoured the selection of resistant bacteria over the existing microbial community (HPC count).

Confirming the efficacy of MBR technology in removing antibiotic resistance determinants, as observed in both this study and previous literature, no significant differences in resistance rates were detected between influents and effluents in the WWTP2 for any monitored ARB. This finding highlights that the MBR treatment did not promote the selection of resistant bacteria over heterotrophic bacteria. This could be attributed to the operating principle of MBR, which relies on size-based physical separation, leading to a comparable removal of both ARB and non-antibiotic-resistant heterotrophic bacteria (Rizzo et al., 2020). However, it is important to note that some studies have reported that membrane filtration systems can serve as substrates for biofilm formation, acting as reservoirs for ARB and ARGs (Luo et al., 2021).

According to the results obtained for absolute ARB abundance, the ARB relative abundance did not show significant variations across different sampling points.

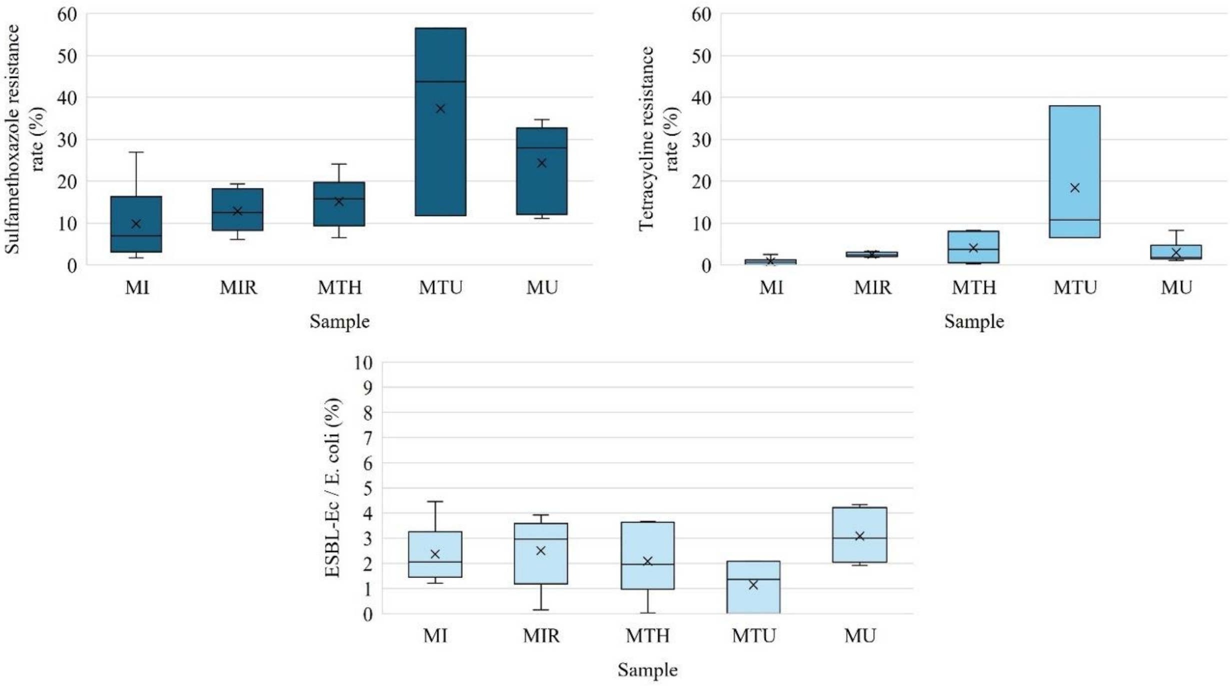


Fig. 4. Antibiotic resistance rate of tetracycline-, sulfamethoxazole-resistant bacteria and extended-spectrum β-lactamase *E. coli* in the different steps of the WWTP1. Values are normalised to HPC abundances for tetracycline- and sulfamethoxazole-resistant bacteria and to *E. coli* for extended-spectrum β-lactamase *E. coli*. Box plots represent median and range values. WWTP: wastewater treatment plant; HPC: total heterotrophic count; different WWTP steps: influent (MI), effluent (MIR), effluent after filtration (MTH), effluent after hydrogen peroxide treatment (MTU) and effluent for reuse after the UV treatment (MU).

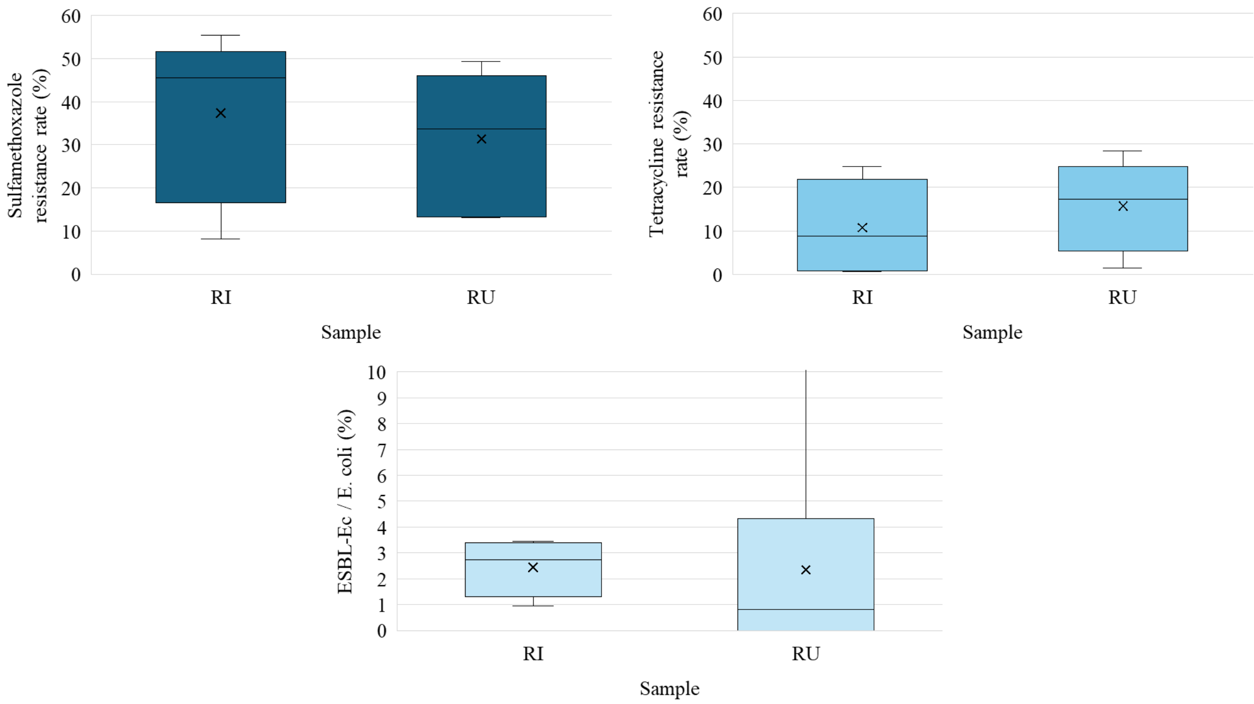


Fig. 5. Antibiotic resistance rate of tetracycline-, sulfamethoxazole-resistant bacteria and extended-spectrum β-lactamase *E. coli* in the different steps of the WWTP2. Values are normalised to HPC abundances for tetracycline- and sulfamethoxazole-resistant bacteria and to *E. coli* for extended-spectrum β-lactamase *E. coli*. Box plots represent median and range values. WWTP: wastewater treatment plant; HPC: total heterotrophic count; different WWTP steps: influent (RI) and effluent for reuse (RU).

2.2.3.2. Antibiotic resistant genes (ARGs)

ARGs (*sul*I, *sul*II, *tet*A, *tet*W, bla_{OXA-48}, bla_{CTX-M}, and *intI*1) detected in the different steps of the WWTP1 are reported in Fig. 6; whereas ARGs detected in the influent and effluent of the WWTP2 are reported in Fig. 7.

The absolute concentrations of the *sul*I, *sul*II, *tet*A, bla_{OXA-48}, bla_{CTX-M}, and *intI*1 genes were similar ($p > 0.05$) in the influents of both WWTPs (~5.5, ~5, ~4.5, ~4.4, ~2.7, and ~5.3 log g.c./mL, respectively), except for the *tet*W gene (4.84 WWTP2 *vs* 4.01 log g.c./mL WWTP1), highlighting comparable ARG levels between the influents of the two WWTPs. Similar concentrations (Oliveira et al., 2020; Bonetta et al., 2022; Cuetero-Martínez et al., 2024; Macrì et al., 2024) or higher ones (Bonanno Ferraro et al., 2024) have been reported in the influents of WWTPs in other studies.

In the effluent samples intended for reuse, no significant differences were observed between the two WWTPs, indicating that the employed technologies achieved comparable performance. Moreover, the removal efficiency reached the same order of magnitude for all genes in both plants, unlike the trends observed for ARB.

For both plants, ARG concentrations significantly decreased in the reuse effluents (MU and RU samples) compared to the influents (MI and RI samples) for all monitored genes ($p < 0.001$), confirming, as previously observed for ARB, the efficiency of the treatment process. Nevertheless, ARGs associated with antibiotic resistance were still detected in all effluent samples intended for reuse.

The dissemination of ARGs in WWTP effluents is well-documented in the literature (Cacace et al., 2019). Reported concentrations vary, but MBR technology generally provides better results than traditional disinfection methods (Rizzo et al., 2013). However, in the present study, both technologies achieved comparable results.

Regarding the WWTP1, it is important to note that the tertiary treatments employed did not significantly improve the quality of the effluent for reuse in terms of ARGs, as no statistically significant differences were found between the MIR (WWTP effluent) and MU (UV-treated reuse effluent) samples ($p > 0.05$). Moreover, no statistically significant differences were observed among other intermediate reuse production steps (MTH post-filtration and MTU post-hydrogen peroxide) and the final reuse effluent (MU), except for the post-filtration sample for the *intI1* gene compared to the MTU and MU samples ($p < 0.05$).

The inefficacy of the monitored treatments, particularly disinfection and UV treatment, in reducing ARG loads has also been reported in the literature (La Rosa et al., 2025). The effectiveness of UV treatment remains controversial; for instance, Guo et al. (2013) and McKinney and Pruden reported efficient ARG removal with variable UV doses (range 5-400 mJ/cm^2), which was neither observed in the present study nor in other studies (Munir et al., 2011; Di Cesare et al., 2016a). Furthermore, although laboratory studies (Zhang et al., 2016; Li et al., 2022) have demonstrated a substantial ARG reduction (2.63–3.48 log reduction) through UV treatment combined with hydrogen peroxide, the results obtained at the WWTP1 highlight the overall inefficacy of this treatment in ARG removal.

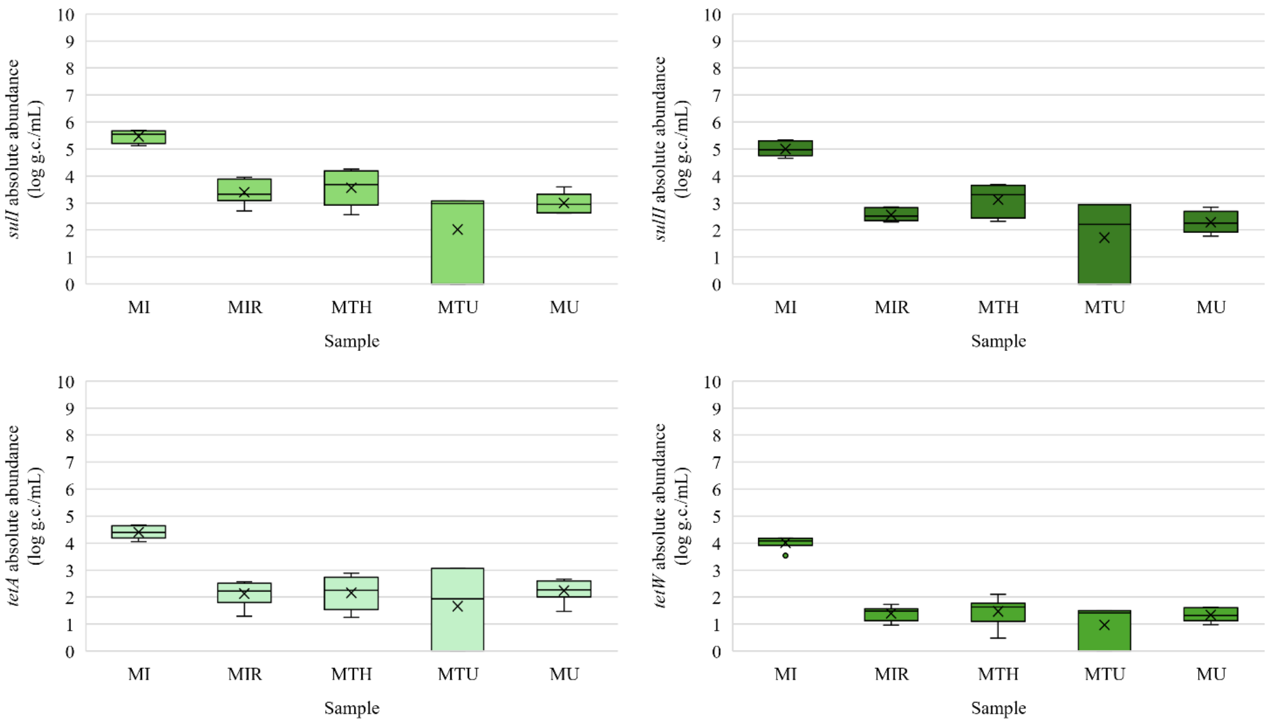
However, it is essential to note that the evaluation was based on data from only 3 out of 6 sampling campaigns due to technical issues with the hydrogen peroxide dosing system during the monitoring campaign.

Regarding MBR treatment, the literature presents conflicting findings on ARG removal efficiency. Wang et al. (2020) observed complete removal of some ARGs (including *intI1* and *tet*W) in a full-scale MBR wastewater treatment plant. On the contrary, although Lin et al. (2021) reported a 98.4% reduction in ARGs in MBR-treated effluent, 35 ARGs were still detected in all analysed samples, including the final effluent. Additionally, various studies highlight the inability of MBR treatment to remove mobile genetic elements (MGEs), such as *intI1* and plasmids, as they can pass through ultrafiltration membrane pores (Arkhangelsky et al., 2011) and reach the final effluent without effective removal (Yang et al., 2013; Drane et al., 2024).

These observations suggest that treatment plants using MBR technology can be effective in reducing ARGs; however, the treated effluent may still contain significant concentrations of ARGs (Lin et al., 2021).

In both WWTPs, the most prevalent gene was *intI1*, closely followed by *sul* genes, as also observed for ARB concentrations. This trend is well documented in the literature, as both genes are considered indicators of anthropogenic contamination (Domingues et al., 2012; Djordjevic et al., 2013; Eckert et al., 2018; Cacace et al., 2019).

None of the absolute abundances of the monitored ARGs showed significant variations across different sampling events.



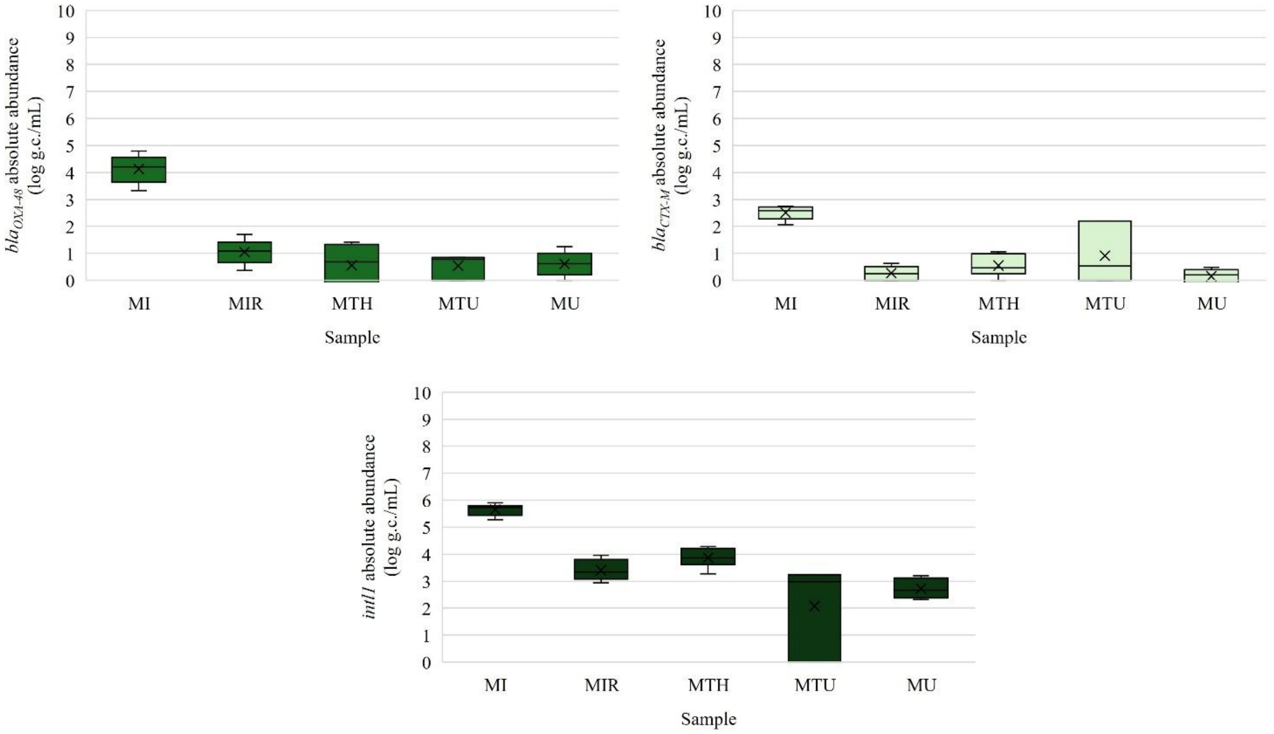
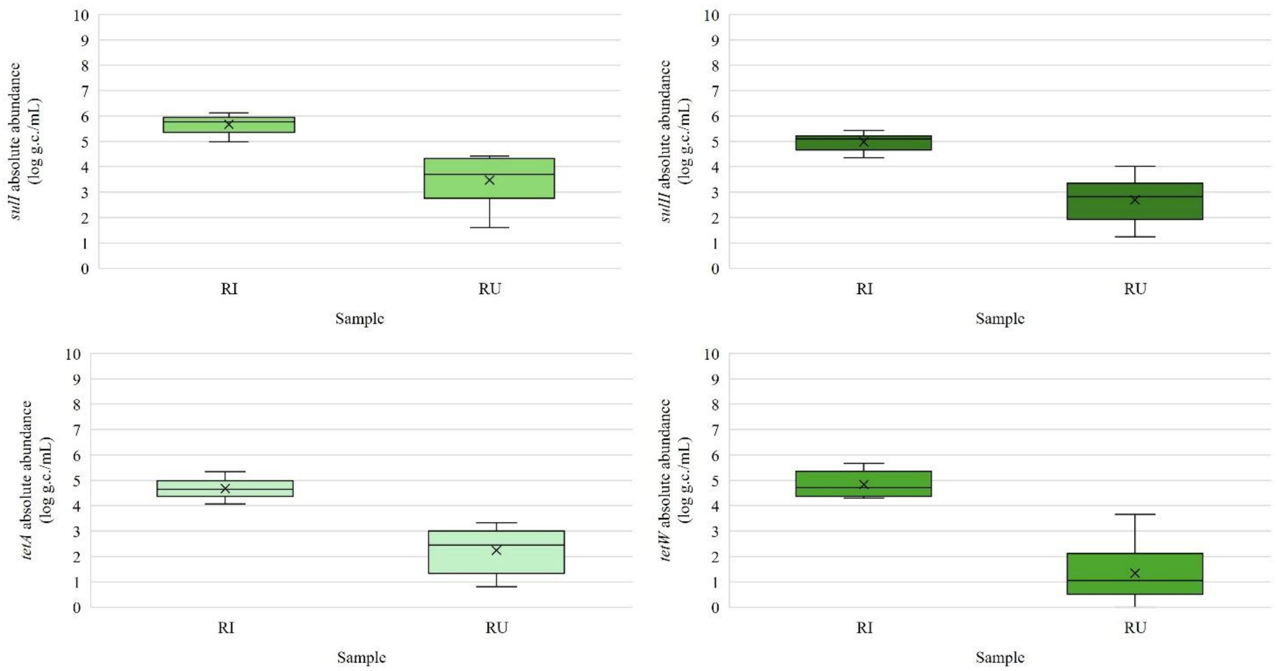


Fig. 6. Absolute abundances of *sul*I, *sul*II, *tet*A, *tet*W, *bla*$_{OXA-48}$, *bla*$_{CTX-M}$, and *intI1* genes detected at WWTP1. WWTP: wastewater treatment plant; different WWTP steps: influent (MI), effluent (MIR), effluent after filtration (MTH), effluent after hydrogen peroxide treatment (MTU) and effluent for reuse after the UV treatment (MU); g.c.: gene copies.



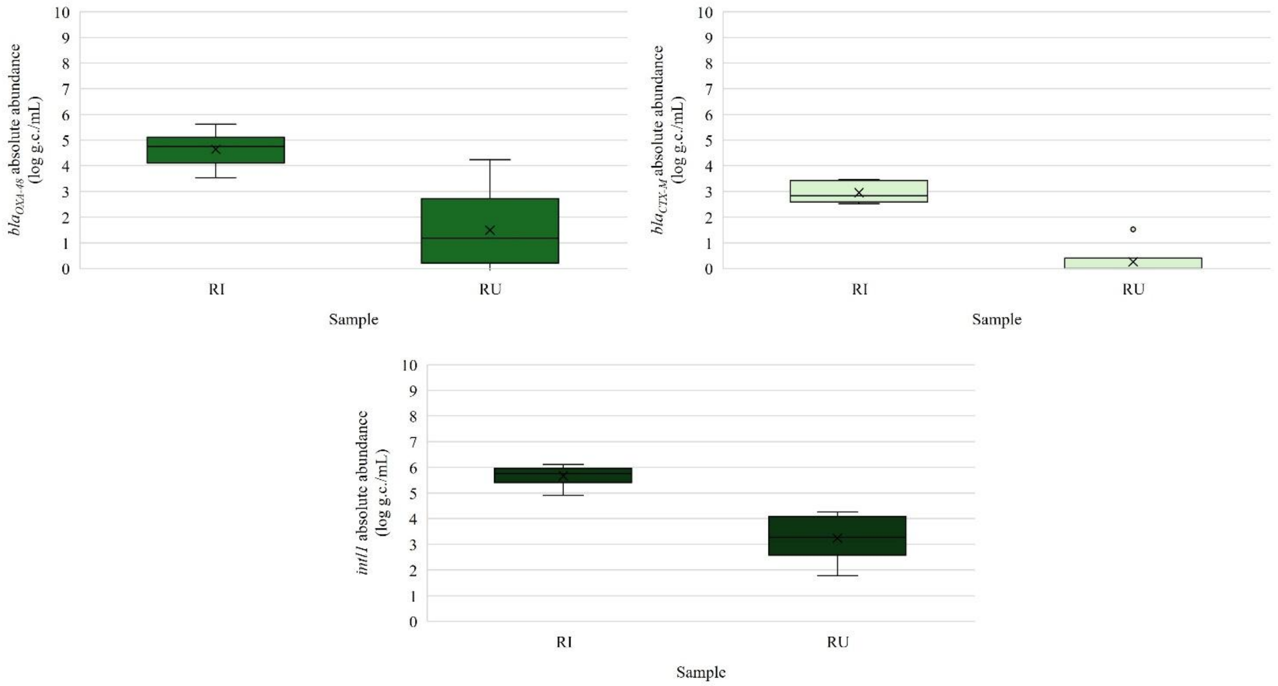


Fig. 7. Absolute abundances of *sul*I, *sul*II, *tet*A, *tet*W, *bla*$_{OXA-48}$, *bla*$_{CTX-M}$, and *intI1* genes detected at WWTP2. WWTP: wastewater treatment plant; different WWTP steps: influent (RI) and effluent for reuse (RU); g.c.: gene copies.

ARG relative abundance, i.e. normalised to 16S rRNA gene quantification (ARGs or *intI1* g.c./16S rRNA g.c.), in the different steps of the WWTP1 and WWTP2 are reported in Fig. 8 and in Fig. 9, respectively.

In WWTP1, the relative abundance of *bla*$_{OXA-48}$ and *tet*W genes was significantly reduced at all treatment stages compared to the plant influent ($p < 0.05$), indicating a decrease in ARGs throughout the treatment process relative to the total microbial population. This contrasts with the trend observed for ARB, for which an increase was detected in the post-hydrogen peroxide treatment phase (MTU).

A statistically significant reduction in relative abundance was also observed in the effluent compared to the influent at the WWTP2 for the *bla*$_{CTX-M}$, *tet*A, *tet*W, and *intI1* genes.

This suggests that the treatment processes applied at both WWTPs do not promote the persistence of ARGs relative to the heterotrophic microbial community, in contrast to findings reported in previous studies (Mao et al., 2015; McConnell et al., 2018). Conversely, Bonanno Ferraro et al. (2024) observed an increase in the relative abundance of ARGs in effluents compared to influents in treatment plants employing disinfection methods. Similarly, Zhuang et al. (2015) highlighted that low doses of UV radiation can increase the relative abundance of certain selected ARGs.

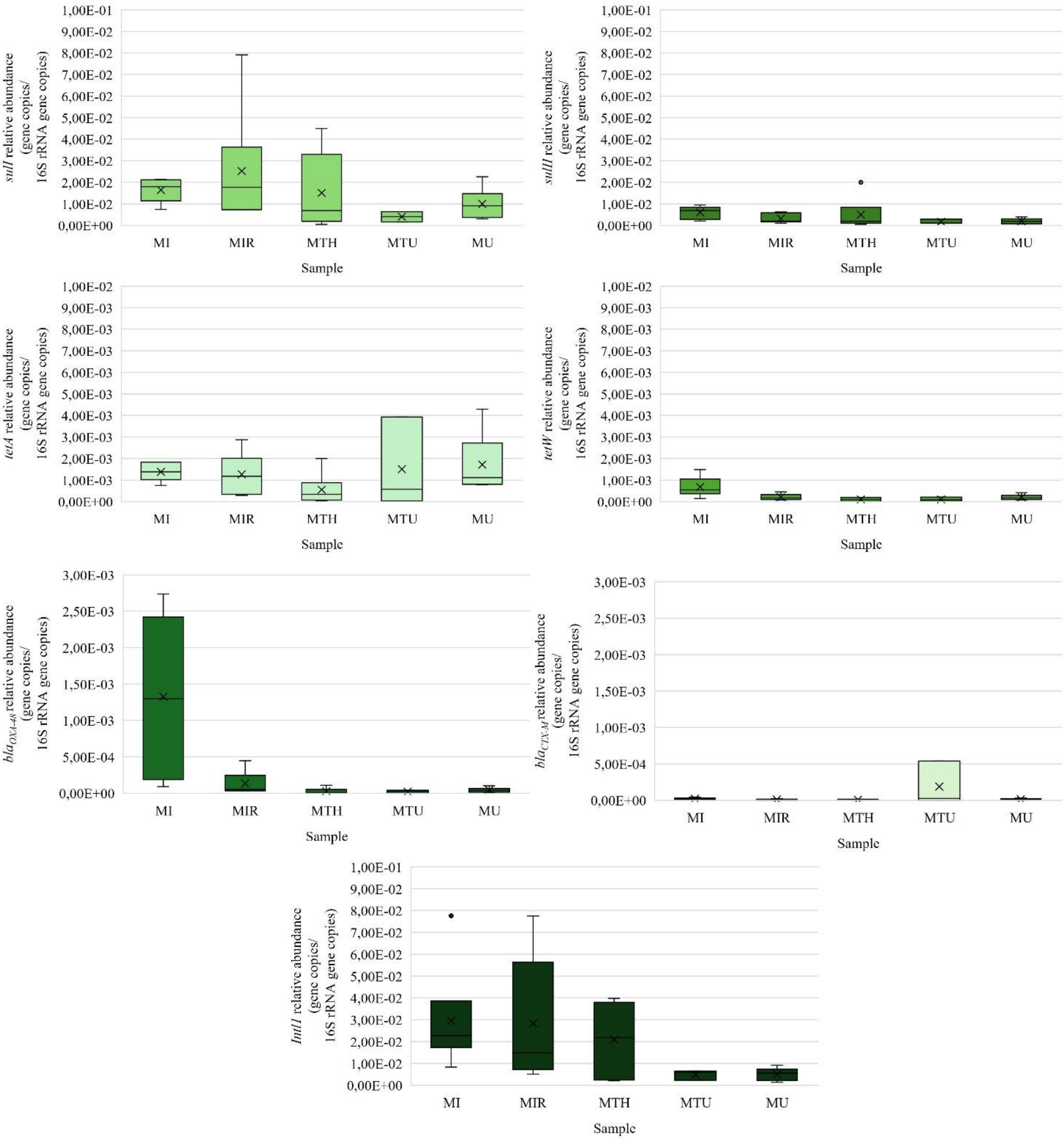


Fig. 8. Relative abundances of *sul*I, *sul*II, *tet*A, *tet*W, *bla*$_{OXA-48}$, *bla*$_{CTX-M}$, and *intI1* genes detected at WWTP1. All values are normalized to 16S rRNA gene copy. WWTP: wastewater treatment plant; different WWTP steps: influent (MI), effluent (MIR), effluent after filtration (MTH), effluent after hydrogen peroxide treatment (MTU) and effluent for reuse after the UV treatment (MU).

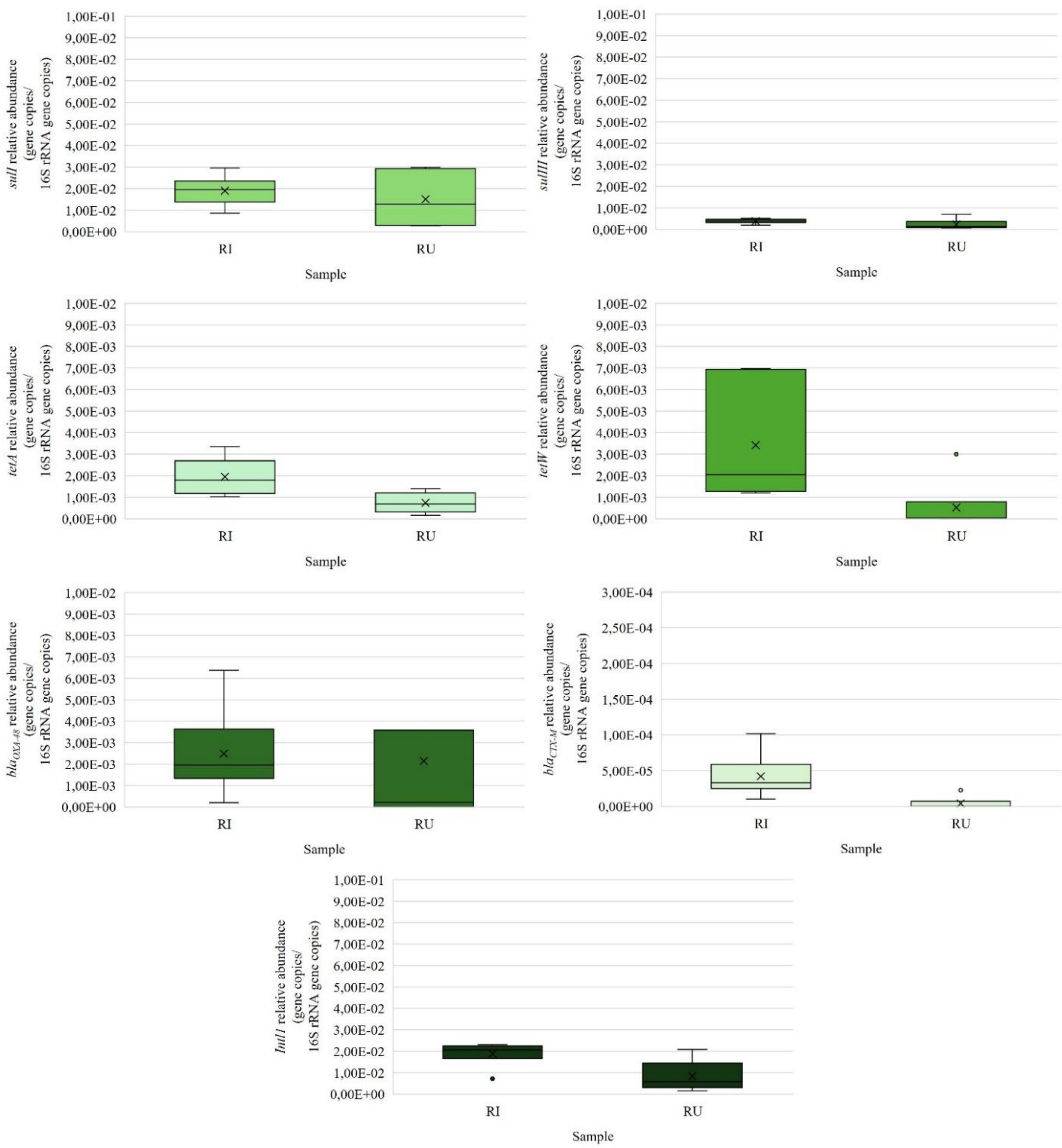


Fig. 9. Relative abundances of *sul*I, *sul*II, *tet*A, *tet*W, *bla*$_{OXA-48}$, *bla*$_{CTX-M}$, and *intI1* genes detected at WWTP2. All values are normalized to 16S rRNA gene copy. WWTP: wastewater treatment plant; different WWTP steps: influent (RI) and effluent for reuse (RU).

2.2.3.3. Association within antibiotic resistance determinants and with physicochemical parameters

The results of the investigated wastewater samples from the two different WWTPs were analysed for possible significant associations between the presence of ARB, ARGs and chemical parameters that were above the limit of quantification (Table 2 for WWTP1 and Table 3 for WWTP2).

Some relevant associations were found between cultural parameters. In particular, the absolute concentration of all the ARB monitored (SRB, TRB and ESBL-Ec) results correlated with the others ($r > 0.7$, $p < 0.001$) in WWTP1. In WWTP2, instead, SRB resulted correlated with both TRB and ESBL-Ec ($p > 0.6$, $p < 0.01$). In both WWTPs, ESBL-Ec resulted strongly correlated with other ARB.

This confirms that these bacteria can be used as an indicator of antibiotic resistance dissemination in WWTPs (Li et al., 2023).

Moreover, regarding the association between cultural and molecular parameters in WWTP1, SRB concentration resulted correlated with one of the respective monitored ARGs, *sul*II ($r > 0.7$, $p < 0.001$) and ESBL-Ec resulted correlated with all the ARGs monitored (including *bla*$_{CTX\text{-}M}$, a common ESBL-Ec marker) and *intI1* ($r > 0.7$, $p < 0.001$). In WWTP2, SRB resulted correlated with both the respective monitored ARGs, *sul*I and *sul*II ($r > 0.7$, $p < 0.001$) and, as observed in WWTP1, ESBL-Ec resulted strongly correlated with all the ARGs monitored and *intI1* ($r > 0.9$, $p < 0.001$).

The correlation between cultural and molecular parameters regarding the resistance to sulfamethoxazole is not surprising since, especially in wastewater treatment settings, *sul*I and *sul*II are among the most widespread genes (Berglund, 2015; Macrì et al., 2024) and they are able to confer resistance to bacteria that were sensitive, increasing their concentration in wastewater. Moreover, ESBL-Ec resulted correlated also with ARGs confirming that they can be used also as indicator of ARG spreading. For this reason, they are considered a One Health antimicrobial resistance surveillance target (Oliveira et al., 2023, WHO, 2021).

Furthermore, *intI1* resulted strongly and positively correlated with all other ARGs in both WWTPs ($r > 0.8$, $p < 0.001$). This association was already demonstrated in other studies (Lupan et al., 2017; Shamsizadeh et al., 2024), since integrases are often involved in ARG acquisition from the environment and are considered indicators of anthropogenic impacts.

The absolute and relative abundance data of ARB and ARGs were correlated also with the physicochemical parameters monitored to identify potential indicators useful for tracking antibiotic resistance during wastewater treatment for reuse.

In the WWTP1, a positive correlation was observed between BOD and COD and all absolute concentrations of ARB and ARGs, including *intI1* ($r > 0.7$, $p < 0.01$). In contrast, correlations were less evident in the WWTP2, where only COD showed a correlation with the absolute concentration of ESBL-Ec and all ARGs (except *bla*$_{OXA\text{-}48}$) as well as *intI1* ($r > 0.7$, $p < 0.05$).

This association has also been reported in other studies (Yuan et al., 2014; Harnisz et al., 2020; Yoo & Lee, 2021). The organic load, represented by parameters such as BOD and COD, may play a significant role in shaping the bacterial community during the treatment process.

Moreover, also total phosphorus and total and ammoniacal nitrogen resulted correlated with most of absolute quantifications in WWTP1 (Table 2); instead, in WWTP2 the significant correlation revealed were mainly with ammoniacal nitrogen (Table 3).

This association is not surprising since it was already detected by other studies in literature (Choi et al., 2020; Reichert et al., 2021; Wang et al., 2021). All these elements serve as nutrient source for various bacterial species and influence growth of the microbial community, including ARB, that could contribute to increased ARG spreading (Basil et al., 2024).

Beyond BOD and COD, other biotic and abiotic factors, such as heavy metals, may influence the presence and spread of ARB and ARGs. The correlation results with heavy metals for the WWTP1 plant are presented in Table 2, while those for the WWTP2 are shown in Table 3.

The correlation analysis between ARB, ARGs, and heavy metals yielded results that are difficult to discuss. A correlation was observed between the absolute concentration of certain ARB and ARGs and Ba and Fe in both WWTPs. Additionally, in the WWTP1, a correlation was found between the absolute concentration of *tet*W and both the absolute and relative concentrations of $bla_{OXA\text{-}48}$ with Ni. Various correlations between certain ARB, ARGs, and metals were observed, but these are challenging to interpret due to the lack of a consistent pattern across different bacteria and resistance genes.

The association between heavy metals and the abundance of ARB and ARGs in wastewater has been documented in the literature for over a decade. However, the range of studied metals remains relatively limited (Gao et al., 2015; Xu et al., 2017; Wu et al., 2020; Gao et al., 2022; Macrì et al., 2024). Environmental pollutants such as heavy metals can exert selective pressure and are often linked to an increased abundance of ARB and ARGs. Furthermore, the combination of heavy metals and antibiotics can lead to co-resistance to both types of contaminants.

Although a considerable number of studies have been published on this topic, the set of investigated heavy metals is still incomplete, making it difficult to obtain comprehensive insights into all detectable metals in wastewater. Most studies have reported associations between ARB, ARGs, and specific heavy metals, such as Cu, Co, Ni, and Zn. In this study, only the correlation between *tet*W and $bla_{OXA\text{-}48}$ with Ni was observed. Previously, correlations with Ni had been reported for *sul* genes (Di Cesare et al., 2016b; Hubeny et al., 2021; Macrì et al., 2024), which, as mentioned earlier, are typically among the most abundant resistance genes.

As a comprehensive literature on this subject is still lacking, it is currently not possible to provide a precise explanation for the individual correlations observed.

	Antibiotic resistant bacteria (ARB)						Antibiotic resistance genes (ARGs)													
	SRB		**TRB**		**ESBL-Ec**		***sul*I**		***sul*II**		***tet*A**		***tet*W**		***bla*$_{OXA-48}$**		***bla*$_{CTX-M}$**		***intI1***	
	A	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**
Ba	r = 0.701 p = 0.000	-	r = 0.638 p = 0.000	-	r = 0.673 p = 0.000	-	r = 0.684 p = 0.000		r = 0.726 p = 0.000	-	r = 0.708 p = 0.000	-	r = 0.860 p = 0.000	r = 0.659 p = 0.000	r = 0.850 p = 0.000	r = 0.709 p = 0.000	r = 0.761 p = 0.000	-	r = 0.693 p = 0.000	-
Cr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	r = 0.953 p = 0.000
Fe	-	-	-	-	-	-	-	-	-	-	-	-	r = 0.62 p = 0.001	r = 0.654 p = 0.000	r = 0.612 p = 0.001	r = 0.638 p = 0.000	-	-	r = 0.600 p = 0.001	-
Ni	-	-	-	-	-	-	-	-	-	-	-	-	r = 0.605 p = 0.001	-	r = 0.615 p = 0.001	r = 0.642 p = 0.000	-	-	-	-
Total phosphorus	r = 0.740 p = 0.000	-	r = 0.611 p = 0.001	-	r = 0.753 p = 0.000	-	r = 0.786 p = 0.000	-	r = 0.835 p = 0.000	-	r = 0.845 p = 0.000	-	r = 0.942 p = 0.000	r = 0.706 p = 0.000	r = 0.940 p = 0.000	r = 0.797 p = 0.000	r = 0.880 p = 0.000	-	r = 0.803 p = 0.000	-
Ammoniacal nitrogen	r = 0.781 p = 0.000	-	r = 0.755 p = 0.000	-	r = 0.749 p = 0.000	-	r = 0.866 p = 0.000	-	r = 0.882 p = 0.000	-	r = 0.828 p = 0.000	-	r = 0.877 p = 0.000	-	r = 0.865 p = 0.000	-	r = 0.764 p = 0.000	-	r = 0.870 p = 0.000	-
Total nitrogen	r = 0.678 p = 0.000	-	r = 0.652 p = 0.000	-	r = 0.697 p = 0.000	-	r = 0.729 p = 0.000	-	r = 0.777 p = 0.000	r = 0.724 p = 0.000	r = 0.752 p = 0.000	-	r = 0.784 p = 0.000	-	r = 0.811 p = 0.000	-	-	-	r = 0.700 p = 0.000	-

Table 2. Correlation matrix of microbiological and chemical parameters in WWTP1. SRB: sulfamethoxazole-resistant bacteria; TRB: tetracycline-resistant bacteria; ESBL-Ec: extended-spectrum β-lactamase producing *E. coli*; A: absolute abundance; R: relative abundance. -: not significant.

	Antibiotic resistant bacteria (ARB)						**Antibiotic resistance genes (ARGs)**													
	SRB		**TRB**		**ESBL-Ec**		***sul*I**		***sul*II**		***tet*A**		***tet*W**		***bla*$_{OXA-48}$**		***bla*$_{CTX-M}$**		***intI1***	
	A	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**
Al	r = 0.705 p = 0.034	-	-	-	r = 0.694 p = 0.038	-	-	-	-	-	-	r = 0.692 p = 0.039	r = 0.687 p = 0.041	-	r = 0.760 p = 0.017	-	r = 0.709 p = 0.032	-	-	-
Ba	r = 0.706 p = 0.010	-	-	-	r = 0.675 p = 0.016	-	r = 0.734 p = 0.007	-	r = 0.722 p = 0.008	-	r = 0.773 p = 0.003	r = 0.809 p = 0.002	r = 0.735 p = 0.006	r = 0.805 p = 0.002	r = 0.604 p = 0.038	-	r = 0.760 p = 0.004	r = 0.741 p = 0.006	r = 0.724 p = 0.008	-
Ca	r = 0.643 p = 0.024	-	-	-	-	-	r = 0.614 p = 0.034	-	-	-	r = 0.644 p = 0.044	r = 0.761 p = 0.011	r = 0.673 p = 0.033	-	r = 0.753 p = 0.012	-	r = 0.677 p = 0.031	-	-	-
Cr	-	-	-	-	r = 0.640 p = 0.046	-	r = 0.774 p = 0.003	-	r = 0.820 p = 0.001	-	r = 0.844 p = 0.001	r = 0.850 p = 0.000	r = 0.887 p = 0.000	r = 0.857 p = 0.000	r = 0.843 p = 0.001	-	r = 0.768 p = 0.004	r = 0.768 p = 0.004	r = 0.817 p = 0.001	r = 0.593 p = 0.0042
Fe	r = 0.632 p = 0.028	-	-	-	r = 0.774 p = 0.003	-	r = 0.820 p = 0.001	-	r = 0.844 p = 0.001	-	r = 0.844 p = 0.001	r = 0.850 p = 0.000	r = 0.877 p = 0.000	r = 0.857 p = 0.000	r = 0.843 p = 0.001	-	r = 0.896 p = 0.000	r = 0.768 p = 0.004	r = 0.817 p = 0.001	r = 0.593 p = 0.042
Pb	r = 0.773 p = 0.041	-	-	-	-	-	-	-	-	-	-	-	r = 0.828 p = 0.022	-	r = 0.815 p = 0.026	-	-	-	-	-
Ammoniacal nitrogen	-	-	-	-	r = 0.725 p = 0.012	-	r = 0.787 p = 0.004	-	r = 0.786 p = 0.004	-	r = 0.842 p = 0.001	r = 0.759 p = 0.007	r = 0.784 p = 0.004	r = 0.638 p = 0.035	r = 0.693 p = 0.018	-	r = 0.802 p = 0.003	-	r = 0.814 p = 0.002	-

Table 3. Correlation matrix of microbiological and chemical parameters in WWTP2. SRB: sulfamethoxazole-resistant bacteria; TRB: tetracycline-resistant bacteria; ESBL-Ec: extended-spectrum β-lactamase producing *E. coli*; A: absolute abundance; R: relative abundance. -: not significant.

2.2.4. Conclusions

Wastewater is known to contain ARB, sometimes pathogenic, and ARG, raising particular concerns when treated water is intended for reuse. However, it remains unclear which treatments are most effective in mitigating antibiotic resistance.

This study provides an assessment of the fate and behaviour of key ARB and ARG using both culture-based and molecular methods, identifying the most vulnerable stages of the wastewater treatment process.

ARB and ARG were monitored in two different WWTPs which employ distinct advanced treatment technologies to produce high-quality effluent suitable for agricultural and other reuse applications.

A reduction in antibiotic resistance was observed in both monitored plants, indicating that the applied treatments effectively decreased the phenomenon. However, all analysed samples still contained ARB, including ESBL-producing *E. coli* in some cases, as well as genes encoding antibiotic resistance.

Regarding the potential selection of antibiotic resistance by the investigated treatments, the only critical point was identified in the hydrogen peroxide disinfection step at the WWTP1 for ARB. It is important to note that the number of analysed samples was lower than that available for other sampling points.

Correlation analyses confirmed the complex interactions between nutrients, heavy metals, and antibiotic resistance determinants, regardless of the tertiary treatment applied. Additionally, BOD and COD and nutrients parameters were almost always correlated with antibiotic resistance parameters, consistent with findings in the literature.

Overall, these results confirm that wastewater treatment plants can serve as a potential source of antibiotic resistance dissemination in the environment, despite significantly reducing the ARB and ARG load they receive.

Moreover, data on tertiary treatments indicate that they only partially contribute to reducing ARB and ARG levels. Therefore, treatment technologies should be improved, and additional processes could be implemented to mitigate the spread of antibiotic resistance in the environment. This highlights the need of effective tertiary treatments to protect Public Health and the environment in the context of water reuse.

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2.3. *Period abroad: Analysis of the resistome in three wastewater treatment plants of the region of Murcia (Spain) after the application of different treatments*

2.3.1. Introduction

The present research activity was conducted during the period abroad (February-July 2023) in Murcia (Spain) at Centro de Edafología y Biología Aplicada del Segura (CEBAS) – CSIC with the Grupo de Microbiología y Calidad de Frutas y Hortalizas under the supervision of Prof. Ana Allende Prieto, and is a part of a wider project called "Assessment of efficient water disinfection technologies compatible with sustainable water reuse approaches in circular economy Agri-food production systems (RECWATER)" funded by Ministerio de Transición Ecológica y Reto Demográfico. This research activity is still undergoing and the results here presented are only referred to the period spent at the institute and not to the entire results collected within the project development.

This part of the project focuses on analysing the resistome within various wastewater treatment plants (WWTPs) in the Region of Murcia through metagenomic analysis. The term resistome refers to the complete collection of antibiotic resistance genes (ARGs) present in a specific environment—here, within samples from different WWTPs collected during various sampling events.

Beside this, as an integrated approach, also Extended-Spectrum β-Lactamase-Producing *E. coli* (ESBL-Ec) presence was evaluated with traditional cultural method. ESBL-Ec was used as an indicator of antibiotic resistant bacteria (ARB) spreading, which is important since they are able to actively carry and spread ARGs in the environment.

Understanding the ARG spreading in wastewater is essential, as it not only provides insight into the effectiveness of current treatment methods but also contributes to developing improved strategies to mitigate antibiotic resistance spread.

The sequencing of the resistome in wastewater has emerged as a powerful tool for monitoring antibiotic resistance in environmental and public health contexts. With this more in-depth molecular analysis of wastewater sample, it is possible to detect a wide range of resistance genes, providing insights into the spread and evolution of the phenomena within WWTPs. This approach offers several advantages, including early detection of emerging resistance, cost-effective surveillance compared to clinical sampling, and the ability to track trends over time (Miłobedzka et al., 2022).

Several sequencing technologies are used to characterise the resistome in wastewater, each with distinct strengths and limitations. High-throughput metagenomic sequencing allows for comprehensive analysis of resistance genes, offering a culture-independent method to assess the complex resistome interplay, enabling the identification of novel resistance genes and their associated mobile genetic elements.

However, this technique presents also some disadvantages. For example, it has high operational costs and require high bioinformatic skills. In addition, the data obtained from sequencing are difficult to compare with other literature, since many other studies were performed with different molecular techniques, such as qPCR or ddPCR, or with different sequencing systems (Miłobedzka et al., 2022).

The application of the resistome analysis to the different step within a WWTP, or to compare different WWTPs, allows to elucidate if some treatment favour the selection of ARGs or if a technology is more efficient respect to another one.

In the present study, three different technologies were compared, in particular, ozonation, sodium hypochlorite and UV-C radiation.

Ozone is a powerful oxidizing agent that effectively degrades organic pollutants, inactivates bacteria, and reduces ARGs (Lim et al., 2022). Its strong oxidative potential allows it to break down complex contaminants, although it requires controlled dosing to prevent the formation of harmful byproducts (Hübner et al., 2024). Sodium hypochlorite, commonly used for chlorination, provides a cost-effective disinfection method by disrupting microbial cell walls and denaturing essential proteins (Collivignarelli et al., 2017). However, its application must be carefully managed to minimize the formation of chlorinated byproducts, such as trihalomethanes, which can pose environmental and health risks (Golfinopoulos et al., 2024). Finally, UV-C irradiation offers a chemical-free alternative by disrupting microbial DNA, preventing replication and effectively inactivating pathogens. This method is particularly advantageous as it does not produce harmful residuals, but its efficacy depends on water clarity and proper exposure time (Rizzo et al., 2020).

By identifying ARG prevalence and assessing the efficacy of different disinfection methods, including their ability to remove ESBL-Ec, this study aims to generate data that could inform Public Health policies and strengthen preventative measures within water treatment practices. The objective is to assess three distinct disinfection techniques, providing crucial insights into their efficiency. This information is increasingly critical, given the growing challenges posed by antibiotic resistance on both local and global scales.

2.3.2. Materials and methods

2.3.2.1. Wastewater treatment plant descriptions

Three urban WWTPs (plants A, B and C) with different treatment systems located in the south of Spain, region of Murcia (Fig. 1) were monitored in this study. WWTP A (Abanilla, Fig. 2a) treated approximately 618,430 m^3 per year of mixed wastewater (urban, containing both a hospital and a healthcare residence for elderly, and industrial with vegetables and meat facilities) from a population equivalent (p.e.) of about 12,042. WWTP B (Alhama de Murcia, Fig. 2b) treated approximately 1,321,086 m^3 of urban wastewater per year for a p.e. of about 28,204. The amount of mixed (urban and industrial) wastewater from WWTP C (Cieza, Fig. 2c) was about 2,437,937 m^3 per year for a p.e. of about 58,323 inhabitants.

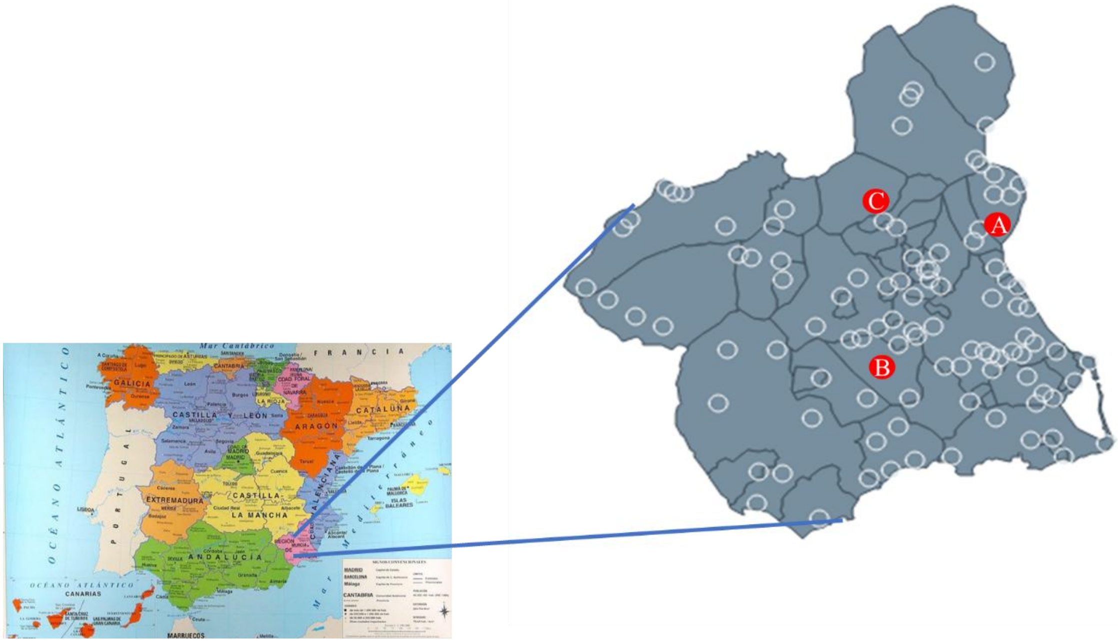

Fig. 1. Wastewater treatment plant (WWTP) localisations. WWTP A: Abanilla (ozone disinfection); WWTP B: Alhama de Murcia (sodium hypochlorite); WWTP C: Cieza (UV-C radiation).

In general, wastewater is pre-treated through separation of suspended solids and particles, desanding-grease removal and a primary setting tank of different dimensions, except WWTP 1 that has no primary setting tank. The secondary treatment consists of a biological aerobic or anaerobic process in a secondary setting including coagulation/flocculation (only in WWTP 3) and extended aeration.

The tertiary treatment of the effluent for reuse is different according to the plant considered:

- WWTP A: Sand Filtration, ozonation and UV disinfection.
- WWTP B: Sand Filtration, sodium hypochlorite and UV disinfection
- WWTP C: Sand Filtration and medium pressure UV-C radiation.

Fig. 2. Different areas of the wastewater treatment plant (WWTP) investigated. A: Abanilla, pre-treatment area; B1: entries of Alhama de Murcia WWTP; B2: chloration area in Alhama de Murcia WWTP; C: entries to Cieza WWTP.

2.3.2.2. Wastewater sample collection

The sampling dates correspond to the months of March and April for sampling 1, and May for sampling 2 in the year 2023, so both cover the spring season. The overall project is wider and cover an entire year of samplings.

During both sampling events, water samples were collected from the influent (raw untreated water) as well as from the effluent (water after undergoing the various treatment processes). The samples from the influent (1 L) were collected before any treatment was applied, while those from the effluent (2 L) were taken after the water had been subjected to the respective treatments at each WWTP. Following the sampling, the analysis was conducted to examine the presence of ESBL-Ec with cultural method and the ARG presence through metagenomic analysis to assess and compare the resistome profiles, providing insight into how these specific disinfection methods affect their abundance and diversity in treated wastewater.

2.3.2.3. E. coli and ESBL-Ec cultural analysis

A standard plate count method on CHROMagar ESBL (CHROMagar, Paris, France) plates was used for the enumeration of ESBL-Ec in all wastewater samples. Chromocult® media was used to enumerate *E. coli*. Serial dilutions were prepared in sterile 0.2% buffered peptone water (BPW, Scharlab, Barcelona, Spain) and, subsequently, spread plating (0.1 mL) or membrane filtration (1, 10 and 100 mL) methods were used. Samples were concentrated with filtration through 0.45 µm cellulose nitrate membrane filters (Sartorius, Madrid, Spain) using a filter holder manifold (Millipore, Madrid, Spain). Plates were then incubated for 24 h at 37 °C, and dark pink-reddish colonies were counted.

The analysis was performed in duplicate, and the results were expressed as Log colony-forming unit (CFU) / 100 mL.

2.3.2.4. Metagenomic analysis of the resistome

The workflow of the metagenomic analysis is shown in Fig. 3. A volume from all the samples (ranging from 20 to 500 mL) were filtered using a nitrocellulose filter with a pore size of 0.2 µm to remove larger particles and retain bacterial and genetic material. For DNA extraction, the DNeasy PowerWater Kit was used. After extraction, the DNA was prepared for sequencing by creating the corresponding libraries, utilising the Native Barcoding Kit 24 V14 (SQK-NBD114.24) to enable multiplexing and accurate sample identification. Finally, the prepared libraries were sequenced on an Oxford Nanopore Technologies MiniON MK1C sequencer, allowing for high-throughput, long-read sequencing of the environmental DNA samples.

The reads generated in the FASTQ files were then processed and analysed bioinformatically entirely at the CEBAS-CSIC institute by the trained Ph.D. student Jesús López.

First, a quality analysis was performed using the NanoPlot tool, which allowed for an evaluation of the overall quality and length distribution of the sequencing reads. Subsequently, all adapters and barcodes used in the library preparation process were removed using Porechop. Afterward, a quality filtering step was carried out using NanoFilt, applying a minimum Phred score of 10 to ensure that only high-quality reads were retained for downstream analysis. Subsequently, a second quality analysis was performed, and the reads with optimal quality were selected for the corresponding resistome analysis. This step ensured that only high-confidence reads were used, allowing for a more accurate and reliable identification of ARGs present in samples. These reads were used for the search of ARGs present in the different samples by using the ResFinder database. ResFinder is a widely used tool that allows for the identification and characterisation of known ARGs based on sequence similarity, enabling a comprehensive analysis of resistance profiles in environmental samples.

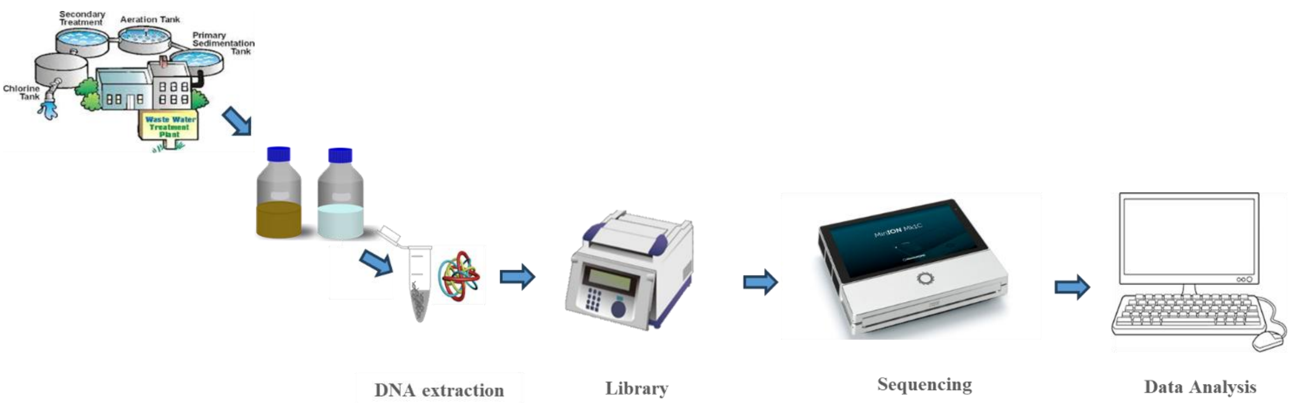

Fig. 3. Schematic representation of the metagenomic analysis performed on WWTP samples.

2.3.3. Results and discussion

2.3.3.1. Occurrence of E. coli and ESBL-producing E. coli in the different wastewater samples

The results of *E. coli* and ESBL-Ec are reported in Fig. 4.

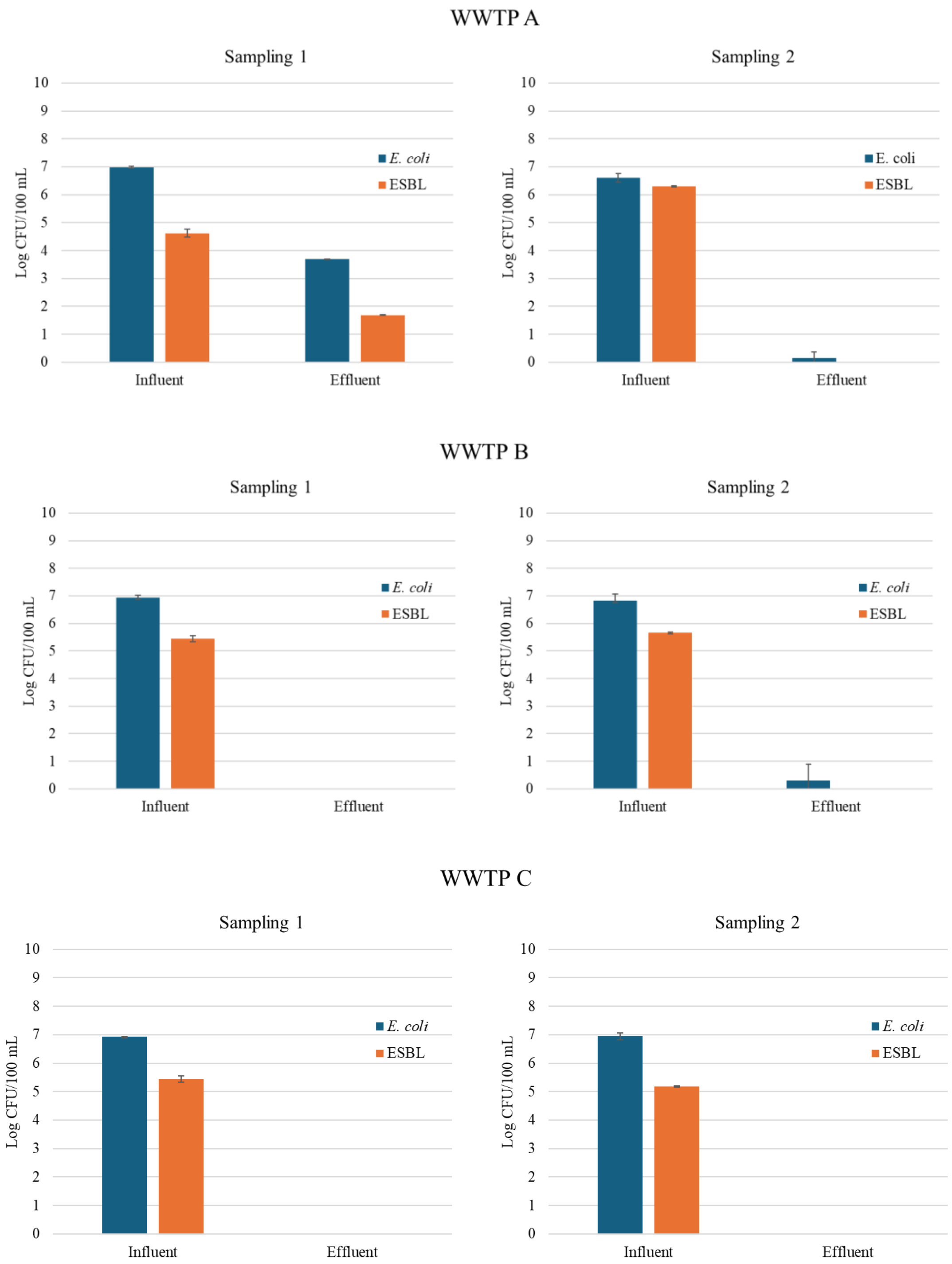

Fig. 4. Distribution of *E. coli* and ESBL-Ec (Log CFU/100 mL) in influent and effluent samples of the three WWTPs monitored between March and May 2023. The results are expressed as mean of the duplicates ± standard deviation (SD).

No significant variation among the different WWTPs in the influent *E. coli* counts was observed (Fig. 4). ESBL-Ec counts ranged from 4.62 to 6.30 Log CFU/100 mL in WWTP A, 5.44 to 5.66 Log CFU/100 mL in WWTP B and 5.19 to 5.44 Log CFU/100 mL in WWTP C. In general, the ESBL-Ec counts observed in the influent samples were similar to other studies present in literature (Gumede et al., 2021), with an overall mean of about 5.44 Log CFU/100 mL, demonstrating that urban wastewater is an important reservoir of these bacteria. Similar concentrations of ESBL-Ec in untreated urban wastewater were observed by Oliveira et al. (2023) in the same geographical area and by Haberecht et al. (2019) in the USA, whereas Schmiege et al. (2021) reported higher ESBL-Ec counts in Germany.

Regarding the effluent samples, significantly lower concentrations of ESBL-Ec were observed, since across all WWTPs and sampling only one resulted positive with very low count; i.e., WWTP A, sampling 1, 1.69 Log CFU/100 mL. This demonstrates that wastewater reclamation processes, including primary, secondary and tertiary treatments, were able to reduce significantly ESBL-Ec counts. Similar results were also obtained by Oliveira et al. (2023) in the same geographical area. These results were not always in accordance with literature, since in other studies from different world areas the ESBL-Ec were still detectable in the WWTP effluent samples (Nzima et al., 2020; Gumede et al., 2021; Li et al. 2023). Moreover, in the research period conducted in the company during the present Ph.D., in the two monitored WWTP effluent samples (see paragraph 2.2) the ESBL-Ec were still present and detectable after similar treatment was applied (UV radiation) and even after higher quality tertiary treatment (Membrane Bio Reactor, MBR).

2.3.3.2. Resistome analysis

2.3.3.2.1. Relative Abundance of ARGs: distribution by antibiotic type in influent and effluent samples

A detailed analysis of the ARG relative abundance (out of 100) was conducted for the samples collected during both sampling events, including those from the influent and effluent. This analysis was performed based on the specific types of antibiotics to which the ARGs confer resistance, covering a total of 17 different antibiotic types. The relative abundance of each ARG was calculated by comparing the prevalence of each gene in relation to the others within the samples. The results of this comprehensive analysis are presented in Fig. 5. In samples corresponding to the influent, a greater variety of ARGs is observed, which is represented by the different colours. This indicates that a higher number of distinct ARGs are present in the raw water before any treatment has been applied. In contrast, the effluent samples show a noticeably lower diversity of ARGs, suggesting that, after the application of the various disinfection methods, some types of ARGs have been effectively removed or reduced. This reduction in diversity reflects the effectiveness of the disinfection processes in eliminating certain resistance genes from the wastewater.

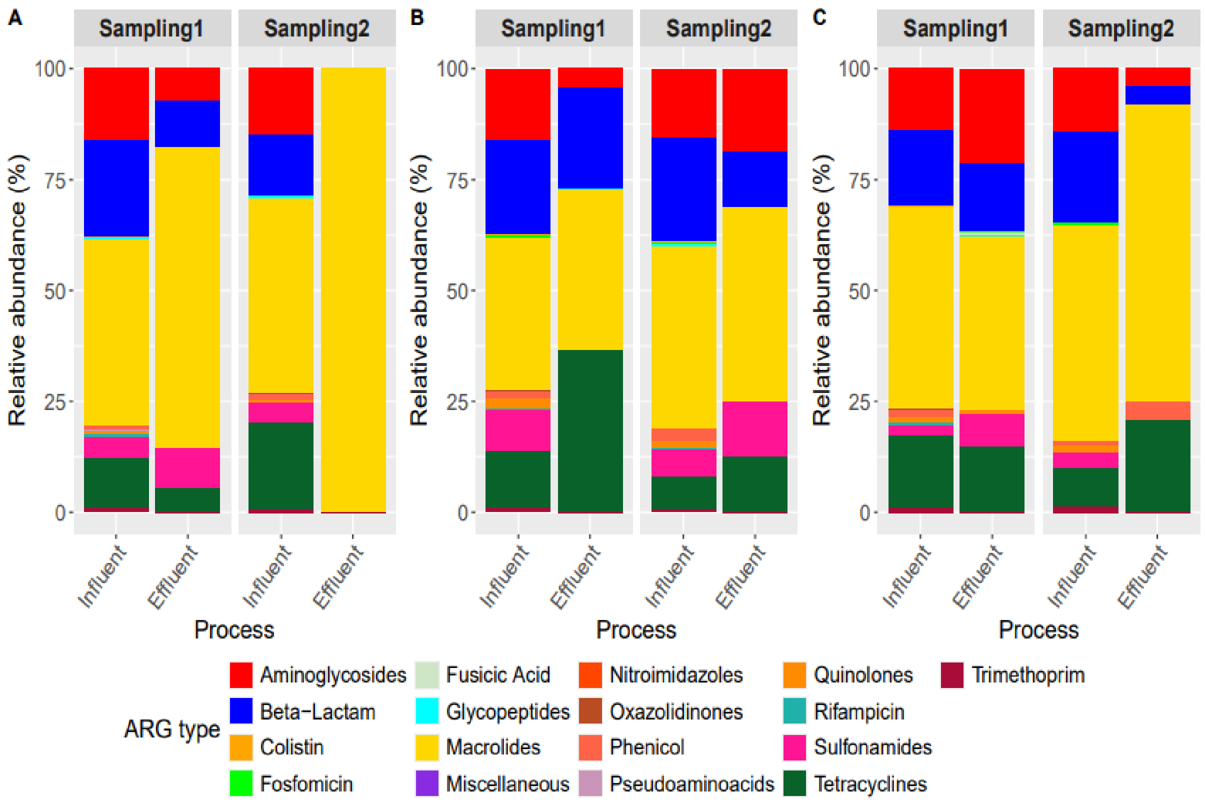

Fig. 5. Relative abundance of antibiotic resistance genes categorised by the specific antibiotics to which they confer resistance. A: WWTP A; B: WWTP B; C: WWTP C.

Samples corresponding to the WWTP A (Fig. 5a) show that, after applying the disinfection methods, the ARGs that tend to prevail in the treated effluent are those against macrolides (Sampling 1: from 42% to 68%; Sampling 2: from 44% to 100%). In the samples corresponding to the WWTP B (Fig. 5b), differences are observed between both samplings. After applying the appropriate disinfection, there is an increase in the abundance of ARGs against tetracyclines in Sampling 1 (from 12.9% to 36%), while in Sampling 2, this increase is observed in ARGs against sulphonamides, tetracyclines and aminoglycosides (sulphonamides: from 6% to 12.5%; tetracyclines: from 7.5% to 12.5%; aminoglycosides: from 15.6% to 18.75%). Finally, with reference to the WWTP C (Fig. 5c), there are also differences between both samplings. In Sampling 1, the ARGs against aminoglycosides and sulphonamides increase in abundance after applying the disinfection (aminoglycosides: from 13.7% to 21.3%; sulphonamides: from 2.5% to 7.3%), while in Sampling 2, those against macrolides and tetracyclines show an increase (macrolides: from 49% to 67%; tetracyclines: from 8.4% to 21%).

Despite some ARG classes remained dominant from the influent to the final effluent, the overall relative abundance of ARGs decreased. This is in accordance with the study of Garner et al. (2024) in which they observed the same trend in WWTPs that have similar tertiary treatments (i.e., ozonation, free chlorine and UV radiation). Moreover, also in the study of Shi et al. (2018) in which they monitored 18 WWTPs, some of them with UV radiation and chlorine disinfection methods, found total ARGs relative abundances in the influent significantly higher than those in the effluent across all WWTPs. Moreover, in the study of Majeed et al. (2021), in which they evaluated a WWTP that applies UV disinfection, was found that relative abundance of total ARGs decreased by ~50% from the influent to the effluent (Majeed et al., 2021).

In addition, some of the prevalent ARG types were sulphonamide, aminoglycoside, macrolide, beta-lactam and tetracycline along with what observed in other studies (Begmatov et al., 2024; Chen et al., 2024).

The comparison of relative abundances across different studies may not be entirely linear, as the variables involved can differ. These variables include factors such as the reference databases used, the units of ARG abundance, and sequencing depth. Overall, the data consistently suggest that the general trend is a reduction in ARG variety during wastewater treatment, rather than a complete elimination.

2.3.3.2.2. Analysis of ARG abundance: copies per million reads (CPMs) in influent and effluent samples from WWTPs

In following section, a detailed analysis of the number of copies of ARGs, expressed as copies per million reads (CPMs), is performed for each of the different samplings (Fig. 6). This includes samples from both the influent and effluent at each of the WWTPs, allowing for a comparison of the ARGs distribution before and after the treatment processes.

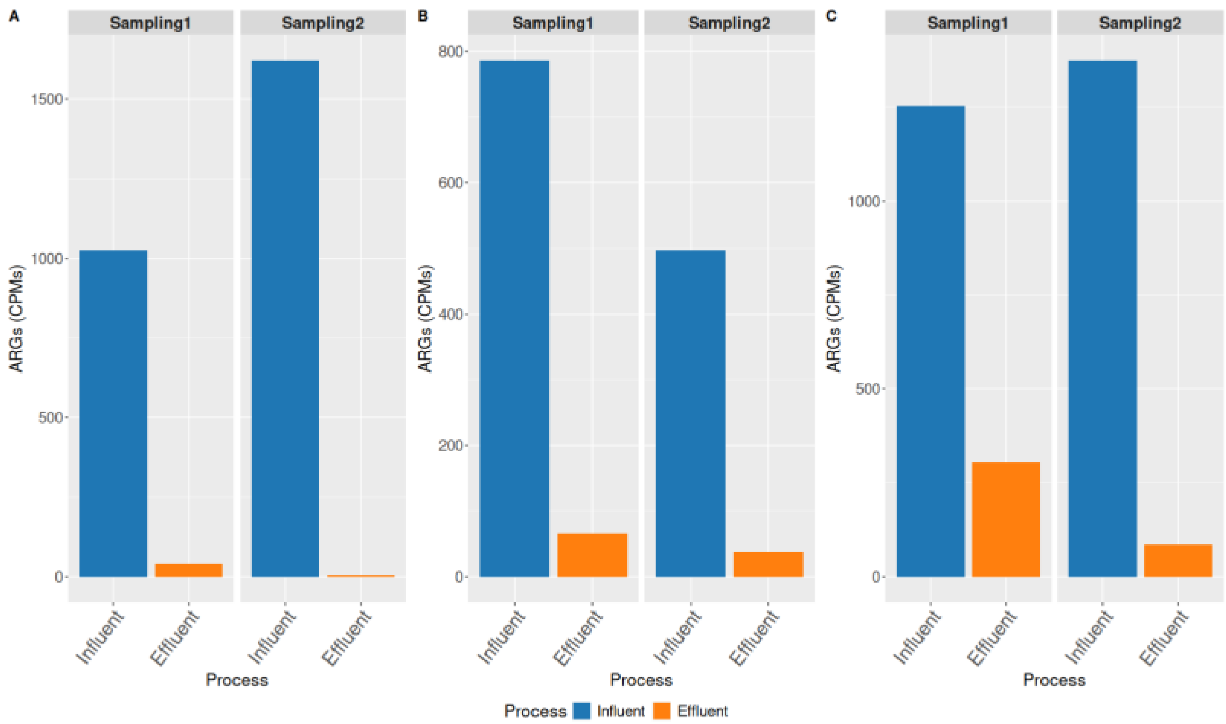

Fig. 6. Number of copies per million reads of the ARGs present in the different samples, divided by sampling type. A: WWTP A; B: WWTP B; C: WWTP C.

In all the samplings from the analysed WWTPs, a clear decrease in the number CPMs of ARGs is observed, which suggests that the disinfection methods applied are functioning effectively in reducing the abundance of these resistance genes in the treated wastewater. The WWTPs A and C are the ones that showed the highest CPMs of ARGs in their influents (over 1000 in both samplings; Sampling 1: 1024 CPMs and 1253 CPMs, Sampling 2: 1621 CPMs and 1374 CPMs, respectively), compared to the WWTP B, where the number did not exceed 800 (Sampling 1: 786 CPMs; Sampling 2: 497 CPMs). When examining the CPMs corresponding to the effluents, it is observed that all the samples, with the exception WWTP C in Sampling 1, show values below 100. This suggests that the disinfection processes were effective in reducing the number of ARGs in the effluent.

A significant decrease in the effluent samples was also observed by other studies that evaluated the effect of UV disinfection method in terms of number of ARG copies (Lira et al., 2020; Majeed et al., 2021). Conversely, in the study of Keenum et al. (2024), investigating two different WWTPs that produce effluents intended for reuse, they found that abundances of total ARGs/clinically-relevant ARGs were reduced during primary and secondary stages of wastewater treatment, but to a lesser extent during the tertiary water reuse treatments. In particular, ozonation tended to enrich multi-drug ARGs.

Globally, the number of ARGs resulted decreased in literature within the wastewater treatment as observed in the present study. Despite this, as observed for the relative abundance, ARGs were still detectable in all sampling in all the WWTPs monitored.

2.3.3.2.3. Analysis of the 25 most prevalent ARGs: variations in copy numbers across treatment methods

The following section presents an in-depth analysis of the 25 most prevalent ARGs identified across both samplings (Fig. 7). This analysis explores the differences in the copy number (CPMs) of specific ARGs between the influent and effluent samples, highlighting how each of the three treatment methods - ozone, sodium hypochlorite and UV-C radiation - impact the presence and reduction of these genes.

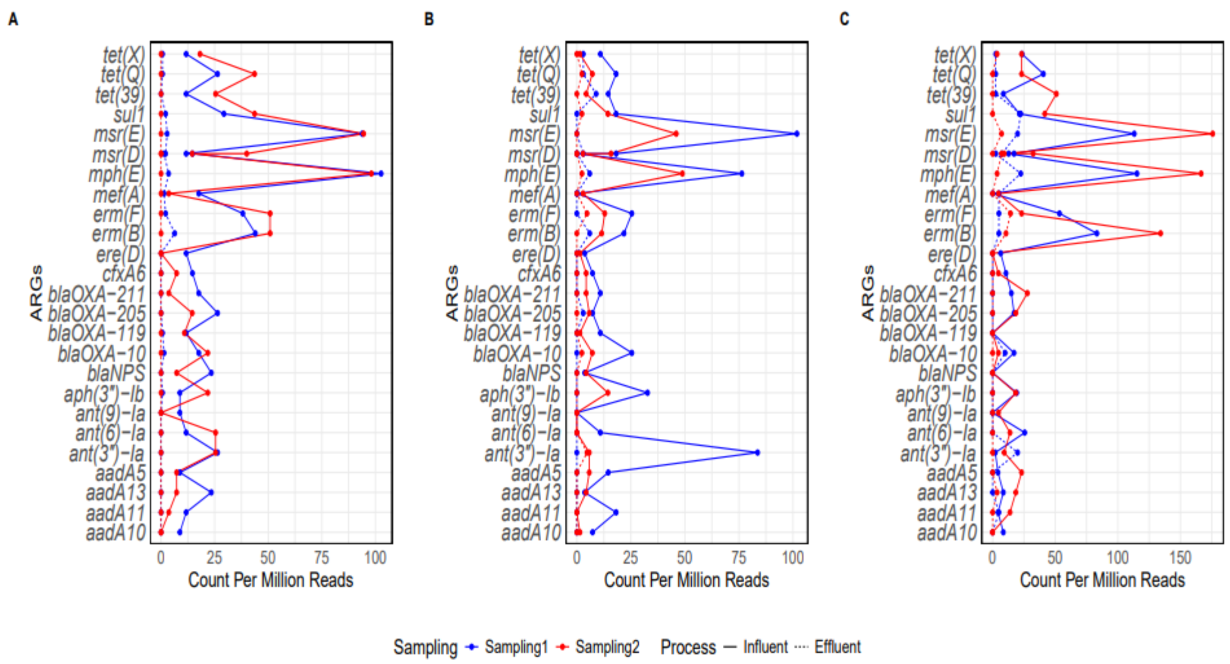

Fig. 7. Variation of the 25 most prevalent ARGs identified in both samplings, showing differences in abundance between influent and effluent samples after treatment. A: WWTP A; B: WWTP B; C: WWTP C.

A clear variation in the number of CPMs is revealed between the influent and effluent samples across the three analysed WWTPs, highlighting the effectiveness of the applied treatment processes in reducing resistance genes. In general, a substantial presence of the ARGs *msr(E)*, *mph(E)*, *erm(B)*, and *erm(F)* is observed in influent. Furthermore, in the WWTP A and B (Fig. 7a and Fig. 7b, respectively), there is evidence of bla_{OXA} genes, which confer resistance to beta-lactams, and for WWTP B also *ant* genes, which confer resistance to aminoglycosides. On the other hand, in effluent, the copy numbers of the genes tend to decrease, exhibiting lower and more similar values across different ARGs. This suggests a reduction in gene abundance following the treatment processes.

According to the results of a worldwide analysis of wastewater resistomes in large cities (Munk et al., 2022), *mph*(E), *msr*(E), *tet*(A), and *sulI* were among the most ubiquitous ARGs. Moreover, in the study of Begmatov et al. (2024) performed in WWTPs located in Moscow (Russia) they found, as the most ten abundant ARGs, *qacE, sulI, ampC, blaOXA, msr(E), erm(B), mph(E), tet(C), aph(3")-Ib* and *aph(6)-Id* in accordance with the present study. The same results were also obtained by Wardi and collaborators (Wardi et al., 2024) in WWTPs from Morocco.

These results suggest that the patterns of ARG presence could be conserved across different regions of the world, although their prevalence may vary locally in WWTPs depending on social, economic, medical, and environmental factors.

2.3.4. Conclusions

In conclusion, despite the data here presented are not intended to be representative of the entire study, they provide preliminary important insights into the presence and variation of ESBL-Ec (intended as a model for ARB detection and quantification) and ARGs in WWTPs.

A bioinformatics pipeline ensured sequencing data quality and high-quality sequences were then screened for ARGs. This approach enabled the identification of a wide array of resistance genes in the influent and effluent samples across the different WWTPs. The analysis revealed a higher abundance and diversity of ARGs in influent water. The results of this study showed the effectiveness of wastewater treatment processes in reducing the environmental load of ARGs and ESBL-Ec. The application of various disinfection methods, including ozone, sodium hypochlorite and UV-C radiation, significantly decreased the abundance and variety of both ESBL-Ec and ARGs in effluent samples. This finding highlights the role of these treatment processes in mitigating the spread of antibiotic resistance elements in the environment. However, the persistence of certain ARGs, particularly those associated with macrolides and beta-lactams, in some effluent samples suggests that some resistance mechanisms are more resilient to treatment. This is a critical observation, as it indicates that further optimisation of the treatment processes may be necessary to achieve more complete removal of resistant bacteria and genes. The continued monitoring of these genes is essential to assess the long-term effectiveness of treatment strategies. Overall, the findings of this study contribute valuable information to the ongoing efforts to reduce the environmental impact of antibiotic resistance. By understanding the dynamics of ARGs in wastewater treatment systems, it is possible better inform policy and improve the management of antibiotic resistance in the environment. The persistence of certain resistance genes in effluent highlights a growing public health concern, as these genes can be transferred to environmental microbiomes, potentially leading to the spread of resistant pathogens. Future research should focus on refining treatment methods and exploring new strategies to reduce the persistence and spread of ARGs in effluent water, which is crucial for protecting both human and animal health.

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2.4. *Second published manuscript: A systematic scoping review of antibiotic-resistance in drinking tap water*

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Review article

A systematic scoping review of antibiotic-resistance in drinking tap water

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2.4.1. Abstract

Environmental matrices have been considered of paramount importance in the spread of antibiotic-resistance; however, the role of drinking waters is still underexplored. Therefore, a scoping review was performed using a systematic approach based on PRISMA guidelines, with the aim of identifying and characterizing antibiotic-resistance in tap water, specifically, water treated at a potabilization plant and provided for drinking use through a water distribution system. The review included 45 studies, the majority of which were conducted in upper-middle-income economies (42.2%), mainly from the Western Pacific region (26.7%), followed by Europe (24.4%). Most of the papers focused on detecting antibiotic-resistant bacteria (ARB), either alone (37.8%) or in combination with antibiotic-resistant genes (ARGs) (26.7%). Multidrug-resistance profile was often identified in heterotrophic bacteria, including various species of nontuberculous mycobacteria, Pseudomonas spp., and *Aeromonas* spp., which were especially resistant to penicillins, cephalosporins (including 3rd-generation), and also to macrolides (erythromycin) and tetracyclines. Resistance to a wide range of antibiotics was also prevalent in fecal bacteria, e.g., the Enterobacteriaceae family, with common resistance to (fluoro)quinolones and sulfonamide groups. ARGs were investigated either in bacterial strains isolated from tap waters or directly in water samples, and the most frequently detected ARGs belonged to β-lactam, sulfonamide, and tetracycline types. Additionally, mobile genetic elements were found (i.e., *int*1 and *tnp*A). Sulfonamides and macrolides were the most frequently detected antibiotics across countries, although their concentrations were generally low (<10 ng/L) in Europe and the United States. From a health perspective, tap water hosted ARB of health concern based on the 2024 WHO bacterial priority pathogens list, mainly *Enterobacteriaceae* resistant to 3^{rd}-generation cephalosporin and/or carbapenem. Despite the fact that tap water is treated to meet chemical and microbiological quality standards, current evidence suggests that it can harbor antibiotic-resistance

determinants, thus supporting its potential role in environmental pathways contributing to antibiotic resistance.

2.4.2. Introduction

Antimicrobial resistance (AMR) is a global public health threat and one of the greatest worries about AMR is represented by antibiotic resistance phenomenon, because antibiotics are widely used for clinical and prophylactic treatments in human health systems, but veterinary medicine also relies heavily on antibiotics (O'Neill, 2014). Antibiotic-resistance has been recently considered a quintessential One Health issue, given the interconnection among human, animal and ecosystem domains in the antibiotic-resistance spread (WHO, 2022a) and the awareness of the environmental role in such triad is progressively increasing (UNEP, 2022, 2023). It is well demonstrated that antibiotic-resistance determinants may not be entirely removed from wastewater treatment plants (WWTPs), and they can behave as hotspot sources of antibiotic-resistance bacteria (ARB), antibiotic resistance genes (ARGs) (Rizzo et al., 2013; Gwenzi et al., 2020; Bonetta et al., 2023), and also antibiotics (Sanseverino et al., 2018).In turn, surface water as well as groundwater receiving sublethal concentrations of antibiotics (in the order of ng/L; Sanseverino et al., 2018) can promote the selection of ARB and ARGs. Once in the drinking water supplies, antibiotic-resistant determinants can pass through drinking water treatment plants (DWTPs), because some treatments show very little ARG abatement (and even promotion effect of ARGs), as in the case of sand filtration toward chloramphenicol resistant genes and activated carbon filtration toward ARGs providing resistance to sulfonamide and quinolone (Zheng et al., 2018; Su et al., 2018). Moreover, chlorination, frequently used as final disinfection stage for potable water production, could enhance dissemination of antibiotic resistance by increasing total relative abundance of various ARGs (Guo et al., 2014; Jia et al., 2015). Such effect mainly happens at low concentration of chlorine, that could exert co-selection mechanisms and improve horizontal gene transfer (HGT) as a result of cell membranes permeabilization that increases both transformation (acquisition of extracellular ARGs) and conjugation (ARG exchange among different bacteria genera) phenomena (Sanganyado and Gwenzi, 2019; Gao and Sui, 2021). These literature data are also confirmed by a scientific report to the Water Research Commission, that addressed the monitoring of waters at the inlet and at the outlet of various DWTPs with different treatment schemes; it showed that various bacterial species isolated from the raw waters and the finished drinking waters had similar antibiotic resistance and virulence phenotypes (Bezuidenhout et al., 2019). Therefore, populations can be exposed to antibiotic-resistant determinants through the ingestion of household waters. In fact, in many countries throughout the world, the household waters are suitable for human consumption and people cover most of their daily water requirement by drinking water directly from the tap (ECORYS, 2015). Health effects of antibiotic-resistance in drinking waters are still largely unexplored, although WHO suggested three possible adverse outcomes (WHO, 2015). The first is human infection by ARB, as confirmed by some outbreaks where the integration between epidemiological surveillance data and environmental monitoring of drinking waters confirmed the role of such a matrix as the vehicle of the resistant pathogens, e.g., *Shigella sonnei* resistant to azithromycin and 3rd generation cephalosporin in China (Ma et al., 2017) and multidrug resistant *Salmonella typhi* in Nepal (Lewis et al., 2005). The second mechanism is the gut colonization by resistant microbes (e.g., Escherichia coli), that is supported by an epidemiological evidence from Coleman et al. (2012), who performed a cross-sectional study showing an association between drinking water consumption and the presence of β-lactam resistant *E. coli* in human feces (namely resistance to, e.g., penicillin and cephalosporins). The third mechanism is ARG transfer to normal microflora, according to a hypothesis early suggested by Salyers et al. (2004) who highlighted the increase of tetracycline- and erythromycin-resistant *Bacteroides* spp. in human stools comparing community colon isolates before the use of antibiotics in human medicine and during the antibiotic era. Such hypothesis has been recently demonstrated through in vitro and in vivo experiments. In particular, Zhou et al. (2022) found that extracellular ARGs showed high gene horizontal transfer potential passing through an artificial digestive tract, especially ARGs against tetracyclines; similar results were obtained by Khan et al. (2020) who spiked the feeding waters of mice with colistin-resistant Bacillus cereus and found that such resistance had been transferred to *Enterococcus hirae*, that is an intestinal indigenous bacterium. Therefore, antibiotic-resistant bacteria and genes in tap waters can pose potential risks to human health. Although to date, there is no legislative requirement for testing drinking waters for antibiotic-resistant determinants, the scientific interest on this topic is rapidly increasing. Thus, this work was aimed at collecting the available evidences on ARB, ARGs, and antibiotics in drinking tap waters.

2.4.3. Material and methods

2.4.3.1. Review type and research team

The scientific literature has been investigated through a scoping review (ScR; Peters et al., 2020), which is suited to identify and describe relevant evidence on an existing or emerging topic using a broader research question, as described by the Joanna Briggs Institute (e.g., Peters et al., 2015, 2020; Munn et al., 2018). To increase methodological transparency and uptake of research findings, the ScR was conducted following the Preferred Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines, adapted for ScR by Tricco et al. (2018) and already applied for knowledge synthesis of environmental science topics (Corrin et al., 2024). Prior to conducting the ScR, a multidisciplinary team (represented by the authors of the present paper) with expertise in environmental hygiene, public health, microbiology, and evidence synthesis discussed and approved a protocol that included the following information: research question, literature search strategy (search string and database), inclusion/exclusion criteria, and data charting form for the extraction of the information from the papers, as detailed below.

2.4.3.2. Research question and eligibility criteria

The goal of the ScR was to investigate the current evidence that address the research question:

Which ARB, ARGs or antibiotics have been detected in drinking tap waters?

The inclusion criteria were:

(1) Publication date: no time limitation

(2) Language: literature published in English

(3) Document type: Primary research, namely monitoring studies where the authors collected and analyzed their own data

(4) Type of waters: waters that received potable treatment and that were distributed to the communities via drinking water distribution systems.

Moreover, we considered methodological rigor as a further eligibility criterion, based on a Kmet checklist (Kmet et al., 2004) for evaluating primary research papers from various fields. In particular, we considered eligible for the inclusion in the present ScR, the papers that reported the question/objective sufficiently described, the study design evident and appropriate, subject characteristics sufficiently described, and sample size appropriate (> or = 10 tap water samples analyzed, to provide adequate representativeness of such matrix), analytical methods described/justified and appropriate, and results reported in sufficient details. Exclusion criteria are reported below.

• Articles not published in English

• Reviews and non-primary literature (e.g., commentaries, opinions, letter to the editors)

• Articles on untreated or partially treated waters within a DWTPs (e. g., water supplies, either fresh or ground waters, water at the exit of each treatment step) or treated but not piped into a distribution system (e.g., water immediately at the exit of final disinfection)

• Articles on waters used for human consumption but that either i) did not receive any treatment (e.g., well or bottled waters), or ii) it was not clear whether they had received treatment or not • Articles reporting aggregated data, namely data on tap waters were presented as a whole with results on other analyzed environmental matrices (e.g., raw water supplies, surface waters, spring waters, sewages, water at different stages of the potabilization treatment) thus hampering the extraction of data specific to tap waters

• Articles whose methodology was not compliant with the criteria selected from Kmet et al. (2004), namely small sample size (<10 samples), analytical methods not adequately described, such as the lack of limit of detection (LOD) and limit of quantification (LOQ) for papers on antibiotics, and results not clearly explained (e.g., information needed to be inferred from figures).

2.4.3.3. Literature search strategy and study selection

The search string includes the following terms ("antibiotic-resistant bacteria" OR "antibiotic-resistant gene" OR ARB OR ARG OR antibiotic) AND ("tap water" OR "potable water" OR "finished water" OR "drinking water"). Searches were conducted on February 6th, 2023, using three electronic databases: PubMed (search field = all fields), Scopus (search field = article title, abstract, and keywords), Web of Science (WoS) core collection (search field = topic). Searches were then updated on August 28th, 2024 using identical search string. The records were cleaned of duplicates using the Zotero platform (Corporation for Digital Scholarship) (Fernandez, 2011). The study selection was performed in two stages (e.g., Boehm et al., 2018). The initial screening for eligibility entailed reading of the title and abstract. It was purposely inclusive, therefore if the abstract was not available and/or the relevance of the article could not be determined from the title, the document was retrieved for full reading. Each article that passed initial screening was subjected to full-text screening. The two-step study selection was done by five reviewers. At each stage, a selection of 20 articles was reviewed by all the above-mentioned investigators to reach consensus about applying the eligibility criteria. After that, each investigator independently assessed the retrieved articles (Lenzen et al., 2017). Any doubt raised during the screening process was resolved through periodic on-line meetings among the entire research team.

2.4.3.4. Data collection and management

A data-charting form was jointly developed by the research team to determine which variables to extract from the articles that met the eligibility criteria. In particular, the following variables were considered: year of publication, country, sample size, sample volume, targets of the monitoring (separately for ARB, ARGs, and antibiotics), analytical methods, and results (types and occurrence of ARB, ARGs, or antibiotics). The data charting process was performed independently by the abovementioned reviewers, who periodically discussed their results and continuously updated the data-charting form.

The results were globally presented in terms of geographical area based on WHO region (WHO, 2024a) and the United Nations Statistics Division (UNSD, 2024), income level (World Bank, 2024), and temporal distribution of the included articles. Then, information on methodological approaches and main findings were synthesized separately for ARB, ARGs, and antibiotics. For the purpose of the present study, ARB were divided into fecal and environmental types, based on the matrix where they are mostly detected. Thus, fecal ARB included bacteria from Enterobacteriaceae family and Enterococcus genus, and the other types of microbes, including heterotrophic plate count (HPC) flora, were considered environmental ARB. Moreover, the occurrence of ARB of particular health concerns was also considered and discussed, given the relevance of tap waters for public health. For the classification of these types of ARB, the recent update of the WHO Bacterial Priority Pathogens List (BPPL) was considered (WHO, 2024b), that spans different families of antibiotic-resistant bacterial pathogens. This list classifies the ARB into 3 priority groups depending on the need of research and development of new antibiotics, given their global impact in terms of burden and issues related to transmissibility, treatability, and prevention options. The scheme of WHO BPPL used for classifying the health-relevance of ARB detected in tap waters is reported in Table S1.

2.4.4. Results and discussion

2.4.4.1. Search results and overall study characteristics

The search of the published literature yielded 11,993 articles: 3277 from Pubmed; 4623 from Scopus; 4093 from WoS (Fig. 1). A total of 152 papers were selected for retrieval on the basis of the inclusion criteria. Of these, 107 were excluded after full reading for the following reasons: 21 articles not focused on drinking tap water (e.g., water at various stages of drinking-water supply chain, well waters, bottle waters, tap waters not used for drinking purposes); 9 articles did not make it clear whether the tap water was treated or not; 18 articles reporting aggregated data; 41 articles provided a methodology that was not considered eligible for the present ScR, mainly given the small sample size. Moreover, 18 papers were also excluded because they were not relevant for the ScR, since they showed different goals related to tap waters (e.g., investigating the role of biofilm, studying the antibiotic-resistance determinants in microcosms) ("Other" category in Fig. 1). The studies that did not meet the criteria are listed in Table S2 with the reasons of exclusion. Overall, 45 articles were included in the review and considered eligible for the assessment, the main characteristics of which are summarized in Table S3. The first paper appeared in 1988, and the publishing rate showed a gradual increase starting from 2005. Then, since 2017, the number of papers has markedly increased (Fig. 2), probably as a result of the global concerns and awareness about antimicrobial resistance, as shown by internationally relevant documents, such as the 2016 United Nations political declaration on AMR (UN, 2016) and the first BPPL released by WHO in 2017 on ARB for which the development of new antibiotics is urgently needed (WHO, 2017). The geographical distribution indicates the predominance of the papers carried out in Asian countries, in fact, in terms of UNSD regions, the most representative were Eastern Asia (24.4%), followed by Southern Asia (13.3%) and Western Asia and Eastern Europe (8.9%, each). In fact, considering WHO regions, the most frequently represented areas were Western Pacific region (26.7%) and Europe (24.4%). The most represented countries were China (10/45, 22.2%) and Poland (4/45, 8.9%). Most of the studies were performed in upper-middle-income economies (42.2%) followed by high-income and lower-middle countries (28.9%, each), but none were conducted in low-income economies (Table S4; Fig. 3). Regarding the aim of the monitoring, in most cases, the studies were aimed at investigating ARB alone (17/45, 37.8%) or in combination with ARG (12/45, 26.7%), while the rest of the papers were focused only on the monitoring of ARGs (10/45, 22.2%) and antibiotics (6/45, 13.3%). Therefore, the investigation of tap waters for antibiotic-resistance is performed preferentially using culture-based methods for establishing phenotypic profiles of ARB. Such a result differs from data reported by Siri et al. (2023) in a water environment that showed a widespread use of molecular methods.

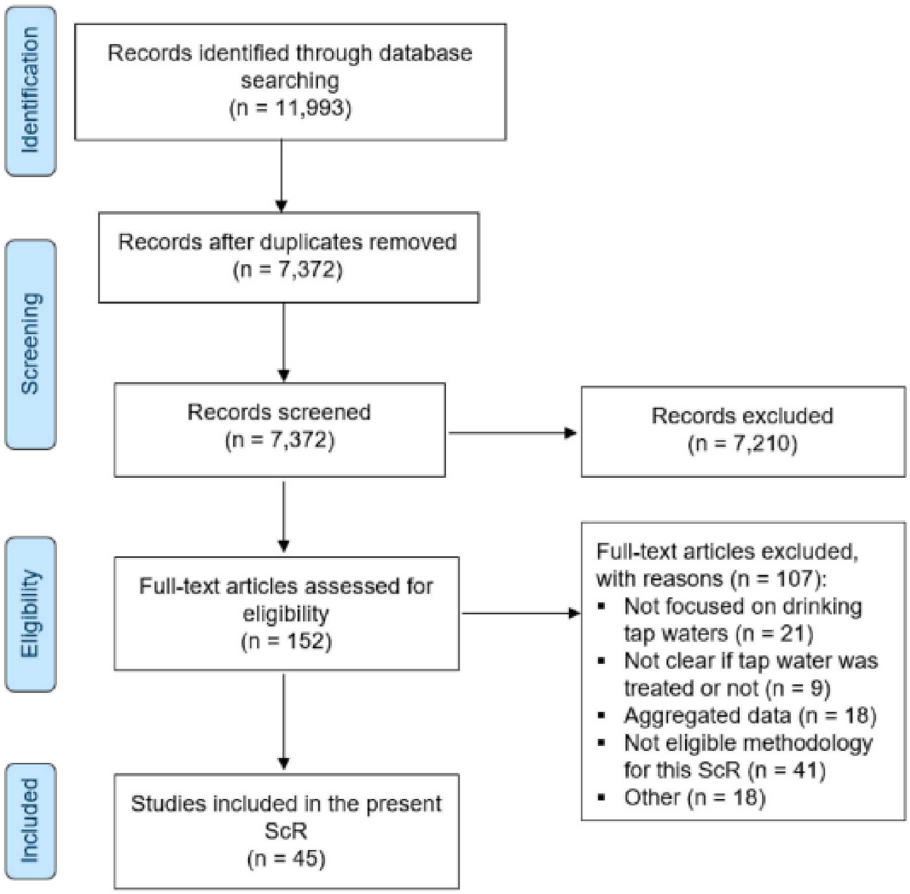

Fig. 1. PRISMA flow diagram of the articles of the scoping review process on antibiotic-resistance in tap waters.

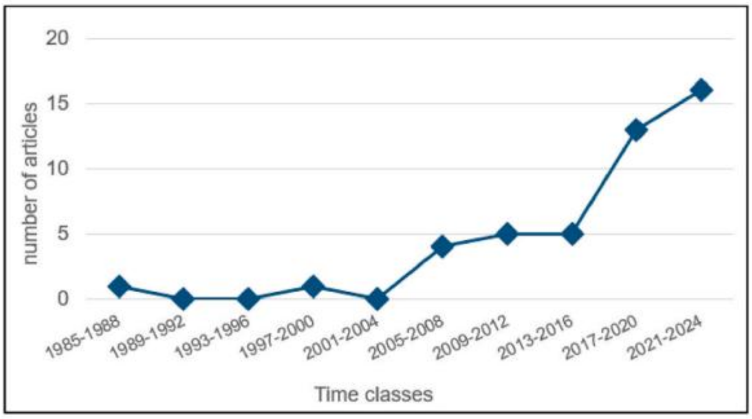

Fig. 2. Time-trend of the included articles (4-year classes). The first article has been published in 1988. For 2024, the search is limited to eight months.

2.4.4.1.1. Tap water definition

Given the heterogeneous origin of the selected papers, water quality at the point of use needs to be further explored since tap water requirements can vary in different countries. At the international level, the reference for the establishment of national/regional regulations for water safety is represented by the guidelines for drinking-water quality (GDWQ), released by the WHO since 1958 and recently updated (WHO, 2022b). The verification and surveillance of microbial water quality are based on the monitoring of fecal indicators, namely E. coli or thermo-tolerant coliforms, that should be absent per volume in all waters intended for human consumption as well as treated water at the entrance and at the exit of the distribution system (guidelines value 0/100 mL; WHO, 2022b). Nevertheless, GDWQ represents guidance for the development of countries' own regulations, therefore they can be adapted to local conditions, circumstances, needs, and resources of countries (WHO, 2021). As an example, some countries fail to meet the requirement for microbial water safety and allow that coliform bacteria may be detected in samples on occasions, also considering that drinking-water of a particular quality may lead to different health effects in different populations, given the variable susceptibility to pathogens (WHO, 2022b). In developed countries, the loss of compliance occurs occasionally and can be the result of, e.g., deterioration in source water quality, failures associated with treatment processes or the integrity of distribution systems, and inadequate disinfection (CDC-EPA-AWWA, 2016; Galway, 2016). In low and middle-income countries, deterioration of tap water is frequent, especially owing to the lack of adequate supply infrastructure for water distribution. In these areas, even if the water is treated adequately by the potabilization facility, low and intermittent water pressure within the piped water supply system is common as a result of water shortage and rupture of the distribution networks, thus drawing the surrounding contaminants into the water supply (Mermin et al., 1999; Shakya et al., 2022), but also cross-contamination of drinking water with sewage (Qamar et al., 2018; Lewis et al., 2005). When tap water exceeds microbial water quality standards, point-of-use household water treatments (e.g., boiling) are recommended by water suppliers and public health authorities before drinking, thus reducing the exposure to pathogens via ingestion (WHO, 2015; WHO, 2022b). Nevertheless, unsafe tap water can be used for other household purposes, e.g., showering, washing clothes, toilet flushing, which could lead to exposure to microbes via accidental swallowing, inhalation or contact with intact skin or wounds.

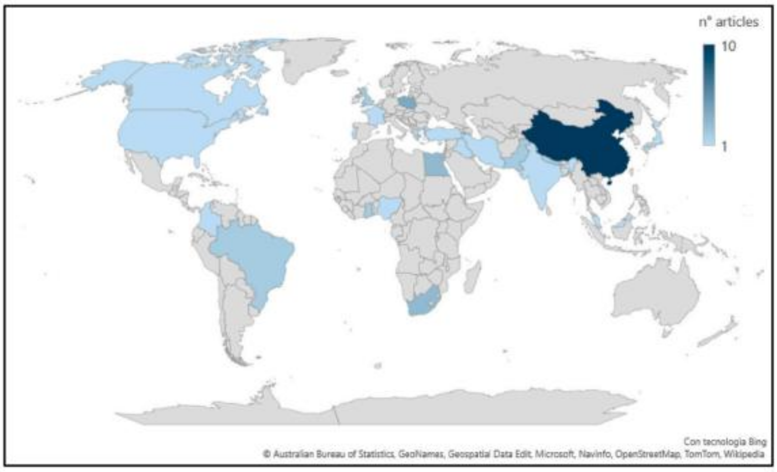

Fig. 3. Geographical distribution of the included articles (the darkest the color the highest the number of the articles published by the country).

2.4.4.2. Antibiotic resistant bacteria

Most of the reviewed articles (29/45) were focused on the investigation of ARB, of which fourteen investigated environmental ARB (Ateba et al., 2020; Davalos et al., 2021; Emekdas et al., 2006; Ezzat, 2014; Furuhata et al., 2006; Leginowicz et al., 2018; Moghaddam et al., 2022; Molale-Tom et al., 2024; Siedlecka et al., 2020, 2021; Scoaris et al., 2008; Vaz-Moreira et al., 2012; Walsh et al., 2011; Zhang et al., 2018), ten monitored fecal ARB and staphylococci (Adzitey et al., 2016; Akbar et al., 2022; Bhatta et al., 2007; Elmi et al., 2021; Elmonir et al., 2020; Hamza et al., 2020; Kinge et al., 2010; Papandreou et al., 2000; Santos et al., 2023; Subba et al., 2013) and five articles investigated both environmental and fecal ones (Ahmed et al., 2022; Adesoji et al., 2017; Borjac et al., 2023; Jazrawi et al., 1988; Siedlecka and Piekarska, 2019). The characteristics of such articles are summarized in Table 1 and described below. The presence of ARB was evaluated in less than 50 samples in more than half of the reviewed articles (16/29) (Table S3). Sample analysis and bacterial isolation methods relied mostly on the membrane filtration technique, which represents the standard procedure for bacteriological examination of drinking waters. Briefly, water samples are filtrated on a 0.45 µm pore size membrane filter, then the membrane is incubated on a selective agar plate, then single colonies are selected for subsequent analysis of identification and antibiotic susceptibility testing. In some cases, authors introduced modifications to the procedure, such as the pore size (0.22 µm) or the usage of agar media already supplemented with an antibiotic. Only few studies applied different sample analysis approaches, such enrichment culture method following by culturing onto selective media and bacterial precipitation by centrifugation (Table 1). The identification of the isolates occurred mainly via biochemical techniques, frequently followed by genotypic identification of the selected isolates by sequencing the 16S rRNA gene. In the case of HPC, the identity of the isolates was determined using only a genotypic approach (Table 1). The preferred method for evaluating antibacterial activity was the agar disk-diffusion method, which is a well-known procedure, where a filter paper disk containing a desired concentration of a certain antibiotic is placed on the surface of agar plates inoculated with the tested bacteria. In particular, this method has been applied by all the reviewed articles focusing on fecal ARB, while the analysis of antibiotic-resistance in environmental strains was performed also using the dilution method. The dilution method involves the incorporation of the desired antibiotic in a growth medium (agar or broth) containing the bacterial inoculum and allows the testing of several dilution of the desired antibiotic, thus allowing the determination of the Minimum Inhibitory Concentration (MIC) value. Overall, the authors distinguished the isolates in resistant or susceptible to a given antibiotic according to international committees (i.e., Clinical and Laboratory Standards Institute – CLSI, or the European Committee on Antimicrobial Susceptibility Testing - EUCAST), which provides guidelines for interpreting the MIC break-point or the inhibition zone diameter breakpoint. However, this approach is considered with caution by reviewed articles on environmental strains, as detailed in Sect. 2.4.3.2.2.

Table 1
Methodological aspects and types of antibiotic-resistant bacteria detected in the reviewed articles on tap waters.

Authors (country)	Category of bacteria investigated	Sample concentration and bacterial isolation	Methods for bacterial identification	Analytical method for verifying sensitivity to antibiotics	Types of ARB families or genus detected
Adesoji et al., 2017 (Nigeria)	Environmental, fecal	No concentration method. 1-ml samples were serially diluted and cultured on nutrient or selective agar media, depending on the searched microbes	Genotypic techniques	Agar dilution method	Various psychro- and mesophilic bacteria species; various species of *Enterobacteriacee* family
Adzitey et al., 2016 (Ghana)	Fecal	Enrichment culture method	Biochemical techniques	Disc diffusion	Salmonella spp.
Ahmed et al., 2022 (Ghana)	Environmental, fecal	Membrane filtration technique	MALDI-TOF MS	Disc diffusion	*Pseudomonas* spp.; various species of Enterobacteriacee family
Akbar et al., 2022 (Pakistan)	Fecal	Enrichment culture method	Biochemical and genotypic identification techniques	Disc diffusion	*Escherichia coli*
Ateba et al., 2020 (South Africa)	Environmental	Membrane filtration technique	Genotypic technique	Disc diffusion	HPC bacteria
Bhatta et al., 2007 (Nepal)	Fecal	Membrane filtration technique	Biochemical techniques	Disc diffusion	Various Salmonella species
Borjac et al., 2023 (Lebanon)	Environmental, fecal, other	- For psychrophilic bacteria: No concentration method. 1-ml samples were serially diluted and cultured on nutrient agar media (R2A agar) - For other target: Membrane filtration technique	MALDI-TOF MS	Disc diffusion	Various species of psychrophilic bacteria; *Pseudomonas aeruginosa*; *Staphylococcus* spp.; *E. coli*; *Enterobacteriaceae* family
Dávalos et al., 2021 (Colombia)	Environmental	Membrane filtration technique with some modifications	Biochemical techniques followed by genotypic identification	Broth dilution method	Nontuberculous mycobacteria
Elmi et al., 2021 (Malaysia)	Fecal	Enrichment culture method	Biochemical techniques	Disc diffusion	*Escherichia coli*
Elmonir et al., 2020 (Egypt)	Fecal	Multiple fermentation tube technique	Biochemical techniques	Disc diffusion	*Escherichia coli*
Emekdas et al., 2006 (Turkey)	Environmental	Enrichment culture method	Biochemical techniques followed by genotypic identification	Disc diffusion	*Aeromonas* spp.
Ezzat 2014 (Egypt)	Environmental	Membrane filtration technique	Biochemical techniques	Disc diffusion	*Aeromonas* spp.
Furuhata et al., 2006 (Japan)	Environmental	No concentration method. Samples were cultured on nutrient agar media (R2A agar)	Biochemical techniques followed by genotypic	Disc diffusion	*Methylobacterium* spp.
Hamza et al., 2020 (Egypt)	Fecal	Membrane filtration technique followed by enrichment method	Biochemical techniques	Disc diffusion	Various species of Enterobacteriacee family
Jazrawi et al., 1988 (Iraq)	Environmental, fecal	Membrane filtration technique	Biochemical techniques	Disc diffusion	*Pseudomonas* spp., various psychro- and mesophilic bacteria species; various species of Enterobacteriacee family
Kinge et al., 2010 (South Africa)	Fecal	Membrane filtration technique	Biochemical techniques	Disc diffusion	*Escherichia coli*
Leginowicz et al., 2018 (Poland)	Environmental	Membrane filtration technique with some modifications (0.22 μm pore size filters)	Biochemical techniques followed by genotypic identification	Disc diffusion	*Pseudomonas* spp., various psychro- and mesophilic bacteria species
Moghaddam et al., 2022 (Iran)	Environmental	Membrane filtration technique	Biochemical techniques followed by genotypic identification	Broth dilution	Nontuberculous mycobacteria
Molale-Tom et al., 2024 (South Africa)	Environmental	No concentration method. 1-ml samples were serially diluted and cultured on nutrient agar media (R2A agar)	Genotypic technique	Disc diffusion	HPC bacteria
Papandreou et al., 2000 (Greece)	Fecal	Membrane filtration technique	Biochemical technique	Disc diffusion and broth dilution methods	Various species of Enterobacteriacee family
Santos et al., 2023 (Brazil)	Other	Membrane filtration technique	Genotypic technique and MALDI-TOF MS for species identification	Disc diffusion	Coagulase-negative *Staphilococcus* spp.
Scoaris et al., 2008 (Brazil)	Environmental	Membrane filtration technique (0.45 μm)	Biochemical techniques followed by genotypic identification	Disk diffusion	*Aeromonas* spp.

Siedlecka and Piekarska 2019 (Poland)	Environmental, fecal	Membrane filtration technique with modifications (0.22 μm pore size filters; usage of agar supplemented with antibiotic – agar type varies according to the searched microbe)	Not specified	Agar dilution method	HPC bacteria; *Klebsiella pneumoniae*, *Enterococcus faecium*, *Enterococcus faecalis*
Siedlecka et al., 2020 (Poland)	Environmental	Membrane filtration technique with modifications (0.22 μm pore size filters; usage of nutrient agar media (R2A agar) supplemented with antibiotic)	Genotypic technique	Agar dilution method	HPC bacteria
Siedlecka et al., 2021 (Poland)	Environmental	Membrane filtration technique with modifications (0.22 μm pore size filters, usage of nutrient agar media (R2A agar) supplemented with antibiotic)	Biochemical techniques followed by genotypic identification	Agar dilution method	Various psychrophilic bacteria species
Subba et al., 2013 (Nepal)	Fecal	Membrane filtration technique	Biochemical techniques	Disk diffusion	*Escherichia coli*
Vaz-Moreira et al., 2012 (Portugal)	Environmental	Membrane filtration technique	Biochemical techniques followed by genotypic identification	Automatized system	*Pseudomonas* spp.
Walsh et al., 2011 (India)	Environmental	Precipitation by centrifugation	Genotypic technique	Broth dilution method	*Pseudomonas* spp., various psychro- and mesophilic bacteria species
Zhang et al., 2018 (China)	Environmental	No concentration method. 1-ml samples were serially diluted and cultured on nutrient agar medium supplemented with antibiotic	Genotypic technique	Agar dilution method	HPC bacteria

HPC = Heterotrophic Plate Count; MALDI-TOF-MS = matrix-assisted laser desorption ionization–time of flight mass spectrometry.

2.4.4.2.1. Antibiotic-resistance features in environmental ARB

Nontuberculous mycobacteria (NTM) (2/29 articles) were found resistant to different classes of antibiotics, depending on the NTM species. Moghaddam et al. (2022) found that more than half of the tested isolates of *M. aurum* were resistant to 2nd-generation cephalosporin (cefoxitin), *M. phocaicum* to fluoroquinolone class (ciprofloxacin), while *M. mucogenicum* and *M. fortuitum* showed major resistance to tetracyclines (doxycycline) and to carbapenems (meropenem and imipenem). Similar results were obtained by Davalos et al. (2021), who detected NTM species also resistant to aminoglycosides (tobramycin). *Aeromonas* spp. (3/29 articles) exhibited wide antibiotic-resistance patterns toward various compounds. Ezzat (2014) found all the tested isolates (more than 70 isolates) resistant to various types of penicillins (i.e., amoxicillin-clavulanic acid, ampicillin, methicillin, and piperacillin), 1st generation cephalosporins (cephalothin), macrolides (erythromycin), glycopeptides (vancomycin) and lincosamides (clindamycin). These results were in accordance with those observed by Scoaris et al. (2008) and Emekdas et al. (2006), although Scoaris et al. (2008) also found isolates resistant to 3rd generation cephalosporins (cefotaxime) and chloramphenicols. Interestingly, in all three studies *Aeromonas* spp. isolates were susceptible to fluoroquinolones. *Pseudomonas* spp. (6/29 articles) showed wide resistance (>50% of the tested isolates) to monobactams (aztreonam) (Ahmed et al., 2022), 3rd-generation cephalosporins (Borjac et al., 2023; Walsh et al., 2011), tetracyclines and epoxide (Vaz-Moreira et al., 2012). Resistance against penicillin classes varied according to the type of compound, with percentages of resistant isolates varying between 50% and 100% for ampicillin (Jazrawi et al., 1988; Leginowicz et al., 2018) and ticarcillin (Vaz-Moreira et al., 2012) compared to piperacillin (resistance less than 10% of the tested isolates; Ahmed et al., 2022). Similarly, also resistance to fluoroquinolones depended on the type of molecule: 80% of the isolates resulted resistant to nalidixic acid (Vaz-Moreira et al., 2012) and only 5% to ciprofloxacin (Ahmed et al., 2022). Other types of environmental bacteria have been also considered by seven of the reviewed articles that tested various isolates of psychro- and mesophilic aerobic bacteria. They were generally cultured and isolated on nutrient agar media, often R2A agar, because of its low-nutrient and low-ionic strength, then incubated at 22 °C or 37 °C for the investigation of psychrophilic or mesophilic bacteria, respectively. Overall, authors found multi-drug resistant (MDR) profiles (resistance to three or more antibiotics) in numerous groups of Gram-negative and Gram-positive bacteria (e.g., *Bacillus* spp., *Acinetobacter*, *Chromobacterium*, *Lysinibacillus*, *Psychrobacter*, *Brevundimonas*, *Myroides*). As an example, *Bacillus* spp. showed resistance in more than 50% of the tested isolates to 1st and 3rd generation cephalosporins and monobactam (Adesoji et al., 2017; Leginowicz et al., 2018). *Acinetobacter johnsonii* showed resistance to 3rd (ceftazidime, ceftriaxone, cefotaxime) and 4th (cefepime) generation cephalosporins with percentages of resistant isolates ranging from 21% to 36% (Borjac et al., 2023). Some isolates of *Brevundimonas* spp. were resistant to aminoglycosides, penicillins and tetracyclines (Leginowicz et al., 2018) as well as fluoroquinolones and 3rd-generation cephalosporins (Siedlecka et al., 2021). *Methylobacterium* spp. was resistant to various antibiotics, including 3rd-generation cephalosporins, penicillins, macrolides, glycopeptides, phenicols (Siedlecka et al., 2021; Furuhata et al., 2006) and similar resistance pattern was found also for *Afipia* spp. (Siedlecka et al., 2021). Forty percent of the isolates of *Chromobacterium* spp. were resistant to penicillins and aminoglycosides (Jazrawi et al., 1988) and all the tested isolates of *Achromobacter* spp. could be considered resistant to 3rd-generation cephalosporins on the basis of their MIC values (Walsh et al., 2011). Some studies (5/29) analyzed environmental bacteria as a whole, focusing on HPC flora at 22 °C. For HPC flora, antibiotic-resistance was frequently expressed as percentage comparing the number of colonies counted on nutrient agar supplemented with a certain antibiotic with those counted without the antibiotic (negative control or blank). The presence of antibiotic-resistant HPC was found in many samples of the reviewed studies, with resistance percentage up to 98% for β-lactams(e.g., ampicillin cephalothin, penicillin) (Siedlecka and Piekarska, 2019; Molale-Tom et al., 2024), >59% for 3rd generation cephalosporins (ceftazidime) (Siedlecka et al., 2020), and varying from 10% to 50% for fluoroquinolones (norfloxacin or ciprofloxacin; Zhang et al., 2018 and Molale-Tom et al., 2024, respectively) and for sulfonamides (sulfamethoxazole or trimethoprim; Zhang et al., 2018 and Ateba et al., 2020, respectively). Overall, the summarized evidence shows a global interest in understanding the role of environmental bacteria in antibiotic-resistance pathways in tap waters. However, the interpretation of antibiotic-resistance in environmental bacteria strains is still challenging. International committees (EUCAST, CLSI) provide guidelines on antibiotic susceptibility testing only for clinically relevant environmental species (e.g., *Pseudomonas* spp. and *Acinetobacter* spp.) thus they are incomplete for the vast majority of other psychrophilic bacteria. This means that most of the environmental bacteria lack of a methodological standard protocol in terms of, e.g., inoculum preparation, inoculum size, and reading values for MIC or zone diameter breakpoints. Therefore, in the reviewed articles, the Authors frequently adopt breakpoint values established for other species of the same phylum or family or, if they are not available, refer to values already published in literature (e.g., Leginowicz et al., 2018). In some cases, the authors preferred to express only the measured values of MIC, assuming that high MIC values represent patterns of resistance (Furuhata et al., 2006; Walsh et al., 2011).

2.4.4.2.2. Antibiotic-resistance features in fecal ARB and resistant staphylococci

The reviewed articles that found fecal bacteria in tap waters were performed mainly in lower-middle income countries, where a final disinfection by DWTP is not always clearly stated (Table S3) and fecal contamination of tap waters can occur as a result of either heavy pollution of water supplies (that interferes with efficient water treatment) or defects in distribution pipelines that are responsible for post-treatment deterioration of water quality (Ateba et al., 2020; Ahmed et al., 2022; Elmonir et al., 2020; Borjac et al., 2023). A detailed description of the antibiotic-resistant profile for each fecal ARB is provided in Sect. 2.4.3.5 and Table 2, where these bacteria are explored further, given their role in clinical infections. Overall, the total number of resistant isolates in tap waters varies among microorganisms and according to different studies in the literature. For *E. coli*, the number of resistant isolates ranged from 7.1% (Elmi et al., 2021) to 36.7% (Kinge et al., 2010), 62.2–66.7% (Ahmed et al., 2022; Jazrawi et al., 1988), and 81.5% (Akbar et al., 2022). The types of resistance was recorded for tetracycline (73–100% of the isolates) (Akbar et al., 2022; Subba et al., 2013; Elmonir et al., 2020), amoxicillin (50–100%) (Akbar et al., 2022; Subba et al., 2013), cephalosporins (67–100%) (Akbar et al., 2022; Elmonir et al., 2020), including 3rd generation ones (35.7%–50%) (Akbar et al., 2022; Subba et al., 2013; Borjac et al., 2023), nalidixic acid (7–87%) (Kinge et al., 2010; Subba et al., 2013; Elmonir et al., 2020; Elmi et al., 2021). For Salmonella spp., Bhatta et al. (2007) reported a very high number of resistant colonies (97.6%). The antibiotic resistance was reported for vancomycin (100%) and erythromycin (100%) (Adzitey et al., 2016), tetracycline (42.9%–100%), ampicillin (100%), chloramphenicol (62.5–100%), trimethoprim-sulfamethoxazol (71.4–100%), nalidixic acid (57.1% for *S. paratyphi* A, 100% *S. tiphymurium* and *S. enteritidis*) (Bhatta et al., 2007). High resistance levels were observed also for other species of *Enterobacteriaceae* family, such as *Enterobacter cloacae* (65.5%), *Enterobacter agglomerans* (70%), *K. pneumoniae* (75%), and *Serratia odorifera* (53%) (Jazrawi et al., 1988). Some articles investigated also the presence of *Staphylococcus* spp. The species identified by Borjac et al. (2023) (*S. aureus*, *S. pasteuri*, *S. equorum*) were resistant mainly to sulfonamides (trimethoprim-sulfamethoxazole) (50%), followed by 2nd-generation cephalosporin (cefoxitin) (29%), tetracycline (4%), and aminoglycoside (gentamicin) (4%). Similarly, also Santos et al. (2023) found several species of coagulase-negative staphylococci (e.g., *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. warneri*, *S. condimenti*) resistant to various antibacterial compounds, mainly to sulfonamides (sulfazotrin), macrolides (erythromycin), and penicillin (39%–43% of the tested isolates), but also to cefoxitin, tetracycline, and gentamicin (8%–11%).

2.4.4.3. Antibiotic resistant genes

Almost 50% of articles (22/45) studied the presence of ARGs in drinking water, in particular 55% of these manuscripts (12/22) analyzed the presence of ARGs in bacterial strain isolated from tap water (Adesoji et al., 2017; Akbar et al., 2022; Ateba et al., 2020; Borjac et al., 2023; Elmonir et al., 2020; Hamza et al., 2020; Khan and Mustafa, 2021; Khan et al., 2016; Leginowicz et al., 2018; Molale-Tom et al., 2024; Santos et al., 2023; Walsh et al., 2011), while the 45% (10/22) studied the ARG presence directly in water samples (Destiani and Templeton, 2019; Ke et al., 2023, 2024; Li et al., 2023; Liang et al., 2022; Mi et al., 2019; Siedlecka and Piekarska, 2019; Siedlecka et al., 2020; Wang et al., 2023; Zhang et al., 2021a). Fifty-six percent (12/22) considered in parallel also the presence of ARB (Table S3).

2.4.4.3.1. ARGs in bacteria isolated from tap water

Considering all the included studies, in 27% (12/45) the investigation of ARGs was carried out from bacterial suspension cultures that have been obtained from tap water concentration and isolation process, that in most of the articles were already tested for phenotypic resistance to a certain antibiotic through culture-based methods (Sect. 2.4.3.2), except for two studies (Khan and Mustafa, 2021; Khan et al., 2016). Such articles were carried out mainly in lower-middle income countries (83% of the reviewed articles), starting from 2010 (Table S3), the mean number of water samples analyzed was 89, and half of the studies considered a high number of water samples ($n \geq 50$) underlining that the data obtained could be a good indicator of the ARG distribution in tap water (Table S3). In 17% of the study (2/12) (Elmonir et al., 2020; Leginowicz et al., 2018) the water volume analyzed was 1 L, while 3 studies analyzed less than 20 mL of samples (25%) (Adesoji et al., 2017; Molale-Tom et al., 2024; Walsh et al., 2011) and 2 (17%) did not report the volume processed (Akbar et al., 2022; Borjac et al., 2023). Regarding the method used to investigate the ARG presence in isolates, as expected polymerase chain reaction (PCR) assay was the main method performed (11/12) according to that observed by other studies in water environments (Siri et al., 2023). Only in the study by Molale-Tom and collaborators (2024) was the presence of ARGs was monitored using Whole Genome Sequencing (WGS). In almost all the studies (75%) at least three gene targets were investigated, and the most frequently investigated genes were β-lactamase, in particular $bla_{\text{CTX-M}}$ gene. In six articles, the presence of other targets (e.g., sulfonamide resistant genes, tetracycline resistant genes) was deepened (Adesoji et al., 2017; Akbar et al., 2022; Ateba et al., 2020; Khan and Mustafa, 2021; Khan et al., 2016; Molale-Tom et al., 2024). In one article, the target for resistance to quaternary ammonium compounds (*qac*S) was investigated (Khan et al., 2016), while presence of Mobile Genetic Elements (MGEs) (e.g., *int*I, *int*II, *tnp*A) was analyzed in four manuscripts (33%) (Adesoji et al., 2017; Khan et al., 2016; Khan and Mustafa, 2021; Hamza et al., 2020). In all studies, bacteria isolated from tap waters hosted one or more ARGs. Overall, the number of isolates that exhibited the ARGs ranged between 8 and 68. Fifty percent of the articles (5/10) showed at least 30% of isolates resistant predominantly the β-lactamase gene $bla_{\text{CTX-M}}$ (3 out 12 studies, 25%; Akbar et al., 2022; Elmonir et al., 2020; Hamza et al., 2020) and sulfonamide resistant genes (*sul*II and *sul*I) (3 out of 12 studies, 25%; Adesoji et al., 2017; Khan et al., 2016; Khan and Mustafa, 2021). The high prevalence of these genes in isolates was confirmed also in drinking water sources (e.g., lake and river) (Reddy et al., 2022; Ana et al., 2021). The similar gene pattern between water supplies and tap water could be related to the lack of effect of drinking water treatments in the dynamics of different ARB. Considering the MGEs, when they were searched for, they were found by the authors (Akbar et al., 2022; Hamza et al., 2020; Khan et al., 2016; Khan and Mustafa, 2021), according to the results obtained in other studies conducted in Asian water environments (Siri et al., 2023). Considering the drinking water treatments, seven studies report the chlorination as disinfection step utilized for water treatment (Adesoji et al., 2017; Ateba et al., 2020; Borjac et al., 2023; Elmonir et al., 2020; Khan and Mustafa, 2021; Molale-Tom et al., 2024; Santos et al., 2023). Such information underlined that the used treatment seems not to allow an adequate reduction in tap water. It is known that the effect of chlorination on ARGs or ARB can be affected by dosage, nature of chlorination agent, contact time and nature of the ARGs or ARB (Sanganyado and Gwenzi, 2019). Moreover, it is important to note that in almost all of the studies the resistant isolates were potential opportunistic human pathogens such as *Klebsiella* spp., *E. coli*, *Acinetobacter* spp. These isolates have been frequently associated with infections both in clinical and community settings and could represent a public health threat for susceptible subjects (e.g., hospitalized, immunosuppressed) (OECD, 2023) (see Sect. 2.4.3.5 for further information on health-relevance of ARB in the reviewed articles).

2.4.4.3.2. ARGs in tap water samples

In these studies (10/45), the investigation of ARGs was performed directly on the microbiota that is retained by filter membranes with micromeritics pore size, thus without any bacterial isolation step. Such studies were carried out mainly in China (6 out of 10 articles), although one of them also analyzed also few samples from South Africa, USA, Brazil, Taiwan and Singapore (Wang et al., 2023). The other studies were conducted in Poland, Canada and UK. Each article was published after 2019 (Table S3). All the studies included a number of samples < 111, with a mean of 34 samples. The water volume analyzed ranged from 300 to 500 mL up to 2000 L, underlining the variability of volume used for the evaluation. Regarding the methods, all the studies used the filtration method to concentrate the sample for DNA extraction and 80% used a 0.22 μm membrane (Mi et al., 2019; Siedlecka and Piekarska, 2019; Siedlecka et al., 2020; Zhang et al., 2021a; Liang et al., 2022; Ke et al. 2023, 2024; Li et al., 2023), the 10% used a 0.45 μm porosity membrane (Destiani and Templeton, 2019) and the 10% (Wang et al., 2023) used both. The filters with the 0.22 μm pore size were the most used membrane also for the detection of ARGs in other water environments (e.g., wastewater samples) (Miłobedzka et al., 2022). In 20% of the reported methods, additional treatments were applied to filters (e. g., washing of the filters to bacterial recovery) (Liang et al., 2022; Wang et al., 2023). The DNA extraction was carried out for all the studies with commercial kit (e.g., DNeasy PowerWater kit Qiagen or FastDNA SPIN Kit for Soil MP biomedicals). As expected, the molecular analysis was performed with different approaches, conversely to analysis in tap water isolates. In particular, 50% of the articles used quantitative PCR (qPCR) (Destiani and Templeton, 2019; Mi et al., 2019; Zhang et al., 2021a; Liang et al., 2022; Li et al., 2023), followed by 30% metagenomics (Ke et al. 2023, 2024; Wang et al., 2023) and 20% qualitative PCR (presence/absence) (Siedlecka and Piekarska, 2019; Siedlecka et al., 2020). Regarding the target of the analysis, a total of 12 types of antimicrobials to which ARGs confer resistance were found (Fig. 4). Along with ARGs, also MGE subtypes were found (MGE were searched together with ARGs in 50% of the reviewed articles; Destiani and Templeton, 2019; Siedlecka and Piekarska, 2019; Siedlecka et al., 2020; Ke et al., 2023; Ke et al., 2024). Besides ARGs and MGEs, also genes related to other resistance mechanisms, such as transmembrane activity and efflux pumps, were investigated in 30% of studies (Siedlecka et al., 2020; Ke et al. 2023, 2024). In general, all the articles investigated at least five ARGs, and the most monitored classes of ARGs were that encoding for β-lactam and sulfonamide (100% of the studies), tetracycline (90%), macrolide and quinolone (80%) resistance, covering a wide range of genes. Other classes were investigated but they were less represented among the reviewed articles (e.g., rifamycins and aminoglycosides). Besides ARGs, in three different studies (30%) the resistance to quaternary ammonium compounds was investigated (Siedlecka and Piekarska, 2019; Siedlecka et al., 2020; Ke et al., 2023). Among the MGEs, instead, the *int*1 (integrase) and *tnp*A (transposase) were the most investigated genes with a positivity rate of 60% each. Overall, in all the articles at least one ARG was detected in tap water samples. Regarding ARGs, *sul*I (90%), *tet*A (70%), and *bla*TEM (50%) were the most frequently found genes. Interestingly, the ARGs for the β-lactam and sulfonamide resistance are the most frequently detected also in isolates (Sect. 2.4.3.3.1). It is possible to observe a strong similarity between ARGs detected in tap water and those in freshwater (Siri et al., 2023). In fact, conventional DWTPs are generally unable to adequately remove ARB or ARGs from water, so if a water supply (e.g., fresh or ground waters) is contaminated by antibiotic-resistance determinants, they can be found also at the exit of the plant. In fact, in all of the studies that specified the treatment (80%, Destiani and Templeton, 2019; Mi et al., 2019, Siedlecka and Piekarska, 2019; Siedlecka et al., 2020; Liang et al., 2022; Ke et al., 2023; Li et al., 2023; Ke et al., 2024), the final chlorination was not efficient in completely removing the environmental ARGs and MGEs. Moreover, it is important to highlight that the low abatement of ARGs by the chlorination could be related to the disinfection characteristics (e.g., chlorination agent, contact time) (Zhang et al., 2021b).

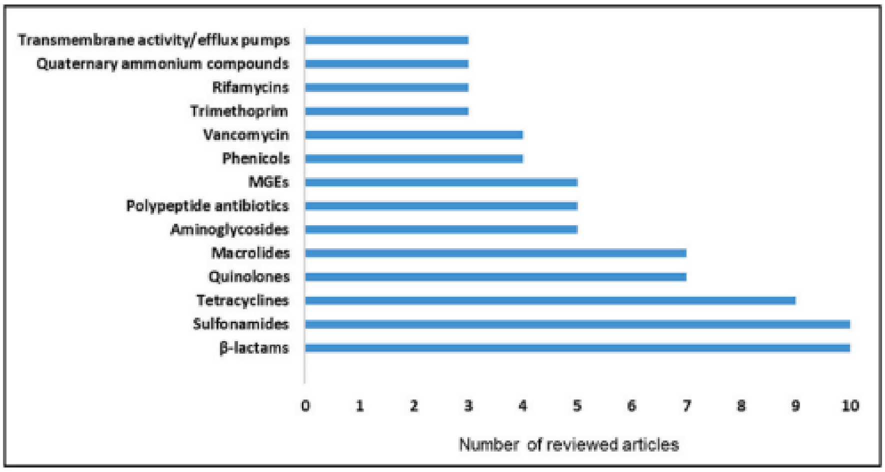

Fig. 4. Occurrence of antibiotic resistance gene (ARG) classes in the reviewed articles. The horizontal axis represents the number of reviewed papers that detected a certain ARG, while the vertical axis lists gene classification, resistance target or mechanisms. MGEs: mobile genetic elements. Polypeptide antibiotics: bacitracin, colistin, polymyxin.

2.4.4.4. Antibiotics in tap waters

Although the detected antibiotics may pose a low risk to human health when considered individually at the residual level in drinking waters, it has become increasingly clear that long term antibiotic exposure could contribute to the evolution of high-level bacterial resistance at concentrations that are several hundred-fold below MICs. Concerning the studies on antibiotic determination in tap waters, screened also taking into account the good practice laboratory and technologies available for the best detection of antibiotics, six studies were considered in the present ScR. Charuaud et al. (2019) monitored water resources and tap water in an intensive husbandry area in France (Brittany region - northwest France). Authors used both water resource samples collected from 25 sites (23 surface waters and two groundwater) intended for tap water production, and 23 samples from corresponding tap water sites. Thirty-eight veterinary drugs were monitored including 21 antibiotics (amoxicillin, ampicillin, cefquinome, chlortetracycline, doxycycline, enrofloxacin, erythromycin, florfenicol, flumequine, lincomycin, marbofloxacin, neospiramycin, oxolinic acid, oxytetracycline, spiramycin, sulfadiazine, sulfadimethoxine, sulfamethazine, tilmicosin, trimethoprim, and tylosin) and 1 antibiotic-metabolite (neospiramicin). Only nine antibiotics were quantified (florfenicol, flumequine, lincomycin, neospiramycin, oxytetracycline, sulfadiazine, sulfamethazine, tilmicosin, trimethoprim) in water samples. Authors found florfenicol which was quantified at 159 ng/L and 211 ng/L and sulfadiazine and tylosin, both in tap waters. As a result of the study, authors reported that 20% of Brittany's tap waters were subject to contamination by residues of veterinary drugs. In tap waters of Cyprus, Makris and Snyder (2010) screened the presence of both trimethoprim and sulfamethoxazole, and no occurrence of the targeted compounds was found. In Asiatic countries, especially in China, Hanna et al. (2018) studied the occurrence of norfloxacin, levofloxacin, ciprofloxacin, enrofloxacin, doxycycline, sulfapyridine, sulfamethoxazole, metronidazole, florfenicol, and chloramphenicol residues in waters from Shandong province (eastern China). In the 47 drinking water samples the drugs detected the reported as median concentrations: sulfapyridine (0.5–0.5) ng/L; sulfamethoxazole 1.7 ng/L (0.3–18.6); ciprofloxacin 21.4 ng/L (0.4–224.4); norfloxacin 1.6 ng/L (0.4–3.6); florfenicol 5.4 ng/L (3.3–26.1). Also, in southern China, Ben et al. (2020) searched for 92 antibiotics in tap waters from the East River (Dongjiang) collected from 10 areas. A total of 58 antibiotics were detected in the filtered tap water and in all samples chlorotetracycline, tetracycline, 4 epi-tetracycline, doxycycline, oxytetracycline, clarithromycin, midecamycin, roxithromycin, ciprofloxacin, enoxacin, enrofloxacin, norfloxacin, ofloxacin, levofloxacin, N-acetylsulfamethoxazole, sulfadiazine, sulfamethoxazole, sulfamethazine, trimethoprim, monensin, and dicloxacillin were detected. The found compounds ranged from 0.021 ng/L for josamycin to 1133 ng/L for dicloxacillin. Nineteen parent compounds (four tetracyclines, three macrolides, six quinolones, four sulfonamides, one β-lactam, and monensin) and two degradation products (4-epitetracycline and N-acetylsulfamethoxazole) were detected in the 36 filtered tap water samples. The total concentration of the detected antibiotics in such water samples ranged from 6.0 ng/L to 1172 ng/L, and 80 had a total concentration of detected antibiotics of greater than 50 ng/L. Ben's study suggested, hence, a complex antibiotic pollution in Chinese drinking water. Again, Jiang et al. (2019) studied the occurrence of 43 antibiotics in tap water both from urban and rural areas of a city of the Yangtze River Delta. A minimum of 7 to a maximum of 25 different antibiotics were detected in various types of drinking water with the total concentration ranging from 6.37 ng/L to 809.28 ng/L, frequently including chloramphenicol, quinolones, sulfonamides, macrolides, and lincosamides. The total concentrations of antibiotics in most drinking water were about 100 ng/L or even higher. Finally, Wang et al. (2011) evaluated the presence of lincomycin, sulfamethoxazole, triclosan, trimethoprim, and tylosin in tap water, where tap water samples were collected from 31 different water treatment facilities of Missouri (USA), and authors showed that antibiotics in tap waters were all below the detection limit. However, in very few samples, lincomycin and sulfamethoxazole were detectable as traces. 3.5. Clinically-relevant ARB in tap waters and public health implication Some of the reviewed articles (16/45) revealed the presence in tap waters of bacteria, whose antibiotic-resistance profile can pose public health threats, e.g., species of the Enterobacteriaceae family, Enterococcus genus, P. aeruginosa. Some of them belong also to the ESKAPE group, namely nosocomial pathogens that exhibit multidrug resistance and virulence, thus of particular high concern given the circulation among vulnerable subpopulations, where disease outcome may be more severe (Mulani et al., 2019). Clinically-relevant ARB were detected mainly in countries with lower-middle income economies (10/16, 62.5%) and the most representative region according to UNSD division was Asia (7/16, 43.8%). Asian countries (7/16, 43.8%), that include Nepal, India, Pakistan (Fig. 5).

2.4.4.5. Clinically-relevant ARB in tap waters and public health implication

Some of the reviewed articles (16/45) revealed the presence in tap waters of bacteria, whose antibiotic-resistance profile can pose public health threats, e.g., species of the Enterobacteriaceae family, Enterococcus genus, P. aeruginosa. Some of them belong also to the ESKAPE group, namely nosocomial pathogens that exhibit multidrug resistance and virulence, thus of particular high concern given the circulation among vulnerable subpopulations, where disease outcome may be more severe (Mulani et al., 2019). Clinically-relevant ARB were detected mainly in countries with lower-middle income economies (10/16, 62.5%) and the most representative region according to UNSD division was Asia (7/16, 43.8%). Asian countries (7/16, 43.8%), that include Nepal, India, Pakistan (Fig. 5).

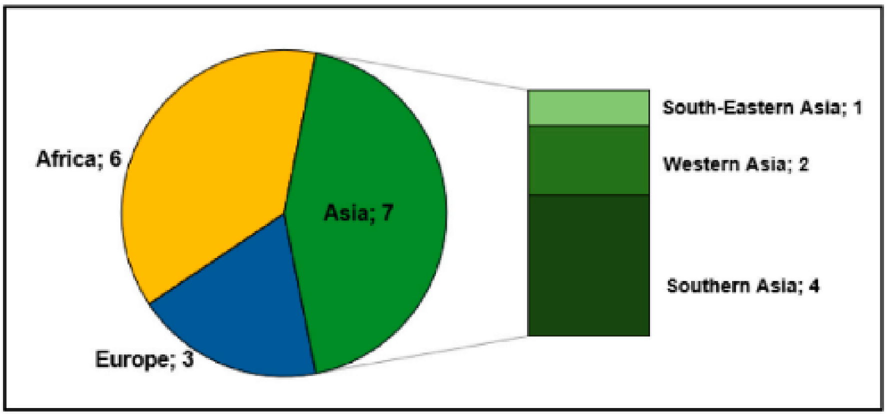

Fig. 5. Geographical location of studies detecting clinically-relevant ARB. The numbers refer to the number of articles published in each area (according to UNSD division; UNSD, 2024).

Table 2
Clinically-relevant ARB detected in tap waters of the revised studies and resistance profile of the analyzed isolates. When only one isolate has been assayed, the profile refers to that specific isolate, thus the percentage was not reported. Antibiotic-resistance profile of concern according to 2024 WHO BPPL are highlighted in bold (WHO, 2024b). References are listed in alphabetical order.

References	WHO-relevant microbes	Type species/group	N. tested isolates	Phenotypic resistance profile of the tested isolates
Adesoji et al. (2017)	Enterobacterales	*Citrobacter freundii*	1	T, S, AM, SXT, N, AMC, SU
		Proteus vulgaris	2	50% FF, 100% T, 100% S, 100% C, 100% AM, 100% SXT, 100% N, 50% AMC, 100% **CEF**, 100% SU, 50% G
		Proteus mirabilis	1	T, S, SXT, SU
		Morganella morganii	1	T, S, **CEF**, AM, SXT, AMC, SU
		Providencia rettgeri [a]	2	50% T, 100% S, 50% C, 50% N, **50% CEF**, 50% AM, 100% SXT, 100% AMC, 100% SU
	Acinetobacter baumannii		1	FF, S, C, AM, SXT, AMC, CEF, SU
Adzitey et al. (2016)	*Salmonella* spp.		6	100% VA, 100% E
Ahmed et al. (2022)	Enterobacterales	*E. coli*	82	91.5% CXM, 65.9% SXT, 54.9% AMC, **34.1% CRO**, 34.1% C, 17.1% CIP, 15.9% AZ, **6.1% ETP**, 3.7% G
	P. aeruginosa		144	52.1% AZ, 11.1% G, 9.7% PTZ, 4.9% CIP
Akbar et al. (2022)	Enterobacterales	*E. coli*	27	100% T, 100% AM, 100% AMC, **100% CTX**, **100% CFX**, **75% CAZ**, **75% IPM**, 75% AMK, 50% G, 50% C, 50% SXT, 50% CIP
Bhatta et al. (2007)	*Salmonella* spp.	*Salm. typhi*	16	100% AM, 62.5% C, 75% SXT, **62.5% N**, 75% T
		Salm. paratyphi A	7	100% AM, 71.4% C, 71.4% SXT, **57.1% N**, 42.9% T
		Salm. typhimurim	16	100% AM, 100% C, 100% SXT, **100% N**, 25% CRO
		Salm. enteritidis	2	100% AM, 100% C, 100% SXT, **100% N**, 100% CRO
Borjac et al. (2023)	Enterobacterales	*E. coli*	8	29% AM, 14% FOX, **14% CAZ**, 14% AZ,
		Other *Enterobacteriaceae*	5	60% AM, **20% FEP**, 100% FOX, **40% CAZ**, 20% AZ, **80% CPD**, **60% CRO**, **20% CTX**, 40% SXT
	P. aeruginosa		11	9% FEP, 9%, CAZ, 18% AZ
	S. aureus		Not specified	21% resistant di cefoxitin as surrogate for methicillin resistance and mecA-positive
Elmi et al. (2021)	Enterobacterales	*E. coli*	14	7.1% T, 7.1% N, 7.1% AM
Elmonir et al. (2020)	Enterobacterales	*E. coli* O26:H11 serogroup	12	83.3% S, 83.3% N, 66.6% SXT, 66.6% T, **58.3% CTX**, 66.6% C, 25% AM, 33.3% K, 25% CIP, 25% G
		E. coli O103:H2 serogroup	1	AM, **CTX**, N, CIP, G, S, C, T, SXT
		E. coli O128:H2 serogroup	2	100% AM, **100% CTX**, 100% N, 100% 100% CIP, 100% S, 100% C, 100% T, 100% SXT, 100% K
Hamza et al. (2020)	Enterobacterales	*E. coli*	22	**100% FOX, 100% CAZ, 54.5% CTX, 81.8% CRO, 54.5% CRE**
		Enterobacter cloacae complex	6	**100% FOX, 100% CAZ, 50% CTX, 100% CRO, 50% CRE**
		Klebsiella pneumoniae	5	**100% FOX, 100% CAZ, 40% CTX, 40% CRO, 40% CRE**
Jazrawi et al. (1988)	Enterobacterales	*Klebsiella pneumoniae*	20	73% AM, 47% CB, 47% CL, 7% K
		Enterobacter cloacae	84	33% AM, 9% CB, 21% CF, 11 CL
		Enterobacter agglomerans	10	71% AM, 43% CB, 57% CF, 11% G, 28% S, 43% SXT
		Citrobacter freundii	21	14% AM, 93% CF, 93% CL
		E. coli	9	50% AM, 50% C, 33% CB, 17% CF, 17% CL, 50% K, 33% S, 17% TE
		Serratia odorifera	17	22% AM, 22% CB, 11% CF, 11% CL, 33% K, 22% S, 11% SXT

Kinge et al. (2010)	Enterobacterales	*E. coli*	60	8.3% K, 31.7% C, 33.3%T, 56.7% AM, 66.7% E, 3.3% S
Leginowicz et al. (2018)	*Acinetobacter baumannii*		1	AMS
Papandreou et al. (2000)	Enterobacterales	*E. coli*	10	50% CF, 20% FOX, 30% AM, 100% CB, 30% TC
		Klebsiella pneumoniae	2	100% AM, 100% CB, 100% TC
		Klebsiella oxytoca	1	CF, CXM, FOX, AM, CB
		Enterobacter cloacae	29	96.4% CF, 24.3% CXM, 96.4% FOX, 71.4% AM, 100% CB, 7.1% TC, 22.5% SU, 3.6% C
		Enterobacter agglomerans	9	22.5% CF, 22.5% CXM, 22.5% FOX, 75% AM, 100% CB, 75% TC, 22.5% SU, 22.5% C
		Enterobacter asburiae	3	100% CF, 100% AM, 100% CB, 33.3% TC, 100% SU, 33.3% SXT
		Enterobacter sakazakii	1	CF, FOX, AM, CB, TC
		Citrobacter freundii	4	100% CF, 100% FOX, 100% AM, 100% CB
		Morganella morganii	1	CF, CXM, AM, CB, T, SU, C
	P. aeruginosa		59	100% CF, 93.2% CXM, 98.3% FOX, 86.4% CTX, 67.8% CRO, 100% CTT, 98.3% CZM, 96.6% AM, 13.6% CB, 100% T, 55.9% SU, 22% SXT, 100% C
Siedlecka and Piekarska (2019)	*Enterococcus faecium* and *Enterococcus faecalis*		not applicable [b]	**VA** (5 out of 16 tap water samples showed growth of resistant colonies)
	Enterobacterales	*Klebsiella pneumoniae*	not applicable [c]	**CRE** (2 out of 16 tap water samples showed growth of resistant colonies)
Subba et al. (2013)	Enterobacterales	*E. coli*	6	100% T, 83% AMX, **50% CFX**, 17% AMK, 16.7% N
		Thermotolerant *E. coli*	8	100% T, 87% AMX, **37.5% CFX**, 25% AMK, 25% N
Walsh et al. (2011)	*P. aeruginosa*		1	CTX, CAZ, **IPM**, **MER**, G, AMK, TO, C

Amino-glycosides group: AMK = amikacin; G = gentamicin; K = Kanamycin; S = streptomycin, TO = tobramycin. **Carbapenems group**: CRE = carbapenems (isolates resistant to at least one of the tested carbapenems, namely: Imipenem, meropenem, ertapenem); IPM = imipenem; ETP = ertapenem; MER = meropenem. **Cephalosporin group**: 1st-generation [CED = Cefradine, CF = cephalothin; CFR = cefadroxil]; 2nd-generation [CXM = cefuroxime, FOX = cefoxitin, CTT = cefotetan]; 3rd-generation [CTX = cefotaxime, CRO = ceftriaxone; CZM = ceftizoxime, CEF = Ceftiofur, CAZ = Ceftazidime, CFX = cefixime, CPD = cefpoxime]; 4th-generation [FEP = cefepime]. **(Fluoro)quinolones group**: N = nalidixic acid; CIP = ciprofloxacin. **Penicillin group**: PEN = penicillin; AM = ampicillin; CB = carbenicillin; AMS = ampicillin/sulbactam; AMC = amoxicillin/clavulanic acid; AMX = amoxicillin; TC = ticarcillin; PIP = piperacillin; PTZ = piperacillin-tazobactam. **Sulfonamides group**: SU = sulfamethoxazole; SXT = trimethoprim-Sulfamethoxazole (or co-trimoxazole). **Others:** C = Chloramphenicol; FF = florfenicol; CL = colistin; OXT = oxytetracycline; T = tetracycline; VA = vancomycin; AZ = aztreonam.

[a] 3rd-generation cephalosporin-resistant *Providencia* spp. was included in the 2017 WHO BPPL (WHO, 2017), but it has been removed from 2024 WHO priority list (WHO, 2024b).

[b] The assay has been performed on CHROMagar™ VRE, and the results were expressed as CFU/500 ml encountered on chromogenic agar media after incubation.

[c] The assay has been performed on CHROMagar™ KPC, and the results were expressed as CFU/500 ml encountered on chromogenic agar media after incubation.

2.4.4.5.1. ARB in the priority list of WHO

In the reviewed papers on tap waters, five types of microbes showed resistance patterns of concern according to WHO BPPL, that ranked into critical and high priority groups (Table 2).

(i) critical group: various species belonging to Enterobacteriaceae family (named Enterobacterales in 2024 BPPL), e.g., *E. coli*, *Citrobacter*, *Serratia*, *Morganella*, *Proteus*, *Providencia* (only in 2017 BPPL), Klebsiella pneumoniae resistant to carbapenem and/or 3rd gen. cephalosporin (Adesoji et al., 2017; Ahmed et al., 2022; Akbar et al., 2022; Borjac et al., 2023; Elmonir et al., 2020; Hamza et al., 2020; Subba et al., 2013; Siedlecka and Piekarska, 2019);

(ii) (ii) high group: fluoroquinolone-resistant Salmonella spp. (Bhatta et al., 2007), carbapenem-resistant P. aeruginosa (Walsh et al., 2011), vancomycin-resistant *Enterococcus faecium* (Siedlecka and Piekarska, 2019), and methicillin-resistant S. aureus (Borjac et al., 2023).

E. coli of critical priority was widespread in the reviewed articles, mainly resistant to 3rd-generation cephalosporin (e.g., ceftazidime, cefotaxime, cefixime, ceftriaxone, ceftiofur, ceftizoxime, cefpoxime) but also to carbapenem. In Pakistan, Akbar et al. (2022) found MDR E. coli in hospital tap water samples, and all the isolates were resistant to various types of 3rd generation cephalosporin, namely cefotaxime (30 µg/mL) and cefixime (5 µg/mL), and 81.5% to ceftazidime (30 µg/mL); moreover, 81.5% were also resistant to carbapenem (imipenem, 10 µg/mL). Interestingly, similar antibiotic-resistant profiles were also obtained from *E. coli* isolates from clinical samples (urine). In Lebanon, *E. coli* isolates, collected at the exit of domestic water storage tanks, were resistant to ceftazidime, but also to other types of β-lactam compounds (cefoxitin, ampicillin, aztreonam) (Borjac et al., 2023). Also in Egypt, all the serotypes of E. coli isolates were resistant to cefotaxime (30 µg/mL); moreover, 7 out of 14 analyzed isolates harbored at least one virulence gene, thus representing an alarming public health threat (Elmonir et al., 2020). Similarly, in Nepal, *E. coli* was resistant to cefixime (5 µg/mL), but also to tetracyclines, fluoroquinolones, and penicillins (Subba et al., 2013). In Ghana, Ahmed et al. (2022) detected MDR *E. coli* of particular concern: 34.1% of the isolates were resistant to ceftriaxone (30 µg/mL) and 6.1% to carbapenem (ertapenem; 10 µg/mL). Similar antibiotic-resistant profile of critical priority was observed for other species belonging to the *Enterobacteriaceae* family. In fish farm of Egypt, Hamza et al. (2020) tested tap waters used by workers for drinking and hand washing, and they found various Enterobacteriaceae (E. coli, Enterococcus, K. pneumoniae) which were all resistant (33 isolates) to 3rd-generation cephalosporin (30 µg/mL) and more than half (51.5%) to carbapenem (10 µg/mL). Resistance of *K. pneumoniae* to carbapenem was observed also in Poland (Siedlecka and Piekarska, 2019). In Nigeria, Adesoji et al. (2017) found several species of *Enterobacteriaceae* (i.e., *Morganella* spp., *Proteus* spp., and *Providencia* spp.) resistant to (ceftiofur, 12 µg/mL). Other authors detected pathogens with resistance pattern of high priority. In particular, in Nepal, Bhatta et al. (2007) found either non-typhoidal and typhoidal Salmonellae, that showed resistance to quinolones (nalidixic acid, 10 µg/mL). Then, in India, Walsh et al. (2011) found one isolate of *P. aeruginosa* with MIC values suggesting resistance to carbapenem, in public drinking tap waters. Finally, in Poland, Siedlecka and Piekarska (2019) found 30 colonies of vancomycin-resistant *Enterococcus faecium* in 6 out of 16 tap water samples. The same Authors searched also for methicillin-resistant *Staphylococcus aureus* in the same samples, but it was not detected. Conversely, Borjac et al. (2023) detected *S. aureus* resistant to cefoxitin agent, that is frequently used as surrogate marker for the detection of methicillin resistance in this species.

2.4.4.5.2. Other clinically-relevant ARB in tap waters

In some studies, the microbes indicated by WHO have been found as MDR, but without the prioritized resistance pattern. In particular, resistance to 3rd-generation cephalosporins and/or carbapenems was tested but not found in *E. coli* isolated from tap water of poultry farms in Malaysia (Elmi et al., 2021) as well as in domestic urban waters in Greece, where also other various MDR Enterobacteriaceae were detected (*Enterobacter* spp., *Citrobacter* spp., *Morganella* spp.) (Papandreou et al., 2000) (Table 2). In other studies, it was not possible to establish if the WHO-relevant species is a priority, because resistance to carbapenems and/or cephalosporin (*Enterobacteriaceae*), carbapenems (*A. baumanii*, *P. aeruginosa*), fluoroquinolone (*Salmonella* spp.) was not tested (Table 2). Nevertheless, Acinetobacter baumannii was resistant to various penicillins, sulfonamides and amphenicols in low-middle income country (Nigeria; Adesoji et al., 2017) as well as high-income country (Poland; Leginowicz et al., 2018). Regarding Enterobacteriaceae, in Iraq, Jazrawi et al. (1988) reported various MDR isolates mostly resistant to two antibiotic classes widely used at the time of the study, namely aminopenicillins (ampicillin, 10 µg/mL) and 1st-generation cephalosporins (cefalotin, 30 µg/mL). Similarly, in South Africa, Kinge et al. (2010) showed *E. coli* resistance to ampicillin, erythromycin, and chloramphenicol. Regarding *P. aeruginosa*, Ahmed et al. (2022) found most than half of the isolates resistant to monobactam (aztreonam), followed by aminoglycosides (gentamicin) and penicillins (piperacillin-tazobactam), while in Greece *P. aeruginosa* (59 isolates) was resistant up to thirteen antibiotics, with more than 90% of the isolates resistant to 1st-, 2nd-, 3rd-generations cephalosporin, tetracyclines, penicillins, and fluoroquinolones (Papandreou et al., 2000). Also in Lebanon, P.

aeruginosa isolates were resistant to β-lactams (penicillin and aztreonam) and 3rd-generation cephalosporin, but also to cefpoxime, a 4th-generation cephalosporin (Borjac et al., 2023).

2.4.4.5.3. Other clinically-relevant ARB in tap waters

Some of the detected ARB are associated with fecal contamination (*E. coli*, *Salmonella*, *Enterococcus faecium*) and they were detected in tap waters of developing countries. The risk associated to such microorganisms can be reduced by improving water quality safety of the drinking waters, especially with regard to water distribution to households. This aspect is extremely relevant in developing countries, where fecal contamination of drinking waters is quite frequent. However, antibiotic-resistance has been revealed also in heterotrophic microorganisms, typically living in the environment (e.g., water, soil), such as *Acinetobacter*, *Klebsiella*, *Serratia*, *Pseudomonas*, that commonly occur in drinking waters, e.g., up to 6% of the HPC flora in drinking-water samples is represented by *Acinetobacter* spp. (WHO, 2022b). In fact, they occur in large numbers in raw water sources, then in drinking-water treatment processes can be reduced by coagulation, sedimentation, and disinfection practices, but they can proliferate in biologically active carbon and sand filtration, and growth rapidly in absence of disinfectant residuals (Shi et al., 2013). Moreover, other microbes belonging to the group of total coliforms, such as the thermotolerant *Klebsiella* and *Citrobacter*, are common in raw waters, and can multiply in the water supply network, especially in the piped distribution system, and may form biofilm (WHO, 2022b). Heterotrophic microorganisms are traditionally used as indicators of effectiveness of disinfection treatment and cleanliness of distribution system during operational monitoring, but their usefulness in verification and surveillance of water quality is limited because they have little representativeness toward fecal pathogen presence (WHO, 2022b). For this reason, WHO guidelines on drinking water did not release specific regulatory value for this parameter (WHO, 2022b) as well as most of the countries worldwide, as highlighted by a recent overview of national regulations and standards for drinking-water quality (WHO, 2021). As an example, European regulation did not pose specific regulatory values for HPC 22 °C, and reports “no abnormal change” for this parameter (Directive EU, 2020/2184). Nevertheless, in this review, we reported MDR strain of heterotrophic bacteria, with some of them also ranking in critical priority according to 2024 WHO BPPL (WHO, 2024b). Therefore, HPC and environmental strains in tap water, even if they are harmLess, could be a threat to human health given their possible role as a reservoir of resistance and ARB dissemination.

2.4.4.6. Limitations

Our ScR has some limitations related to the search strategy used during the ScR process. The electronic search was limited to three relevant databases commonly employed in literature reviews on environmental science topics (Pubmed, Scopus, Web of Science), due to the large volume of literature obtained on this topic from these databases. Although there is inadequate evidence to suggesting a specific number of databases or the necessity of including particular databases (Aromataris et al., 2024), limiting the search to a small number of databases could reduce the comprehensiveness of the current evidence on antibiotic-resistance in tap water. Another limitation is represented by the use of a simple search string. Although this approach was considered appropriate for the focus of the current ScR, which aimed to map available evidence on antibiotic-resistance determinants in tap water, the lack of variations and related terms in the search string could result in an incomplete representation of the relevant articles. Nonetheless, this ScR provided evidence on the breadth of literature in this field of research, thus it can serve as a foundation for future reviews aimed at exploring separately each specific aspect of the phenomenon, namely ARB, ARGs, and antibiotics, by refining and expanding search strategies.

2.4.5. Conclusions

Antimicrobial-resistance is recognized as one of the top global public health threats. In the last years, environmental pathways were demonstrated of paramount importance in the development, transmission and spread of the phenomenon. This scoping review provides an overview of antibiotic-resistance in drinking tap water, revealing its potential role as both a reservoir and vehicle of antibiotic-resistant bacteria and their determinants. In particular, the structured literature investigation on this topic allowed to highlight the following aspects.

I. the presence of multi-drug resistant HPC isolates, regardless of the geographical location, which suggests their environmental role in antibiotic-resistance transmission, despite the HPC parameter being minimally considered in drinking water regulations worldwide;
II. the presence of clinically relevant ARB in tap water, especially in lower-middle-income economies but also in some European countries;
III. the presence of ARG, especially those conferring resistance to sulfonamides, tetracyclines and β-lactamases, as well as trace levels of antibiotics.

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2.4.7. Supplementary materials

Table S1. WHO Bacterial Priority Pathogens List used for classifying the health-relevance of ARB detected in tap waters (WHO, 2024).

WHO priority group	Pathogen families/species	Antibiotic-resistance profile
Critical group	*Acinetobacter baumannii*	Carbapenems
	Enterobacterales (*Enterobacteriaceae* family)	3rd-generation cephalosporin-resistant and/or carbapenem
High group	*Salmonella typhi*	Fluoroquinolone
	Non-typhoidal *Salmonella*	Fluoroquinolone
	Shigella spp.	Fluoroquinolone
	Enterococcus faecium	Vancomycin
	Pseudomonas aeruginosa	Carbapenems
	Neisseria gonorrhoeae	3rd-generation cephalosporin-resistant and/or fluoroquinolone
	Staphylococcus aureus	Methicillin
Medium group	*Streptococcus pneumoniae*	Macrolide
	Haemophilus influenzae	Ampicillin

	Group A Streptococci	Macrolide
	Group B Streptococci	Penicillin

WHO, 2024. WHO Bacterial Priority Pathogens List, 2024: bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance. Geneva: World Health Organization; 2024. Licence: CC BY-NC-SA 3.0 IGO

Table S2. Main reason of exclusion of eligible studies (alphabetical order)

Number	Author, Year	Title	Reason of exclusion	Comments
1	Abada et al., 2019	Molecular identification of biological contaminants in different drinking water resources of the Jazan region, Saudi Arabia	Aggregated data	Data on tap waters aggregated with other drinking water sources (e.g. ground water, water purification shops, commercially bottled water)
2	Abana et al., 2019	Investigating the virulence genes and antibiotic susceptibility patterns of Vibrio cholerae O1 in environmental and clinical isolates in Accra, Ghana	Aggregated data	Data on tap waters aggregated with various water matrices (streams, shallow wells, household storage tanks)
3	Abera et al., 2014	Bacterial quality of drinking water sources and antimicrobial resistance profile of Enterobacteriaceae in Bahir Dar city, Ethiopia	Aggregated data	Data on tap waters aggregated with non-treated water sources (i.e., spring and reservoir)
4	Abera et al., 2016	Extended-Spectrum beta (beta)-Lactamases and Antibiogram in Enterobacteriaceae from Clinical and Drinking Water Sources from Bahir Dar City, Ethiopia	Not eligible methodology for this ScR	Cultural method not clearly described
5	Adesoji et al., 2013	Physicochemical Properties and Occurrence of Antibiotic-Resistant Bacteria in Ife and Ede Water Distribution Systems of Southwestern Nigeria	Not eligible methodology for this ScR	Number of clinical isolates not clearly specified
6	Ahangaran et al., 2022	Tetracycline Resistance Genes in Escherichia coli Strains Isolated From Biofilm of Drinking Water System in Poultry Farms	Other	The sampling matrix consists of biofilms
7	Ahmad et al. 2014	Microbiology and evaluation of antibiotic resistant bacterial profiles of drinking water in Peshawar, Khyber Pakhtunkhwa	Aggregated data	Data on tap waters aggregated with various water sources for drinking purpose (e.g., tube wells, hand pumps)
8	Akinjogunla et al. 2023	Enterobacteriaceae isolates from clinical and household tap water samples: antibiotic resistance, screening for extended-spectrum, metallo- and ampC-beta-lactamases, and detection of bla(TEM,)bla(SHV) and bla(CTX-M) in Uyo, Nigeria.	Not clear if tap water was treated or not	It is indeterminable whether the household water samples have undergone treatment or whether the drinking water is supplied through a distribution system
9	Akoachere et al., 2014	Water sources as reservoirs of Vibrio cholerae O1 and non-O1 strains in Bepanda, Douala (Cameroon):	Aggregated data	Data on tap waters aggregated with well and stream waters

		Relationship between isolation and physico-chemical factors		
10	Alfonso Molina et al. 2024	Bacterial community assessment of drinking water and downstream distribution systems in highland localities of Ecuador	Not eligible methodology for this ScR	Litte sample size (n = 4)
11	Al-Sulami et al., 2013	Isolation and identification of Legionella pneumophila from drinking water in Basra governorate, Iraq	Aggregated data	Data on tap waters aggregated with water purification plants, tankers and plants supplying water by reverse osmosis
12	Amarasiri et al., 2022	Prevalence of antibiotic resistance genes in drinking and environmental water sources of the Kathmandu Valley, Nepal	Aggregated data	Data on tap waters aggregated with various ground and surface waters (i.e., lakes, ponds, raw waters, boreholes, wells, unprotected springs)
13	Amin et al., 2022	Effects of chronic exposure to arsenic on the fecal carriage of antibiotic-resistant *Escherichia coli* among people in rural Bangladesh	Not clear if tap water was treated or not	It is indeterminable whether the household water samples have undergone treatment
14	Amir et al. 2017	Impact of unhygienic conditions during slaughtering and processing on spread of antibiotic resistant *Escherichia coli* from poultry	Not focused on drinking tap waters	Tap waters in the poultry shop were not treated neither used for human consumption (they are used for washing of utensils, tables, cutting knives, and hands)
15	Andrade et al., 2022	The antimicrobial resistance profiles of *Escherichia coli* and *Pseudomonas aeruginosa* isolated from private groundwater wells in the Republic of Ireland	Not focused on drinking tap waters	Monitoring of not treated household waters (well waters)
16	Asaduzzaman et al., 2022	Spatiotemporal distribution of antimicrobial resistant organisms in different water environments in urban and rural settings of Bangladesh	Not focused on drinking tap waters	Monitoring of not treated household waters (untreated tubewell water) and surface waters (pond and river water)
17	Atharinia et al. 2023	Detection of antibiotic-resistant bacteria and resistance genes SHV, TEM, and CTX-M in drinking waters in Tehran, Iran	Not clear if tap water was treated or not	It is indeterminable whether the household water samples have undergone treatment or whether the drinking water is supplied through a distribution system
18	Atobatele and Owoseni 2023	Distribution of multiple antibiotic-resistant Gram-negative bacteria in potable water from hand-dug wells in Iwo, Nigeria	Not focused on drinking tap waters	Monitoring of waters from hand-dug wells
19	Bdewi et al., 2020	Isolation of MRSA from Drinking water Supplies in Al-Anbar Province, Iraq	Not eligible methodology for this ScR	Water concentration techniques not reported
20	Birmingham et al., 1997	Epidemic cholera in Burundi: Patterns of transmission in the Great Rift Valley Lake region	Not eligible methodology for this ScR	Little sample size (n = 6)

21	Brizzotti-Mazuchi et al, 2020	Diversity, seasonality, and antifungal susceptibility of yeasts in the public drinking water supply in a municipality of southeastern Brazil	Other	The study is focused on the characterization of yeast (Candida) isolated from tap water
22	Burke et al. 2016	Investigation of pharmaceuticals in Missouri natural and drinking water using high performance liquid chromatography-tandem mass spectrometry	Not focused on drinking tap waters	Monitoring of drinking water supplies (e.g., groundwaters)
23	Cai et al., 2015	Determination of selected pharmaceuticals in tap water and drinking water treatment plant by high-performance liquid chromatography-triple quadrupole mass spectrometer in Beijing, China	Not eligible methodology for this ScR	The concentration of antibiotics detected in the water samples were reported only as range
24	Castiglioni et al.	Micropollutants in Lake Como water in the context of circular economy: A snapshot of water cycle contamination in a changing pollution scenario	Not eligible methodology for this ScR	Little sample size (n =3)
25	Cheng et al. 2024	Optimized Antibiotic Resistance Genes Monitoring Scenarios Promote Sustainability of Urban Water Cycle.	Other	Set-up of optimized methodology for ARGs search in environmental matrices
26	Chowdhury et al., 2022	Screening and Identification of Antibiotic Resistant Gene int1 in Coliforms Isolated From Drinking Water	Not clear if tap water was treated or not	It is indeterminable whether the household water samples have undergone treatment
27	Daly et al. 2023	Antimicrobial Resistance Is Prevalent in *E. coli* and Other Enterobacterales Isolated from Public and Private Drinking Water Supplies in the Republic of Ireland.	Not focused on drinking tap waters	Monitoring of public and private water supplies
28	Das et al. 2024	Characterization and antibiotic resistance profile of pathogenic bacteria from drinking and surface water in Odisha	Other	It is indeterminable whether the drinking water has undergone treatment
29	Delière et al., 2000	Epidemiological investigation of *Ochrobactrum anthropi* strains isolated from a haematology unit	Not eligible methodology for this ScR	Little sample size (n = 1)
30	Dhengesu et al., 2022	Antimicrobial Resistance Profile of Enterobacteriaceae and Drinking Water Quality Among Households in Bule Hora Town, South Ethiopia	Not clear if tap water was treated or not	It is indeterminable whether the household water samples have undergone treatment
31	Diwan et al., 2009	Detection of antibiotics in hospital effluents in India	Not eligible methodology for this ScR	Little sample size (n = 2); LOQ and LOD were not reported
32	Enayati et al., 2015	Virulence and antimicrobial resistance of *Enterococcus faecium* isolated from water samples	Not focused on drinking tap waters	Monitoring of not treated household waters (well waters)

33	Fernando et al., 2016	Detection of antibiotic resistance genes in source and drinking water samples from a first nations community in Canada	Not eligible methodology for this ScR	Little sample size (n = 7)
34	Ferro et al. 2024	Water quality and phenotypic antimicrobial resistance in isolated of *E. coli* from water for human consumption in Bagua, under One Health approach	Not eligible methodology for this ScR	Litte sample size (n = 6)
35	Ferro et al., 2021	Evolution of gentamicin and arsenite resistance acquisition in *Ralstonia pickettii* water isolates	Not eligible methodology for this ScR	Starting volume not defined, LOD/LOQ not defined
36	Figueira et al., 2011	Diversity and antibiotic resistance of *Aeromonas* spp. in drinking and wastewater treatment plants	Other	No aeromonads were isolated from any sampling point after water chlorination including in household taps
37	Fleres et al., 2019	Detection of a novel mcr-5.4 gene variant in hospital tap water by shotgun metagenomic sequencing	Not eligible methodology for this ScR	Little sample size (n = 8)
38	Fu et al. 2023	A novel culture-enriched metagenomic sequencing strategy effectively guarantee the microbial safety of drinking water by uncovering the low abundance pathogens.	Other	Lab-scale study for optimizing methodology for ARBs detection in drinking waters
39	Gaur et al. 2023	Microbial profile and antibiotic resistance pattern of water supply in a tertiary care hospital of Uttarakhand.	Other	Samples from tap faucet were collected using swab
40	Ghabalo et al., 2022	Monitoring and Evaluation of Antibiotic Resistance Pattern of Escherichia coli Isolated from Drinking Water Sources in Ardabil Province of Iran	Not focused on drinking tap waters	Monitoring of various water sources (e.g., dams, stream sources, rivers, and wells)
41	Ghorbani et al., 2022	Antibiotic resistance's Genotypic and Phenotypic Characteristics and the Frequency of Virulence Factors in *P. aeruginosa* Isolates Isolated from Water Samples in Iran	Not clear if tap water was treated or not	It is indeterminable whether the household water samples have undergone treatment
42	Gilchrist et al., 1986	Detection of *Pseudomonas mesophilica* as a source of nosocomial infections in a bone marrow transplant unit	Other	It was not possible to understand the resistant profile of the bacteria strains
43	Gu et al. 2023	Prevalence of antimicrobial resistance in a full-scale drinking water treatment plant.	Not focused on drinking tap waters	Monitoring of waters at different stages of a drinking water treatment plant
44	Gu et al., 2022	Characteristics of Antibiotic Resistance Genes and Antibiotic-Resistant Bacteria in Full-Scale Drinking Water Treatment System Using Metagenomics and Culturing	Not eligible methodology for this ScR	Little sample size (n = 6)

45	Guo et al., 2022	The persistent, bioaccumulative, toxic, and resistance (PBTR) risk assessment framework of antibiotics in the drinking water sources	Not focused on drinking tap waters	Monitoring of river waters
46	Huang et al., 2019	Diverse and abundant antibiotics and antibiotic resistance genes in an urban water system	Not eligible methodology for this ScR	The number of samples were not reported; only the range of antibiotic concentration detected in the water samples were reported
47	Huang et al., 2021	Dynamics of antibiotic resistance and its association with bacterial community in a drinking water treatment plant and the residential area	Not eligible methodology for this ScR	The data are reported only in the figure
48	Huang et al., 2022	Rapid determination, pollution characteristics and risk evaluations of antibiotics in drinking water sources of Hainan, China	Not focused on drinking tap waters	Monitoring of surface waters (river and lake)
49	Jia et al. 2019	Metagenomic assembly provides a deep insight into the antibiotic resistome alteration induced by drinking water chlorination and its correlations with bacterial host changes	Not eligible methodology for this ScR	Little sample size (n =9)
50	Kaur et al. 2020	Molecular characterization and antimicrobial susceptibility of bacterial isolates present in tap water of public toilets	Not focused on drinking tap waters	Tap waters in the public toilets were not used for human consumption (they are used for used to urinate and defaecate, wash hands, access mirrors, attend to menstrual hygiene needs and access dustbins for waste disposal)
51	Ke et al. 2023	Antibiotic resistome alteration along a full-scale drinking water supply system deciphered by metagenome assembly: Regulated by seasonality, mobile gene elements and antibiotic resistant gene hosts.	Not eligible methodology for this ScR	Litte sample size (n = 8)
52	Ke et al. 2023	Seasonality Determines the Variations of Biofilm Microbiome and Antibiotic Resistome in a Pilot-Scale Chlorinated Drinking Water Distribution System Deciphered by Metagenome Assembly.	Other	Pilot-scale study for undestanding the antiobitic-resistance features of the biofilm in a pilot drinking water distribution system
53	Khaled et al., 2017	Isolation, identification, and characterization of *Bacillus subtilis* from tap water	Not eligible methodology for this ScR	Little sample size (n = 3)
54	Kichana et al., 2022	Prevalence of multidrug-resistant *Escherichia coli* in household drinking water in rural Ghana	Not clear if tap water was treated or not	It is indeterminable whether the household water samples have undergone treatment

55	Kilvington et al., 1990	Laboratory Investigation of *Acanthamoeba keratitis*	Other	The study is focused on the characterization of a protozoa isolated from tap water
56	Kimbell et al. 2023	Impact of corrosion inhibitors on antibiotic resistance, metal resistance, and microbial communities in drinking water.	Other	Lab-scale study on drinking water supply for understanding the role of different corrosion inhibitors on antibiotic-resistance determinants
57	Koskeroglu et al. 2023	Biofilm formation and antibiotic resistance profiles of water-borne pathogens.	Aggregated data	Data on tap waters aggregated with surface waters and pools
58	Li et al. 2023	Extended chloramination significantly enriched intracellular antibiotic resistance genes in drinking water treatment plants.	Not focused on drinking tap waters	Monitoring of waters at the exit of drinking water treatment plants
59	Li et al. 2023	The prevalence of extra- and intra-cellular antibiotic resistance genes and the relationship with bacterial community in different layers of biofilm in the simulated drinking water pipelines	Other	The sampling matrix consists of biofilms
60	Li et al., 2020	Occurrence and fate of antibiotic residues and antibiotic resistance genes in a reservoir with ecological purification facilities for drinking water sources	Not focused on drinking tap waters	Monitoring of various drinking water sources
61	Liu et al. 2024	Prevalence of class 1 integron and its gene cassettes carrying antibiotic resistance genes in drinking water treatment and distribution systems	Not eligible methodology for this ScR	Litte sample size (n = 2)
62	Liu et al., 2021	Occurrence of antibiotics and antibiotic resistance genes and their correlations in river-type drinking water source, China	Not focused on drinking tap waters	Monitoring of various drinking water sources
63	Liu et al., 2022	Investigation on the fate of quinolone antibiotics in three drinking water treatment plants of China	Not eligible methodology for this ScR	Number of samples not clearly specified
64	Lu et al., 2020	Identification and inactivation of Gordonia, a new chlorine-resistant bacterium isolated from a drinking water distribution system	Other	The study is focused on the characterization of chlorine-resistant Gordonia specie isolated from tap water
65	Medina-Pizzali et al., 2022	Whole-Genome Characterisation of ESBL-Producing *E. coli* Isolated from Drinking Water and Dog Faeces from Rural Andean Households in Peru	Not clear if tap water was treated or not	It is indeterminable whether the household water samples have undergone treatment
66	Meng et al., 2020	Genetic Diversity, Antimicrobial Resistance, and Virulence Genes of Aeromonas Isolates from Clinical Patients, Tap Water Systems, and Food	Not eligible methodology for this ScR	Not possible to associate antibiotic-resistance profile to microbes

67	Meng, et al. 2025	Metagenomic perspectives on antibiotic resistance genes in tap water: The environmental characteristic, potential mobility and health threat.	Not eligible methodology for this ScR	Litte sample size (n = 9)
68	Mian et al., 2020	A study of bacterial profile and antibiotic susceptibility pattern found in drinking water at district Mansehra, Pakistan	Aggregated data	Data on tap waters aggregated with non-treated water sources (i.e., storage tanks, tube well water)
69	Miao et al., 2022	Bacterial community assembly and beta-lactamase (bla) genes regulation in a full-scale chloraminated drinking water supply system	Not eligible methodology for this ScR	Number of samples not reported
70	Mir et al., 2021	Isolation of Multi Drug Resistant *Escherichia coli* from drinking water of Lahore City, Pakistan	Not eligible methodology for this ScR	Lack of consistency in the description of antibiotic-resistance results
71	Monteiro et al., 2018	Development and validation of liquid chromatography-Tandem mass spectrometry methods for determination of beta-lactams, macrolides, fluoroquinolones, sulfonamides and tetracyclines in surface and drinking water from Rio de Janeiro, Brazil	Not eligible methodology for this ScR	The concentration of antibiotics detected in the water samples are not reported
72	Narciso-da-Rocha et al., 2014	Genotypic diversity and antibiotic resistance in Sphingomonadaceae isolated from hospital tap water	Not eligible methodology for this ScR	Antibiotic-resistance results on Sphingomonadaceae have been aggregated with data from previous papers, whose origin from tap waters is not clear
73	Olanrewaju et al. 2024	Genome mining of *Escherichia coli* WG5D from drinking water source: unraveling antibiotic resistance genes, virulence factors, and pathogenicity.	Not eligible methodology for this ScR	Number of samples not reported
74	Oluyege et al., 2011	Bacteriological and physico-chemical quality assessment of household drinking water in Ado-Ekiti, Nigeria	Aggregated data	Data on tap waters aggregated with non-treated water sources (i.e., well water, harvested rain water)
75	Oluyege et al., 2019	Water quality assessment and plasmid analysis of multiple antibiotic-resistant *Escherichia coli* O157:H7 from well-water sources in Ado-Ekiti metropolis, Nigeria	Not focused on drinking tap waters	Monitoring of not treated household drinking water (well waters)
76	Onohuean et al., 2022	Antibiogram signatures of Vibrio species recovered from surface waters in South Western districts of Uganda: Implications for environmental pollution and infection control	Aggregated data	Data on tap waters aggregated with various ground and surface waters (i.e., lakes, ponds, raw waters, boreholes, wells, unprotected springs)

77	Paiga et al., 2017	Development of a multi-residue method for the determination of human and veterinary pharmaceuticals and some of their metabolites in aqueous environmental matrices by SPE-UHPLC-MS/MS	Not eligible methodology for this ScR	Little sample size (n = 2)
78	Panagopoulou et al. 2007	Filamentous fungi in a tertiary care hospital: environmental surveillance and susceptibility to antifungal drugs	Other	Target of the research for antibiotic-resistance was not found in tap waters
79	Paopradit et al., 2017	Distribution and characterization of *Stenotrophomonas maltophilia* isolates from environmental and clinical samples in Thailand	Not eligible methodology for this ScR	Not possible to associate antibiotic-resistance profile to microbes
80	Pervaiz et al. 2024	Microbial Contamination and Antibiotic Resistance in Food and Water: Assessing the Threat of *Staphylococcus aureus* in Lahore Metropolitan	Aggregated data	Data on tap waters aggregated with other types of food matrices (e.g., milk, yogurt)
81	Ramalho et al., 2022	The occurrence of antimicrobial residues and antimicrobial resistance genes in urban drinking water and sewage in Southern Brazil	Aggregated data	Data on tap waters aggregated with various sources (pre-treatment/raw and post-treatment water from drinking water treatment plants, water from sewage treatment plants and sewage pumping plants)
82	Ranjbar et al., 2019	Prevalence and Characterization of Plasmid-mediated Quinolone Resistance Genes among *Escherichia coli* Strains Isolated from Different Water Sources in Alborz Province, Iran	Not focused on drinking tap waters	Monitoring of various drinking water sources
83	Salamandane et al., 2021	High Fecal Contamination and High Levels of Antibiotic-Resistant Enterobacteriaceae in Water Consumed in the City of Maputo, Mozambique	Aggregated data	Data on tap waters aggregated with supply wells and home-bottled street water
84	Sarker et al. 2023	Antibiotic-Resistant *Escherichia coli* Isolated from Duck Cloacal and Tap Water Samples at Live Bird Markets in Bangladesh	Not focused on drinking tap waters	Tap waters in the live bird market were not used for human consumption (they were used for drinking the poultry or for washing the customers' hands)
85	Sayimakturk et al., 2012	Determination of microbial quality and plasmid-mediated multidrug resistant bacteria in fountain drinking water sources in Turkey	Other	It was not possible to understand the resistant profile of the bacteria strains, nor how the resistant species (environmental and fecal species) were subdivided in the different samples

86	Scherer et al., 2022	Parasitological, microbiological, and antimicrobial resistance profiles of raw and drinking water in a tourist city in the tri-border region of South America	Not eligible methodology for this ScR	Number of tap water samples not reported
87	Shi et al., 2013	Metagenomic insights into chlorination effects on microbial antibiotic resistance in drinking water	Aggregated data	Data on tap waters aggregated with waters from different stages of the same drinking-water system (filtered waters, disinfected waters, tap waters)
88	Storto et al., 2021	Seasonal Dynamics of Microbial Contamination and Antibiotic Resistance in the Water at the Tietê Ecological Park, Brazil	Other	Target of the research for antibiotic-resistance was not found in tap waters
89	Tanner et al., 2015	Development and field evaluation of a method for detecting carbapenem-resistant bacteria in drinking water	Not eligible methodology for this ScR	It is not possible to associate the antibiotic resistant strains to the samples therefore it is not possible to calculate the number of positive water samples
90	Taviani et al., 2022	Occurrence of waterborne pathogens and antibiotic resistance in water supply systems in a small town in Mozambique	Not clear if tap water was treated or not	It is indeterminable whether the household water samples have undergone treatment
91	Tewari et al., 2013	Plasmid mediated transfer of antibiotic resistance and heavy metal tolerance in thermotolerant water borne coliforms	Not focused on drinking tap waters	Monitoring of various not treated waters for domestic purposes (e.g., piped supplies, hand pumps, and untreated well waters) and surface waters
92	Thakali et al., 2022	Prevalence of antibiotic resistance genes in drinking and environmental water sources of the Kathmandu Valley, Nepal	Aggregated data	Data on tap waters aggregated with various surface waters (i.e., shallow wells, deep wells, stone spouts, and springs)
93	Tiwari et al., 2022	Bacterial Genes Encoding Resistance Against Antibiotics and Metals in Well-Maintained Drinking Water Distribution Systems in Finland	Not eligible methodology for this ScR	The sampling details are not clear
94	Tsholo et al. 2024	Distribution of antibiotic resistance genes and antibiotic residues in drinking water production facilities: Links to bacterial community.	Not focused on drinking tap waters	Monitoring of waters at the exit of drinking water treatment plants
95	Tsvetanova et al. 2023	Antibiotic Resistance of Heterotrophic Bacteria Isolated from Drinking Water – from the Water Source to the Consumers' Taps	Not eligible methodology for this ScR	Litte sample size (n = 4)
96	Tsvetanova et al., 2022	Antimicrobial Resistance of Heterotrophic Bacteria in Drinking Water-Associated Biofilms	Other	The study is lab-scale experiments

97	Tsvetanova et al., 2022	Prevalence of antimicrobial resistance in a Bulgarian drinking water supply system	Not eligible methodology for this ScR	Number of samples not reported
98	Vazquez-Roig P et al., 2012	Risk assessment on the presence of pharmaceuticals in sediments, soils and waters of the Pego-Oliva Marshlands (Valencia, eastern Spain)	Aggregated data	Data on tap waters aggregated with surface and spring
99	Wang et al. 2024	Deciphering the dynamics and driving mechanisms of high-risk antibiotic resistome in size-fractionated bacterial community during drinking water chlorination via metagenomic analysis.	Not eligible methodology for this ScR	Litte sample size (n = 6)
100	Wang et al., 2011	Determination of cephalosporin antibiotics in water samples by optimised solid phase extraction and high performance liquid chromatography with ultraviolet detector	Not eligible methodology for this ScR	Little sample size (n = 5)
101	Wang et al., 2022	Removal and distribution of antibiotics and resistance genes in conventional and advanced drinking water treatment processes	Not eligible methodology for this ScR	Number of samples not reported
102	Wolf-Baca and Siedlecka 2023	Community Composition and Antibiotic Resistance of Tap Water Bacteria Retained on Filtration Membranes	Other	The sampling matrix consists of biofilms collected through dedicated filtration membrane apparatus
103	Yiruhan et al., 2010	Determination of four fluoroquinolone antibiotics in tap water in Guangzhou and Macao	Not eligible methodology for this ScR	LOD was not reported
104	Zamberlan da Silva et al., 2008	Characterisation of potential virulence markers in *Pseudomonas aeruginosa* isolated from drinking water	Aggregated data	Data on tap waters aggregated with mineral water and artesian well water
105	Zhao et al., 2022	Fate, mobility, and pathogenicity of drinking water treatment plant resistomes deciphered by metagenomic assembly and network analyses	Not eligible methodology for this ScR	Number of samples not reported
106	Zhou et al. 2023	Comprehensive profiling and risk assessment of antibiotic resistance genes in a drinking water watershed by integrated analysis of air-water-soil.	Not focused on drinking tap waters	Monitoring of water, air and solid in the same watershed used for drinking water production
107	Zhou et al., 2021	Metagenomic analysis of microbiota and antibiotic resistome in household activated carbon drinking water purifiers	Not eligible methodology for this ScR	Little sample size (n =3)

Table S3. Summary of the key features of the included articles.

Number	Authors	Year	Location	Tap water sample size[a]	Volume of tap water analysed (mL)	Type of disinfection applied by DWTP	Target of the study	ARB of clinical relevance (yes, no, N/A)
1	Adesoji et al.	2016	Nigeria	< 50	1	Chlorination	ARB (environmental and fecal), ARGs (in bacteria isolated from tap water)	yes
2	Adzitey et al.	2016	Ghana	< 50	1	Not specified	ARB (fecal)	yes
3	Ahmed et al.	2022	Ghana	> 100	100	Chlorination	ARB (environmental and fecal)	yes
4	Akbar et al.	2022	Pakistan	< 50	1 - 100[b]	Not specified	ARB (fecal), ARGs (in bacteria isolated from tap water)	yes
5	Ateba et al.	2020	South Africa	< 50	100	Chlorination	ARB (environmental), ARGs (in bacteria isolated from tap water)	no
6	Ben et al.	2020	China	< 50	1,000	Chlorination	Antibiotics	N/A
7	Bhatta et al.	2007	Nepal	> 100	100	Not disinfected (bleaching powder is occasionally used)	ARB (fecal)	yes
8	Borjac et al.	2023	Lebanon	50 - 100	1-mL for psychrophilic bacteria; 250-mL for other bacterial parameters	Chlorination	ARB (environmental and fecal), ARG (only mecA gene in bacteria isolated from tap water)	yes
9	Charuaud et al.	2019	France	< 50	2,000	Chlorination	Antibiotics	N/A
10	Dávalos et al.	2021	Colombia	< 50	2,000	Chlorination	ARB (environmental)	no
11	Destiani and Templeton	2019	United Kindom	< 50	2,000 – 3,000	Chlorination	ARGs (directly in tap water samples)	N/A
12	Elmi et al.	2021	Malaysia	< 50	1 - 100[b]	Not disinfected	ARB (fecal)	yes

13	Elmonir et al.	2020	Egypt	> 100	100	Chlorination	ARB (fecal), ARGs (in bacteria isolated from tap water)	yes
14	Emekdas et al.	2006	Turkey	> 100	1 - 100[b]	Not specified	ARB (environmental)	no
15	Ezzat	2014	Egypt	50 - 100	100	Chlorination	ARB (environmental)	no
16	Furuhata et al.	2006	Japan	> 100	Not specified	Chlorination (residual chlorine in tap water from 0.00 to 0.8 mg/L)	ARB (environmental)	no
17	Hamza et al.	2020	Egypt	< 50	100[b]	Not specified	ARB (fecal), ARGs (in bacteria isolated from tap water)	yes
18	Hanna et al.	2018	China	< 50	1,000	Not specified	Antibiotics	N/A
19	Jazrawi et al.	1988	Iraq	> 100	100[b]	Chlorination (residual chlorine in tap water from 0.1 to 0.3 mg/L)	ARB (environmental and fecal)	yes
20	Jiang et al.	2019	China	< 50	1,000	Chlorination	Antibiotics	N/A
21	Ke et al.	2023	China	< 50	20,000	Chlorine dioxide disinfection	ARGs (directly in tap water samples)	N/A
22	Ke et al.	2024	China	< 50	10,000	Chlorination	ARGs (directly in tap water samples)	N/A
23	Khan and Mustafa	2021	Pakistan	50 - 100	100	Chlorination	ARGs (in bacteria isolated from tap water)	N/A
24	Khan et al.	2016	United Kingdom	> 100	100	Not specified	ARGs (in bacteria isolated from tap water)	N/A
25	Kinge et al.	2010	South Africa	< 50	100[b]	Not specified	ARB (fecal)	yes
26	Leginowicz et al.	2018	Poland	< 50	1,000	Disinfection treatment applied but not specified	ARB (environmental), ARGs (in bacteria isolated from tap water)	yes
27	Li et al.	2023	China	< 50	10,000	Chlorination	ARGs (directly in tap water samples)	N/A

28	Liang et al.	2022	China	< 50	20,000	Chlorination	ARGs (directly in tap water samples)	N/A
29	Makris and Snyder	2010	Cyprus	< 50	1,000	Chlorination	Antibiotics	N/A
30	Mi et al.	2019	Canada	< 50	300 - 500	Chlorination	ARGs (directly in tap water samples)	N/A
31	Moghaddam et al.	2022	Iran	> 100	500	Chlorination	ARB (environmental)	no
32	Molale-Tom et al.	2024	South Africa	< 50	1[c]	Chlorination	ARB (environmental), ARGs (in bacteria isolated from tap water)	N/A
33	Papandreou et al.	2000	Greece	> 100	500	Chlorination	ARB (fecal)	yes
34	Santos et al.	2023	Brazil	> 100	100	Chlorination	ARB (*Staphilococcus* spp.), ARG (only mecA gene in bacteria isolated from tap water)	N/A
35	Scoaris et al.	2008	Brazil	< 50	100[b]	Chlorination (residual chlorine in tap water from 0.00 to 1.2 mg/L)	ARB (environmental)	no
36	Siedlecka and Piekarska	2019	Poland	< 50	250[d] for ARB; 10,000 for ARGs	Chlorination (residual chlorine in tap water from 0.07 to 0.27 mg/L)	ARB (environmental and fecal), ARGs (directly in tap water samples)	yes
37	Siedlecka et al.	2021	Poland	< 50	250	Chlorination (residual chlorine in tap water from 0.00 to 0.31 mg/L)	ARB (environmental)	no
38	Siedlecka et al.	2020	Poland	< 50	250[d] for ARB; 10,000 for ARGs	Chlorination (residual chlorine in tap water from 0.00 to 0.31 mg/L)	ARB (environmental), ARGs (directly in tap water samples)	no
39	Subba et al.	2013	Nepal	< 50	100[b]	Not specified	ARB (fecal)	yes
40	Vaz-Moreira et al.	2012	Portugal	< 50	100	Chlorination	ARB (environmental)	no

41	Walsh et al.	2011	India	50 - 100	15	Not specified	ARB (environmental), ARGs (in bacteria isolated from tap water)	yes
42	Wang et al.	2011	United States	< 50	500	Chlorination	Antibiotics	N/A
43	Wang et al.	2023	Mostly from China (86%) the rest from South Africa, USA, Brazil, Macau, Taiwan, Singapore and Hong Kong	> 100	2,000,000	Not specified	ARGs (directly in tap water samples)	N/A
44	Zhang et al.	2018	China	50 - 100	1	Chlorination	ARB (environmental)	no
45	Zhang et al.	2021	China	< 50	2,000	Not specified	ARGs (directly in tap water samples)	N/A

DWTP = drinking water treatment plant

[a] Sampling size less than 50 refers to the analysis of at least 10 samples, according to inclusion criteria of the present ScR.

[b] This value has been inferred from the articles, that cited they performed the assays according to standard guidelines protocols, e.g., American Public Health Association (APHA) methods.

[c] This value has been inferred from Kritzinger et al. (2019) that has been cited as reference for the microbiological methods (Kritzinger, R.K., 2019. Antibiotic resistant bacteria and-genes in raw water, and the implications for drinking water production. In: MSc. North-West University (South Africa) Potchefstroom).

[d] This value has been inferred from Siedlecka et al. (2021), a research study performed by the same researcher group (Siedlecka, A., Wolf-Baca, M.J., Piekarska, K., 2021. Antibiotic and Disinfectant Resistance in Tap Water Strains - Insight into the Resistance of Environmental Bacteria. Pol. J. Microbiol. 70(1):57-67. doi: 10.33073/pjm-2021-004).

Table S4. General features of the included papers: countries division according to geographical regions defined by WHO or UNSD and to income level (Wang et al. 2023 analyzed samples from various countries, but it is here classified as China, because more than 86% of the analyzed samples came from this country).

Geographic Regions	**Countries included in the revised papers**	**Number of studies (n, %)**
Countries division according to WHO regions (**WHO, 2024**)		
America	Canada, US, Brazil, Colombia	5, 11.1
Europe	Cyprus, Greece, Portugal, France, Poland, UK, Turkey	11, 24.4
Eastern Mediterranean region	Egypt, Iraq, Iran, Lebanon, Pakistan	8, 17.8
African region	Ghana, Nigeria, South Africa	6, 13.3
South-East Asia region	India, Nepal	3, 6.7
Western Pacific region	China, Japan, Malaysia	12, 26.7
Countries division according to geographic regions based on UNSD (**UNSD, 2024**)		
Northern America	Canada, US	2, 4.4
South America	Brazil, Colombia	3, 6.7
Eastern Asia	China, Japan	11, 24.4
Western Asia	Cyprus, Iraq, Lebanon, Turkey	4, 8.9
Southern Asia	India, Iran, Nepal, Pakistan	6, 13.3
South-Eastern Asia	Malaysia	1, 2.2
Northern Africa	Egypt	3, 6.7
Western Africa	Ghana, Nigeria	3, 6.7
Southern Africa	South Africa	3, 6.7
Northern Europe	UK	2, 4.4
Western Europe	France	1, 2.2
Southern Europe	Greece, Portugal	2, 4.4
Eastern Europe	Poland	4, 8.9
Country division according to income level based on World Bank Atlas method (**World bank, 2024**)		
High-income economies	Canada, Cyprus, France, Greece, Japan, Poland, Portugal, UK, US	13, 28.9
Upper-middle-income economies	Brazil, China, Colombia, Iraq, Malaysia, South Africa, Turkey	19, 42.2
Lower-middle income economies	Egypt, Ghana, India, Iran, Lebanon, Nepal, Nigeria, Pakistan	13, 28.9
Low-income economies	none	0, 0.0

UNSD, 2024. Methodology - Geographic Region. United Nations Statistics Division (UNSD). https://unstats.un.org/unsd/methodology/m49/ (last accessed 3 September 2024); WHO, 2024. WHO regional website. Available at: https://www.who.int/countries (last accessed 15 February 2024); World Bank, 2024. Data - World Bank country and Lending groups. Available at: https://datahelpdesk.worldbank.org/knowledgebase/articles/906519-world-bank-country-and-lending-groups (last accessed 3 September 2024).

2.5. *Third in draft manuscript: Prevalence of Antibiotic Resistance Genes in Particulate Matter (PM) Collected from Various Sampling Sites*

2.5.1. Introduction

According to World Health Organization (WHO), One Health *is an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals and ecosystems. It recognizes that the health of humans, animals, and the wider environment are inherently linked and interdependent.*

A typical One Health issue is represented by the ability of antibiotic resistance to develop, spread, and persist in the environment through various pathways, thereby forming a crucial aspect of the human-animal-environment cycle (Wu et al., 2022).

Compared to soil, water, and waste, antibiotic resistance elements in ambient air are more extensively and closely connected to humans (Woolhouse, 2024). Specifically, antibiotic resistance associated with airborne particulate matter (PM) intensifies this health problem because the PM_{10} fraction (diameter ≤ 10 μM) poses significant health risks, as it can penetrate the respiratory system, causing respiratory and cardiovascular diseases, and potentially exacerbating conditions such as asthma and lung cancer. On these particles, different kind of pollutant can be adsorbed (Becsei et al., 2021; Macrì et al., 2023), including antibiotic resistance determinants such as antibiotic resistance genes (ARGs) (Zhou et al., 2021). Inhaled ARGs have been found to expose humans to concentrations of $10^{2\text{-}3}$ copies/m^3-air (Xie et al., 2019), and their pathogenic bacterial hosts could increase the likelihood of resistant infections through airborne exposure.

The profiles of airborne ARGs are typically influenced by the characteristics of their emission sources and atmospheric factors. Areas with great importance for Public Health or known to be source of antibiotic resistance elements should be monitored, such as wastewater treatment plants (WWTPs), hospitals, and livestock farms.

WWTP are considered hotspot of antibiotic resistance spreading in the environments (Bonetta et al., 2023) and the presence of antibiotic resistance elements, such as bacteria (ARB) and ARGs, in WWTPs is well known in the scientific literature (Zhuang et al. 2021), with a high prevalence of those genes that belong to sulphonamide-resistance genes (e.g. *sul*I, *sul*II and *sul*III). Since WWTPs are considered as important hotspots in the ARG spreading in the environment through the discharge of wastewater in receiving water bodies (Pazda et al. 2019), but also during the WWTP effluent reuse practice, it is important to consider their role in ARG dissemination in the air.

Moreover, in farm settings, using high-throughput quantitative polymerase chain reaction (HT-qPCR), amplicon sequencing, and metagenomic sequencing methods, airborne ARB and ARGs have been detected in livestock environments with various farming practices worldwide (Liu et al., 2012; Davis et al., 2018; Franceschini et al., 2019). Specifically, the detected ARGs included *tetC*, *tetG*, *tetM*, *sul1*, *sul2*, *ermA*, *ermC*, *ermF*, *mecA*, *blaSHV*, and *aac(6')-aph(2'')* in animal farms (Liu et al., 2012; McEachran et al., 2015; Gao et al., 2016; Sancheza et al., 2016).

Clinical environments are typically marked by the more frequent use of first-line antibiotics and the high prevalence of human bacterial pathogens (Wu et al., 2022). These challenges are especially significant in large urban hospitals in rapidly developing nations, where antibiotic overuse is widespread (Hvistendahl, 2012), and healthcare-associated infections along with patient overcrowding are often reported to be critical issues. Furthermore, mobile genetic elements (MGEs), e.g. integrases, in the air can promote the spread of ARGs to airborne bacteria through horizontal gene transfer (HGT) (Li et al. 2018). Therefore, the concurrent presence of airborne ARGs, MGEs, and bioaerosols from clinical sources may support the transmission of potential ARB in airborne particles released from hospitals. This scenario warrants in-depth investigation, especially given the lack of studies exploring this aspect in European countries.

The presence of ARGs in urban air is an emerging issue with significant public health implications. PM, generated by human activities such as traffic, industry, and domestic heating, can act as a carrier for microorganisms and environmental DNA containing ARGs. Recent studies have shown that areas with high population density and heavy traffic exhibit higher concentrations of ARGs in the air, highlighting the urgent need for monitoring and mitigation measures to protect human health and reduce the impact of resistant infections. To the best of our knowledge, most of these studies were focused on highly polluted megacities in China (Wang et al., 2019; He et al., 2021; Xie et al., 2022; Wu et al., 2023). The pollution levels in these cities are significantly higher than those reported in European countries, but this could not be correlated with a lower risk for human health.

For these reasons, the present part of the Ph.D. project aimed to investigate the distribution and abundance of representative ARGs in environments critical to human well-being, including urban areas, hospitals, and livestock farms. Special attention was also given to the role of the urban water cycle, as exemplified by WWTPs, in the dissemination of ARGs, also considering the possible reuse of effluent.

2.5.2. Materials and methods

2.5.2.1. Site descriptions and sampling strategy

Two outdoor PM_{10} sampling campaigns were conducted during two different seasons, summer and winter, in Northwest Italy within the Po Valley (Piedmont region), one of the most polluted areas in Europe. The sampling sites included: (i) a hospital site, (ii) an urban site, (iii) a WWTP, and (iv) a livestock farm (Fig. 1). At the hospital site, PM_{10} samples were collected from the courtyard near one of the main entrances of the largest hospital (bed capacity of 1,342) in the Piedmont region. The urban site, located a few kilometres away, was situated in an area characterised by high traffic levels. The WWTP investigated, instead, is farther away, as shown in Fig. 1, and has a capacity of 70,000 population equivalent (p.e.). It treats predominantly domestic wastewater with pre-treatments, primary and secondary treatments followed by a disinfection step, performed with sodium hypochlorite, before the discharge in the receiving water body. The livestock farm site is a recognised private alpine pasture raising around eighty free-grazing specific cattle breeds for the production of dairy-based products.

Fig. 1. Geographical localisation of the sampling sites. A: hospital site; B: urban site; C: wastewater treatment plant; D: livestock farm.

The sampling was conducted by a high-volume sampler (AirFlow HVS Touch, AMS Analitica, Italy) equipped with a PM_{10} fractioning inlet. The air was sampled at an average flow rate of 1.16 m^3/min for 24 h. Particles with diameter ≤ 10 µm were collected on 203,2 x 254 mm Tissuequartz filters (Whatman, U.S.). All the filters were packed in clean aluminium foil and sterilised in a muffle furnace at 500°C for 5 hours prior to sampling. The filter holder and all the tools used to manipulate the filters were cleaned with 75% ethanol. One filter per sample for each sampling campaign was collected. Beside this, also another filter for PM quantification was collected and weight before and after the sampling with mg accuracy. The PM quantification was performed only for hospital and livestock farm, since there were no data on PM_{10} quantities available in these areas. In the urban and WWTP areas, instead, the PM_{10} concentrations were obtained from Regional Agency for Environmental Protection (ARPA Piemonte).

2.5.2.2. Sample pre-treatment and DNA extraction

To deal with the low concentration of DNA on the collected PM samples, an initial phase of protocol optimisation was performed. The final version is subsequently described. According to other relevant literature (Cao et al., 2014; Jiang et al., 2015), 1/4 of the filter was cut and used for the DNA extraction. This part of the filter was placed in 50 mL centrifuge tube filled with sterile PBS buffer supplemented with 0.05% Tween20. The tubes were then sonicated in an ultrasonic water bath for 20 min and subsequently vortexed for 30 min at 2800 rpm. All the suspension collected on the filter was then filtered onto a 0.22 µM polycarbonate filter (Merck, Germany), subsequently the DNA was extracted using DNeasy PowerWater Kit (Qiagen, Germany). The DNA was finally quantified with Quantifluor dsDNA system (Promega Corporation, U.S.). A blank control filter undergone to the same procedure and the DNA extraction of blank control samples resulted in DNA quantification below the detection limit of the instrument.

2.5.2.3. Antibiotic resistance gene abundance

ARGs were chosen based on the literature (Echeverria-Palencia et al., 2017; Liang et al., 2020; Ginn et al. 2021; He et al., 2021). The six selected ARGs included *bla*$_{TEM}$, *bla*$_{CTX-M}$, *sul*I, *sul*II, *tet*A and *tet*W (representing ARGs associated with resistance to β-lactams, sulphonamides and tetracycline, respectively). Additionally, a mobile genetic element (MGE) was included in the quantification to identify a gene that could serve as an indicator of antibiotic resistance, specifically *intI*1.

Gene quantification was performed using droplet digital PCR (ddPCR). 22 µl of reaction mix was prepared, containing QX200 ddPCR EvaGreen Supermix with primers at a concentration of 100 nM, and 5 µL of DNA (or 5 µL of nuclease-free water for the no-template control). The reaction mix (20 µL) and 70 µL of QX200 Droplet Generation Oil for EvaGreen were transferred to a DG8 Cartridge, which was then placed in the QX200 Droplet Generator (BioRad). The generated droplets were subsequently transferred to a 96-well PCR plate for DNA amplification using a T100 Thermal Cycler (BioRad). The amplification program consisted of an initial denaturation at 95°C for 30 seconds, followed by primer-specific annealing at 1-minute intervals with a ramp rate of 2°C/s, 4°C for 5 minutes, and a final step at 90°C for 5 minutes (Table 1). After amplification, the plates were transferred to a QX200 Droplet Reader (BioRad) for gene quantification. Only reactions producing more than 10,000 droplets were considered. Data were analysed using QuantaSoft Analysis Pro Software (BioRad) and expressed as Log gene copies (g.c.) per 1000 m^3 of sampled air and 1000 µg of collected PM.

Gene	**Primer sequence**	**Annealing Temperature (°C) ddPCR**	**Reference**
*intI*1	Fw CTGGATTTCGATCACGGCACG Rv ACATGCGTGTAAATCATCGTCG	60	Wang et al., 2014
bla$_{TEM}$	Fw TCCGCTCATGAGACAATAACC Rv TTGGTCTGACAGTTACCAATGC	55	Reinthaler et al., 2010
bla$_{CTX-M}$	Fw GGAGGCGTGACGGCTTTT Rv TTCAGTGCGATCCAGACGAA	55	Zhu et al., 2013
*sul*I	Fw GTGACGGTGTTCGGCATTCT Rv TCCGAGAAGGTGATTGCGCT	58	Modified from Boerlin et al., 2005
*sul*II	Fw TCCGGTGGAGGCCGGTATCTGG Rv CGGGAATGCCATCTGCCTTGAG	58.5	Di Cesare et al., 2015
*tet*A	Fw GCTACATCCTGCTTGCCTTC Rv CATAGATCGCCGTGAAGAGG	59.6	Petrin et al., 2019
*tet*W	Fw GAGAGCCTGCTATATGCCAGC Rv GGGCGTATCCACAATGTTAAC	60	Aminov et al., 2001

Table 1. Primer pairs and annealing temperatures used to quantify *intI1* and antibiotic resistance genes with droplet digital PCR (ddPCR).

2.5.2.4. Statistical analysis

The statistical analyses were performed with SPSS software (version 30.0.0). ARG concentrations were converted to log10 (log gene copies / 1000 m^3 or log gene copies / 1000 µg). Differences in PM or ARG concentration between the different sites were analysed by ANOVA followed by Tukey's post-hoc test. Differences between the two different seasons in terms of both PM and ARG concentration were analysed by T-test. Potential correlations between ARGs and PM concentrations and between different genes were investigated by Pearson's correlation. P-value was considered significant when $p < 0.05$.

2.5.3. Results and discussion

2.5.3.1. PM_{10} concentration

The PM_{10} concentration detected in the four sites considered is reported in Table 2. Statistical analysis revealed a statistically significant difference between summer and winter sampling ($p < 0.05$), whereas no statistically significant differences were observed considering the sites. For almost all the sites, as expected, it is possible to observe a higher concentration of PM_{10} in winter respect to summer, except to the livestock farm. This is due to the geographical localisation of hospital, urban and WWTP sites because they are all located in an urban landscape; instead, the livestock farm is in a rural area that does not suffer of typical seasonal trend of main pollutants caused by both anthropic sources (e.g., industrial and vehicular emissions) and meteoclimatic conditions. In fact, the decrease of PM_{10} in the warm season has been generally observed in urban environments (Bonetta et al., 2019). The highest PM_{10} concentration was observed in the urban site during winter sampling, whereas the lowest was observed always in the urban site during summer sampling.

Site	**PM_{10} (µg/m^3) concentration in different seasons**	
	Winter	***Summer***
Hospital	35	24.69
Urban	56	9
Wastewater treatment plant	50.86	21
Livestock farm	11.1	11.47

Table 2. PM_{10} concentration (µg/m^3) detected in the four sites.

2.5.3.2. ARG abundances

The abundances of ARGs normalised per 1000 m^3 or per PM mass (1000 µg) are presented in Fig. 2 and Fig. 3, respectively. The results expressed per m^3 reflect the concentration of pollutants (i.e., PM or ARGs) in a given volume of sampled air. The results expressed per µg represent the concentration of pollutants (i.e., PM or ARGs) in a fixed mass of particulate matter, this allows for a direct comparison of the quantity of ARG contained in a given amount of PM, irrespective of the volume sampled.

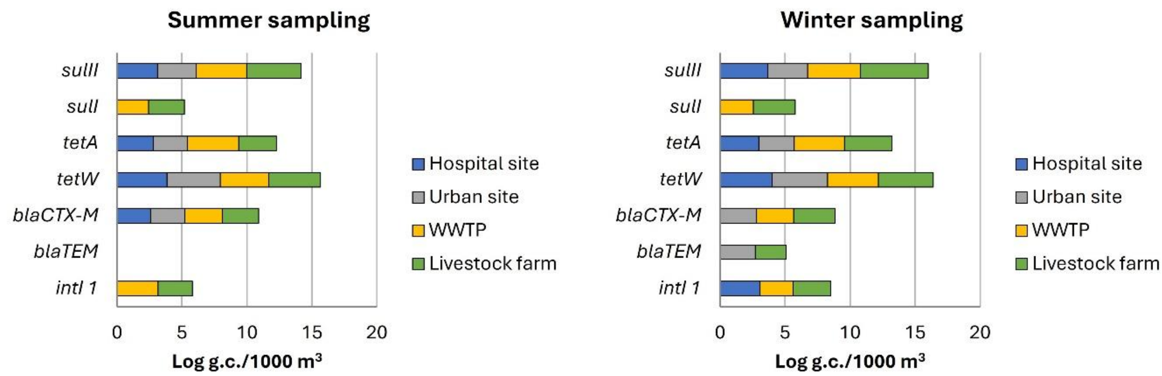

Fig. 2. Antibiotic resistance genes (ARGs) expressed as Log gene copies (g.c.)/1000 m^3 in the different sites considered.

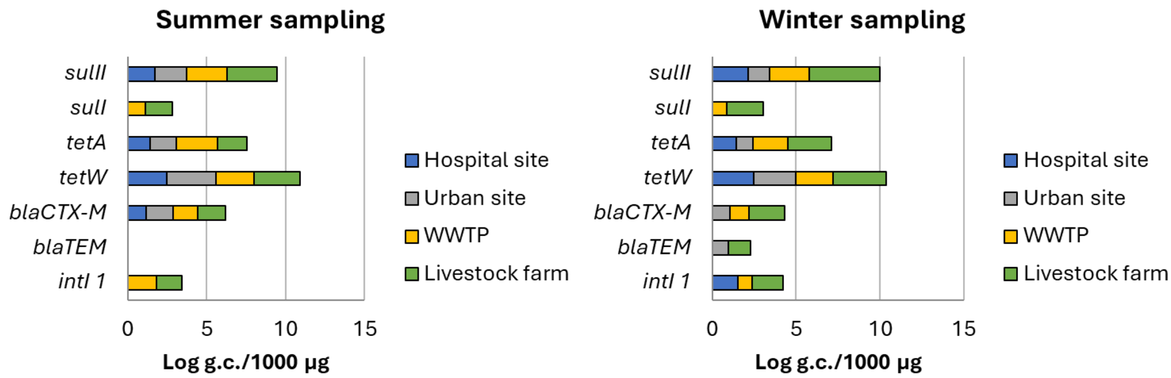

Fig. 3. Antibiotic resistance genes (ARGs) expressed as Log gene copies (g.c.)/1000 µg in the different sites considered.

The most important contribution, in terms of ARG load, was given by livestock farm in winter and from WWTP in summer season respect to volume of sampled air (Fig. 2). With the data expressed as PM mass, instead, the livestock farm contributed with the highest ARG load for both seasons (Fig. 3); this is due to the lower PM mass detected in this site, that contributes to increase the ARG concentration contained in the PM collected.

Globally, the most widespread genes were *sul*II and *tet*W for both seasons, with highest abundance in livestock farm and urban sites, respectively, even if not statistically significant. In fact, there are no statistically significant differences between the two seasons in terms of ARG concentration.

Among the ubiquitous ARGs (i.e., genes that were detected in all sites and seasons), the only detected statistically significant difference ($p < 0.05$) was reported for *tet*A gene between urban (mean 2.69 Log g.c./1000 m^3) and WWTP (mean 3.90 Log g.c./1000 m^3) sites.

*sul*I gene was never found in hospital and urban sites, but only in WWTP and livestock farm, that in fact represented the most ARG polluted sites. *sul*I gene has proven to be strongly correlated with human activities globally and can serve as indicators of anthropogenic sources (Pruden et al. 2020). In addition, it is often detected on the sequence binding with class 1 integrons, mobile genetic elements (MGEs) that can spread gene cassettes with site-specific recombination, this means that these ARGs are able to participate in horizontal gene transfer, leading to their dissemination in environments (Deng et al. 2015).

In our study, the contribution of β-lactam resistance genes to the total ARG load represented a low amount since *bla*$_{TEM}$ gene that was not detected at all in summer sampling and *bla*$_{CTX-M}$ was found in hospital site only during summer. This is in contrast with other studies in which β-lactam resistance genes, among others, were the most abundant in outdoor atmosphere (Li et al., 2018) and $PM_{2.5}$ emitted from an hospital site (Wu et al., 2022). Despite this difference, it is important to highlight that in different sites or geographical areas, distinct genetic patterns may emerge even when the site type remains the same (e.g., hospital *vs* hospital).

Although some differences in the presence of ARGs across different seasons, Pearson's correlation analysis showed no significant correlation between PM_{10} and ARG concentrations. In fact, the site with the highest ARG load was the livestock farm, which exhibited a very low PM_{10} concentration. This is in contrast with the study of Gao et al. (2018), since they assumed in their study that the high concentration of PM detected in an area of a composting plant was the responsible of high concentration of genes since microorganism present in the air are usually attached to the surface of PM (Yamamoto et al. 2012), but this is not the only mechanism since airborne bacteria and consequently ARGs can be found also in other sources, such as dust, water droplets, fumes, and other microscopic particles.

The *intI1* gene, used as an indicator of antibiotic resistance, was never found in urban site, and in hospital site was found only during winter sampling. Moreover, the correlation analysis revealed an association between *intI1* and *sul*II, *sul*I and *tet*A ($r > 0.71$ and $p < 0.05$) genes, indicating that *intI1* could be a useful indicator of ARG presence but not for all the subtypes of genes.

The presence of ARGs also in the air is a recent awareness and, for this reason, the literature is still lacking, even if the interest is growing nowadays. Most of the study about the presence of antibiotic resistance in air are focused on highly polluted area like China and most of them are focused on metagenomic approach and for this reason, the direct comparison of the results of the present study with literature is difficult.

The ARG air pollution monitoring in the urban environments is of primary importance, since more than 4 billion people – more than half of the world – live in urban areas (World Bank, 2023) and they could be potentially exposed to ARG inhalation, beside other pollutants. In our study, the urban site did not represent the most ARG polluted site, and also the ARG variability detected was lower respect the other sites monitored. But this is not true for other world areas. In literature, the ARGs targeted and those reported to be most abundant were largely inconsistent across the studies, but some trends were observable (Kormos et al. 2022). *sul*I, *intI*1, certain β-lactam ARGs, and certain tetracycline ARGs were consistently dominant, which is not surprising because *sul*I, *intI*1, and *tet*W are well established as indicators of anthropogenic sources of antibiotic resistance. Surprisingly, *sul*I and *intI1* in the urban site monitored in the present study were never detected, this means the urban area specificity of the ARG pattern, and it is important also to underline that none of the studies included in the aforementioned review of the literature were conducted in Europe.

In relation to hospital sites, these environments were monitored with a metagenomic approach in different studies located in China (He et al., 2020; Wu et al., 2022) in which they found the abundance of ARGs generally at higher magnitude in comparison with urban ambient air $PM_{2.5}$. This is not the same trend observed in our study, since hospital and urban areas showed similar levels of ARG pollution. These discrepancies could be due to the different methodological approach used (in the present study we used a quantitative approach that is ddPCR) beside the different landscapes in which the hospitals were located: a megacity with high levels of air pollution *vs* a city with significant levels of pollution in Italy but not comparable to the pollution levels observed in China. Few studies evaluated this aspect with a qPCR approach finding results partially in accordance with the present study, i.e. *tet*W as the most abundant ARG in this setting (Kormos et al. 2022). Moreover, despite a direct comparison is not possible we found another result in contrast to previous research: in the study of Wang et al. (2019), among different sites monitored, they found that the hospital site has the highest ARG diversity, but in our study this characteristic can be attributed to livestock farm.

The livestock farms are characterised by the presence of animals and animal manure, that is considered as a major source of ARGs (Gao et al., 2018). The ARGs could spread, from animal manure to air through aerosolization and aerosolised ARGs can travel considerable distances and pose a potential risk for environment and human health, since the inhalation of aerosol represent an important pathway for exposure to ARGs and also pathogens (Létourneau et al., 2010). In the study by Gao et al. (2018), which assessed the abundance and diversity of antibiotic resistance genes in a composting plant using livestock manure, the highest concentration was found for the *intI1* gene. In contrast, the present study did not identify *intI1* as the gene with the highest concentration. Among other ARGs, tetracycline and sulphonamide resistance genes are ubiquitous in livestock manures (Qiao et al., 2018) and are detected at high levels in farms (Gao et al., 2017), and this is in accordance with our study even if at lower extent.

The other site that contributed significantly to the ARG load was the WWTP site. This reflects the ability well-known in literature of WWTPs to spread in the environment the antibiotic resistance (Miłobedzka et al., 2022). This characteristic is extensively studied in the treated wastewater, but the same aspect is less studied in the air surrounding the WWTPs. In the study of Gaviria-Figueroa et al. (2019), they evaluated ARGs in the aerosol of upwind and downwind samples near an activated sludge tank in a WWTP (located in the U.S.). They found the β-lactam resistance genes the most abundant in these samples, differently from our study in which the most abundant ARGs in WWTP site were *tet*A, *tet*W and *sul*II. Moreover, they found also that the downwind samples had similar ARG profiles as those generated from liquid activated sludge samples while the upwind profiles showed a distinct difference, this indicates a clear contribution of WWTP processes to ARG distribution in air. In the study of Yen et al. (2025), in which they investigated the resistome contained in the $PM_{2.5}$ collected above an aeration tank of a WWTP, they found *sul*I as the most abundant ARG, apart from multidrug resistance.

Regarding the ARG concentration, in the study of Ginn et al. (2021) they analysed with ddPCR specific ARGs dispersed near wastewater flows in urban area and found *intI1*, *tet*A and bla_{TEM} gene copies/m^3 significantly higher respect to our study. The same trend can be observed in the study of Gao et al. (2018) performed in a composting plant in China, since they found quantities of ARGs (among others, *intI1*, *tet*W, bla_{TEM}, *sul*I and *sul*II genes) significantly higher than those reported in our study, even in the office area.

The main reason for these discrepancies could be attributed to different site of sampling but mainly to the extremely low level of pollution detected respect to other world areas. Despite this, ARGs were present and detectable, and this aspect deserve greater attention.

2.5.4. Conclusions

This study demonstrated that the atmosphere represents a significant and underexplored reservoir of ARGs, especially the air surrounding farm and WWTP.

There is a pressing need for research focused on developing a quantitative comprehension of ARG dissemination within the atmosphere. The preliminary results obtained in the present work respond to one of the concerns raised in the literature review by Kormos and colleagues (2022): i.e., most studies reported ARGs in terms of relative abundance through metagenomics, which is helpful for identifying "hot spots" but concentration data (ARGs per volume of air) are needed to inform emission and exposure rates.

Both approaches are fundamental since they give complementary information and, for this reason, the same samples analysed in the present study will be undergone also to DNA sequencing in order to deeply investigate the bacterial population composition.

An integrated approach to linking diverse ARG data will be instrumental in mitigating the burden of antibiotic resistance, ultimately promoting the health of humans, animals, and the environment, according with the One Health approach.

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3. *Pathogens*

3.1. *Forth in draft manuscript: Detection of Legionella spp. and Legionella pneumophila in water intended for reuse*

3.1.1. Introduction

The genus *Legionella* consists of facultative intracellular Gram-negative bacteria commonly found in both natural aquatic environments and anthropogenic aquatic environments (e.g., pipelines, rainwater storage tanks, water cisterns, aeration systems, etc.). Approximately 60 different species have been identified, over 20 of which are classified as human pathogens. The species of greatest relevance in healthcare is *Legionella pneumophila*, which is further divided into 15 serogroups with varying pathogenic characteristics. The most dangerous serogroup of *L. pneumophila* for humans is serogroup 1 (SG1), responsible for more than 90% of confirmed cases of Legionnaires' disease (LD) and 10–15% of the mortality associated with it (Zayed et al., 2020).

Infections caused by *Legionella* develop exclusively through the inhalation of aerosolized particles that penetrate the lungs, attacking lung tissue and producing various outcomes depending on the host's health condition. The most severe cases develop LD, which manifests as severe pneumonia and can lead to death in vulnerable individuals, while milder cases develop Pontiac fever, a set of flu-like symptoms that usually do not require hospitalisation (Blanky et al., 2017).

According to a report published by the European Centre for Disease Prevention and Control (ECDC) in 2023, over 10,700 cases of LD were recorded in Europe in 2021, an increase compared to previous years, but consistent with epidemiological projections. Most cases were reported in France, Spain, Italy, and Germany (75% of total cases), with over 2,700 cases (4.6 cases per 100,000 inhabitants) in Italy (ECDC, 2023). Individuals most vulnerable to developing severe forms are males over 65 years of age, smokers, and those with other underlying conditions, such as diabetes (Maisa et al., 2015). Furthermore, diseases caused by *Legionella* follow a seasonal pattern, with higher incidence during the summer due to increased temperatures and humidity, which are key factors for the growth of this organism.

Legionella is a ubiquitous pathogenic microorganism widely distributed in aquatic environments. From natural reservoirs (spring and thermal waters, surface waters), these pathogens can spread and proliferate in artificial aquatic environments, such as water distribution systems and water storage structures in large buildings (e.g., hospitals, hotels), cooling towers, etc., creating a potential health risk. However, there is limited information regarding the presence of *Legionella* spp. in non-potable water sources, which can still be an important source of contamination, such as wastewater. Some studies have shown that in wastewater treatment, specifically in biological oxidation tanks, microclimatic conditions may develop that could favour the growth and development of *Legionella* (Garner et al., 2018; Kulkarni et al., 2018; Caicedo et al., 2019).

Additionally, it has been observed that some forms of *Legionella* can resist standard disinfection methods using chlorine-based products commonly used in wastewater treatment plants (Lesnik et al., 2016). Studying how *Legionella* spp. proliferates and persists in wastewater treatment plants is therefore essential for developing effective preventive and control strategies. The importance of the *Legionella* spp. detection in treated wastewater is further emphasized by the fact that effluents may be reused for irrigation, which could pose a health risk due to the potential inhalation of aerosolized water (Caicedo et al., 2019).

With these premises, it is crucial to pay more attention to the evaluation of health risks posed by these microorganisms and to the pathogen removal capacity in treatment plants, in order to identify potential critical issues, particularly with respect to promoting the reuse of effluents. Recent European regulations (EU Regulation 2020/741) and Italian regulations (Legislative Decree 39/2023) focus on this aspect by introducing the evaluation of *Legionella* as one of the minimum parameters required for water reuse. However, these evaluations currently only involve a quantitative assessment (CFU/L) of *Legionella* spp. in treated WWTP effluent using culture-based methods and do not include the use of molecular methods.

For these reasons, the aim of the present research work is to assess the presence of *Legionella* spp. and *L. pneumophila* at the influent, during the various stages of treatment, and at the effluent of a WWTP producing effluent for agricultural reuse, by comparing the results obtained using traditional culture-based methods and molecular methods (qPCR). Studying how *Legionella* spp. proliferates and persists in WWTP is important for developing effective preventive and control strategies. It is equally important to assess the risks associated with the presence of this pathogen in the effluent in case of agricultural reuse, to safeguard the health of consumers and workers (Caicedo et al., 2019).

3.1.2. Materials and methods

3.1.2.1. Sample collection

The investigated WWTP was the same of the first published manuscript (paragraph 2.1.). The samplings were performed as described in 2.1.3.1. paragraph. Briefly, the wastewater treatment plant (WWTP) serves 280,000 population equivalent (p.e.) and processes 19 million m^3 per year, with 5.5 million m^3 annually reused for agricultural irrigation. The influent consists of mixed wastewater, with industrial effluent contributing 10% of the total. Upon entry, the wastewater is split into three treatment lines, with the third line being the largest, handling about 40% of the flow, until secondary treatment. Afterward, the three lines are merged into one, producing the WWTP effluent, which undergoes further treatments (sand filtration, hydrogen peroxide -H_2O_2, and UV treatment) to make it suitable for reuse.

In this study, six distinct 24-hour composite samples were collected at the following stages: A (WWTP influent); B (primary treatment inlet); C (biological treatment inlet); D (secondary treatment effluent); E (WWTP effluent); F (effluent for agricultural reuse). The B, C, and D samples correspond to the third treatment line. The samples were collected during six monthly sessions (January–June 2022) (n=36 samples). A 2 L volume of each sample was taken from each point, transported under refrigeration to the laboratory, and processed within 24 hours.

3.1.2.2. Culture-dependent method

For the determination of *Legionella* spp. and *Legionella pneumophila*, the culture method described in the ISO 11731:2017 standard "Water quality – Enumeration of *Legionella*" was used.

As the first step, the samples from the effluent for reuse (F) and from D and E were concentrated by centrifugation at 3,000 g for 30 seconds, repeated twice consecutively. Each sample was then divided into two aliquots, one untreated (NT) and one heat-treated (T). The heat treatment was performed at 50°C for 30 minutes, to reduce the presence of microorganisms that could interfere with the growth of *Legionella*. Each aliquot was then used to serial dilutions.

For each dilution, 500 µL of the sample was inoculated onto GVPC Agar (Glycine Vancomycin Polymyxin Cycloheximide). The plates were incubated at 36 ± 2°C for 10 days, after which colony counts were performed. *Legionella* appears as circular colonies that are grey or white, although colour variations may occur, such as blue, purple, yellow, green, or pink. Colonies with the typical morphology were isolated by inoculating the same colony onto BCYE agar plates without L-cysteine and BCYE agar plates with L-cysteine. All plates were incubated at 36 ± 2°C for 10 days. This procedure is performed because *Legionella* is a cysteine-dependent microorganism, meaning it requires the presence of L-cysteine for growth; therefore, it only grows on media containing L-cysteine. Colonies that grew only on plates with L-cysteine were confirmed biochemically using a latex agglutination test (Legionella Latex Test, Oxoid), which can distinguish *Legionella pneumophila* colonies in various serogroups from those of *Legionella* spp. The results are expressed as CFU/L.

3.1.2.3. Culture-independent method

For molecular analysis, different volumes of the sample (ranging from 20 to 200 mL) were filtered onto polycarbonate filters with a porosity of 0.22 µm. The filters were stored at -20°C until DNA extraction, which was performed using the DNeasy PowerWater Kit (Qiagen) according to manufacturer's instructions.

For the detection of *Legionella* spp. and *Legionella pneumophila* DNA, a Real-Time PCR (Quantitative PCR or qPCR) was performed using fluorescent hybridization probes. The target genes used were 16S rRNA for *Legionella* spp. and the *mip* gene for *L. pneumophila*, which is involved in the virulence, infection, and life cycle of the bacterium (Shevchuk et al., 2011).

For Real-Time PCR, the iQ-Check® Quanti *Legionella* spp. Real-Time PCR Quantification Kit and iQ-Check® Quanti *L. pneumophila* Real-Time PCR Quantification Kit (BioRad) were used. A mix containing the amplification solution (primers, dNTP, Taq polymerase, $MgCl_2$, PCR buffer, and UDG), fluorescent probes, and an internal plasmid DNA control was prepared. A total of 45 µL of this mix was distributed into the wells of the plate, and 5 µL of the previously extracted and diluted DNA or standards (Qs1, Qs2, Qs3, and Qs4) containing serial dilutions of the target DNA genomic units were added, along with a blank (sterile water).

The amplification reaction was performed using the CFX 96 C1000 instrument (Biorad), and the results were interpreted using the quantification curve based on the four standards. The results were expressed in genomic units (GU) per liter of sample (GU/L).

3.1.2.4. Statistical analysis

Statistical analysis was performed using the SPSS software (version 28.0.0). Data distribution was assessed using the Shapiro-Wilk normality test. To evaluate potential differences in contamination levels observed at different stages of the treatment process, a one-way ANOVA test followed by the Tukey post-hoc test for normally distributed data was applied, and the Kruskal-Wallis test for non-normally distributed data. P was considered significant when < 0.05.

3.1.3. Results and discussion

The culture-dependent method for *Legionella* detection revealed the presence of *Legionella pneumophila* SG 2-14 only in one influent (A) sample (5.7 log CFU/L). No other colonies attributed to *Legionella* spp. or *L. pneumophila* were detected in any of the other analysed samples.

Low percentages of *Legionella* positivity have also been reported in other studies using the culture method in wastewater treatment plants. In a study by Bonetta et al., conducted to assess the abatement efficiency of three Italian WWTPs, the culture-dependent method monitored *Legionella* spp. in just one influent sample (Bonetta et al., 2022). Similar results were found by Medema et al., who did not detect *Legionella* in any of the effluent samples analysed (Medema et al., 2004). Likewise, in the study by Caicedo et al., which evaluated the presence of *Legionella* in the secondary treatment tanks of various WWTPs, no positivity for *Legionella* spp. or *L. pneumophila* was found in any of the samples analysed (Caicedo et al., 2016). Lund et al. observed a low positivity rate for *Legionella* (3%) using the culture-dependent method, even in samples from Norwegian WWTPs (Lund et al., 2014).

In contrast, the culture-independent analysis (molecular analysis performed with qPCR) detected the presence of *Legionella* DNA at various stages of wastewater treatment (Fig. 1).

Fig.1 shows the average concentrations obtained using the molecular method for *Legionella* spp. and *Legionella pneumophila* in the influent wastewater samples, the effluent samples, and at different stages of the treatment process.

Specifically, all analysed samples tested positive for *Legionella* spp., and 66% of the samples were also positive for *Legionella pneumophila* (50% of the A samples, 67% of the B samples, 83% of the C samples, 83% of the D samples, 100% of the E samples, and 17% of the F samples).

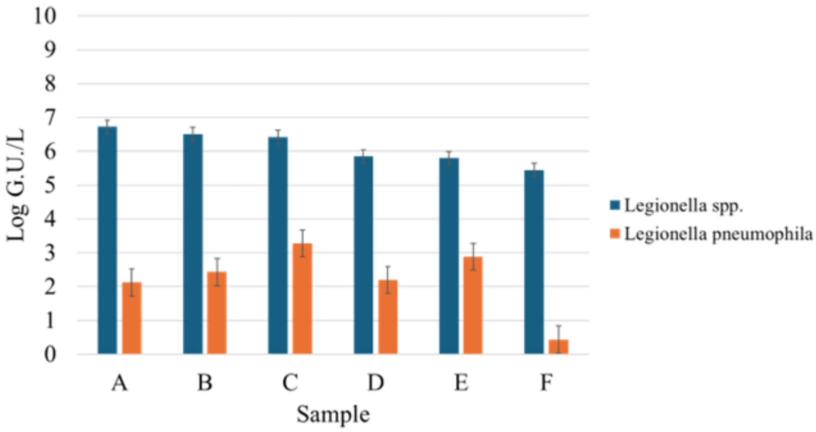

Fig. 1. Results obtained from the molecular analyses performed to detect *Legionella* spp. and *L. pneumophila* at the different sampling points; results are expressed as the logarithm of genomic units per liter of sample (log GU/L). A (WWTP influent); B (primary treatment inlet); C (biological treatment inlet); D (secondary treatment effluent); E (WWTP effluent); F (WWTP effluent for agricultural reuse).

The positivity rates found using the molecular method in this study is in agreement with those observed in other studies. In the study by Bonetta et al., the molecular method identified *Legionella* spp. in 100% of pre- and post-treatment samples from all monitored plants, while *L. pneumophila* was found in 57% to 100% of pre-treatment samples and in 0% to 50% of post-treatment samples (Bonetta et al., 2022). Medema and colleagues detected the presence of *Legionella* spp. in 100% of the analysed samples (Medema et al., 2004) and Caicedo et al. identified *Legionella* spp. in 20% to 80% of the samples from the WWTPs investigated (Caicedo et al., 2016). Lund and colleagues also found *Legionella* spp. and *L. pneumophila* in 98% and 30% of the analysed samples, respectively (Lund et al., 2014).

As observed from the results, the positivity rates obtained using the culture-independent method were significantly different from those obtained with the culture-dependent method. As highlighted in other studies, the culture-dependent method can underestimate the *Legionella* presence in wastewater, while the culture-independent method, conversely, can overestimate it. Several factors can contribute to the underestimation of *Legionella* detection using the cultural method, including the presence of abundant or competitive bacterial flora that inhibits microorganism growth, and the inability of the method to detect viable but non-culturable (VBNC) forms. The ability of *Legionella* to survive stressful conditions by entering a VBNC state, during which it exhibits low metabolic activity and cannot reproduce, is well-documented in the literature. A factor contributing to the transition to the VBNC form is exposure to high temperatures (50°C), which are used in treatments defined by ISO 11731:2017 (the reference standard for *Legionella* quantification in water) in the preparation of samples, necessary to reduce interfering flora. Additionally, the cultural method faces challenges in obtaining reproducible results (Caicedo et al., 2019).

Culture-independent techniques (qPCR), on the other hand, can overestimate health risks because they detect the genetic material of dead or damaged microorganisms, which are not capable of causing disease. These differences between culture-dependent -independent methods have been highlighted in several studies. In the Netherlands, all raw effluent samples analysed by qPCR (n = 7) resulted contaminated by *Legionella* spp., whereas the culture-dependent method never detected this microorganism; this was attributed to the growth of interfering flora on the BCYE agar plates used for analysis (Medema et al., 2004). Similar issues were observed in the monitoring of three active sludge plants in Germany receiving domestic and/or industrial effluents, due to the growth of contaminating flora on the GVPC agar plates (Caicedo et al., 2016). Similar results were also observed in the studies by Bonetta et al. (Bonetta et al., 2022) and Lund et al. (Lund et al., 2014).

Regarding *Legionella* spp., the contamination levels ranged from 5.02 log G.U./L to 7.25 log G.U./L. The highest value was found in an influent sample (B), while the lowest value was detected in an effluent sample intended for reuse (F) (Fig. 1).

The levels of *Legionella* spp. recorded at the influent of the treatment plant (A) were similar to those observed by Bonetta et al., who detected maximum concentrations of 7.2 log G.U./L at the influent of an Italian wastewater treatment plant (Bonetta et al., 2022). Lower contamination levels were found in the study by Caicedo et al., who observed maximum concentrations of 3.5 log G.U./L at the influent of three German wastewater treatment plants (Caicedo et al., 2016).

The *Legionella* spp. levels monitored in the effluent samples from the WWTP were similar to those reported by Bonetta et al., who found values of 5.1 log G.U./L at an Italian treatment plant (Bonetta et al., 2022). Higher concentrations of *Legionella* spp. were observed in the effluents of two Norwegian treatment plants (6.9 and 9.1 log G.U./L) (Lund et al., 2014) and in the effluents of a treatment plant in France (range 6–7.5 log UG/L) (Brissaud et al., 2008). Lower values were recorded in the effluents of the treatment plants analysed by Caicedo's group, with a maximum concentration of 3.4 log. A possible explanation for these differences could be related to the type of treatment applied: the wastewater treatment plants analysed by Caicedo et al. (Caicedo et al., 2016) perform more advanced treatments compared to those in the other studies, and consequently, they appear to achieve a higher reduction in *Legionella* concentrations.

In our study, the influent samples (A) and those collected during the early stages of wastewater treatment (B and C) showed statistically significant higher levels of *Legionella* spp. contamination compared to the effluent samples (D, E and F) ($p < 0.05$). The data indicated a significant reduction in contamination starting from the effluent samples after secondary sedimentation, whereas no significant reduction was observed during the early stages of the treatment process or between the effluent samples (E – effluent vs F – reuse effluent). This trend highlights that secondary treatments are necessary to reduce *Legionella* spp. contamination, while tertiary treatments, although required for achieving high-quality effluent, do not appear to significantly reduce the contamination levels.

Furthermore, the results showed that none of the intermediate stages of treatment seemed to lead to a significant increase in *Legionella* spp. concentration. This finding contrasts with some studies in the literature, although knowledge on the progression of *Legionella* contamination levels in relation to the treatment process stages is still limited. In the study by Caicedo et al., a significant increase in the presence of *Legionella* was observed at the end of the secondary treatments (Caicedo et al., 2016). Similarly, the Kulkarni group observed an increase in

Legionella during the intermediate stages of the treatment process, with concentrations higher than those in the influent samples (Kulkarni et al., 2018). An increase in *Legionella* concentration after primary and secondary treatments was also highlighted in the study by Palmer et al. (Palmer et al., 1993).

The differences observed between the results of this study and those reported in other works could be partly related to the type of treatment used in the intermediate stages, but also to the physicochemical parameters characterising the process and the effluent (e.g., pH, temperature) as well as the microbiological characteristics of the effluent. In fact, some studies have highlighted that high microbial load, temperatures between 30 and 40°C, and high contamination by protozoa and amoebae in bacterial biofilms may create ideal conditions for the proliferation of *Legionella* (Caicedo et al., 2018).

Regarding *L. pneumophila*, the contamination level was rather low in all stages of the treatment plant (Fig. 1). In fact, the positive samples showed concentrations always below the method quantification limit. These results align with those observed in other studies. Brissaud et al. detected *L. pneumophila* values below the quantification limit in most of the samples analysed (Brissaud et al., 2008). Similar results were obtained by Bonetta et al., who observed values below the quantification limit in all samples that tested positive for the pathogen (17 out of 21 influent samples and 4 out of 21 effluent samples) (Bonetta et al., 2022). In the study by Lund et al., analysing effluents from Norwegian treatment plants, the concentrations of *L. pneumophila* were above the quantification limit but still relatively low, ranging from 2.4 to 5.3 log G.U./L (Lund et al., 2014).

Regarding the contamination levels observed at different treatment stages, statistical analysis showed a significant decrease in contamination only for the effluent samples treated for reuse (F), while no significant differences in contamination were observed between the other stages of the treatment plant. The positives found in the various samples were also sporadic, especially at certain points, making it difficult to trace a clear trend during the treatment process, in contrast to *Legionella* spp., which was present in all the analysed samples.
These results are consistent with those observed in other studies. The Kulkarni group, in fact, observed a significant reduction in the concentration of *L. pneumophila* only in the effluent samples from the treatment plant (Kulkarni et al., 2018). Similarly, in the study by Caicedo et al., a significant reduction in *L. pneumophila* concentration was observed in the final stages of the treatment process, although the values were still higher than those recorded in this study (Caicedo et al., 2016).

For the purposes of wastewater reuse, current regulations (EU Regulation 741/2020) require the detection of *Legionella* to define the quality of the effluent. According to the regulations, the maximum allowable concentration of *Legionella* spp. in treated wastewater intended for reuse is 1,000 CFU/L (3 log CFU/L). The results of our study, based on culture-based analysis, show the presence of *Legionella* colonies in only one influent sample, indicating that the treated effluent is suitable for irrigation uses, including spraying. However, the high average concentrations of *Legionella* spp. detected by qPCR in the effluent samples, although partially attributable to the potential presence of non-viable microorganisms, necessitate further investigation if the treated effluent is to be used in agriculture.

Additionally, since current regulations require the detection of *Legionella* using the culture method, future studies should also address methodological issues related to the detection of *Legionella* spp. This will help better understand the reasons for the significant disparity observed between the results obtained with the two methods and prevent underestimating the health risks associated with the presence of this pathogen in treated wastewater intended for reuse.

3.1.4. Conclusions

One of the possible solutions to water shortage induced by climate change could be the reuse of treated wastewater, a technique that presents numerous advantages, both economic and environmental, but also various critical issues to consider, particularly from a Public Health perspective (Mishra et al., 2023). In addition to the chemical issues, it is undeniable that potential risks also include those of microbial origin. Indeed, if treated effluents are reused for agricultural purposes, it is necessary to consider that the irrigation method used (e.g., spray irrigation) could promote the dispersion of aerosols containing pathogens such as *Legionella*. Although it is known that water can be an important source of *Legionella*, only a few studies have focused on the presence of this bacterium in WWTPs. The results obtained in this study showed that none of the stages of the investigated wastewater treatment plant seem to promote the proliferation of *Legionella*. However, considering that the chemical-physical parameters characterising the process and the effluent (pH, temperature), as well as the microbiological characteristics of the effluent, may favour *Legionella* growth in certain treatment stages, there is a need to further investigate how *Legionella* spp. proliferates and persists in wastewater treatment plants, with the aim of developing effective preventive and control strategies. Regarding the possible reuse of treated wastewater, the effluent samples, analysed using the cultural method as required by current regulations, were found to be suitable for irrigation uses, including spray irrigation. However, the presence of high concentrations of *Legionella* spp. DNA detected by qPCR in the same samples raises the need for further investigation. Moreover, since current regulations require the assessment of *Legionella* presence solely through the cultural method, it will also be necessary to further explore the methodological issues related to *Legionella* spp. detection to avoid underestimating health risks to the population due to the presence of this pathogen in refined water sent for reuse.

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3.2. *Fifth published manuscript: Impact of wastewater treatment and drought in an Alpine region: a multidisciplinary case study*

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Research article

Impact of wastewater treatment and drought in an Alpine region: a multidisciplinary case study

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3.2.1. Abstract

In the context of global climate change, drought occurrence in streams of alpine origin is a recent phenomenon, whose impact is still poorly investigated. In this study, we adopted a three-disciplinary approach to investigate the response of an Alpine river (NW Italy) to severe drought conditions occurred in the year 2022. We hypothesised that the considerable loss in the water flow could exacerbate wastewater treatment plant (WWTP) discharge effects, lowering dilution capacity of the stream system and then increasing chemical/microbial pollution and altering benthic community characteristics. To assess river response to drought conditions of the considered year, the concentration of the main chemical variables, faecal indicator bacteria, pathogen presence and structural/functional organisation of benthic macroinvertebrates and diatom communities were measured monthly in the reaches located upstream and downstream of a WWTP (January–December 2022). Main environmental variables, such as flow velocity, water depth, and flow regime, were also considered. A multivariate analysis approach was then applied to emphasise correlations between selected variables and flow regime. Comparing upstream and downstream sections over the considered year, a common behaviour of chemical/microbiological parameters was observed, with generally higher concentrations of nutrients and bacterial indicators downstream of the local WWTP. Moreover, a positive correlation between water scarcity and nutrients/bacterial concentrations was revealed. The macro-invertebrate communities responded accordingly, both in terms of density and biological metric shift. Interestingly, differences between the two sections were strictly associated with hydrological conditions, with higher dissimilarities found in low-flow conditions. As the magnitude and duration of drought events are projected to increase in the years to come in different parts of Europe, this work can serve as a first building block and as a hint for future

studies aimed at improving our knowledge about the consequences of these events that is pivotal for planning effective management strategies.

3.2.2. Introduction

Climate change is causing significant alterations in natural ecosystems due to global rising temperatures and hydrological regime disruption. These have a profound impact on water resources, affecting both their quantity and quality. The IPCC's Climate Change 2022 Synthesis Report highlights the moderate level of confidence in the observed changes in precipitation patterns and glacier melting, which in turn influence water runoff and hydrological regimes [1]. Rivers represent the most dynamic, pivotal and biodiverse freshwater aquatic environments, but unfortunately, they are also among the most modified and threatened ecosystems in the world [2]. Previous studies highlighted the impact of climate change on river regimes, underlining how alterations in stream flow induced by climate change might vary regionally [3]. In particular, the biodiversity and integrity of riverine ecosystems depend on natural flow regimes [4]. Changes in flow regime can lead to dramatic consequences for stream ecosystems, such as biomass and biodiversity collapse, local extinction, and the invasion of exotic species, particularly for alpine aquatic environments [5]. Indeed, alpine aquatic environments are extremely fragile systems to multiple stressors [6]. Rivers of alpine origin are characterised by a typical hydrological regime in which the flow rate varies significantly throughout the year. During the warmer months, melting snow and ice contribute to increased flow, while in the winter months, the flow may decrease due to reduced melting and possible freezing. This natural regime could be altered by diminution of snowfalls or alteration of snowmelt, that are direct consequences of climate change. In the context of this rapid climate change, point sources of contaminants can play an important role. In particular, the impact of wastewater treatment plants (WWTPs) may be exacerbated by climate change as a consequence of the lower water flow, resulting in a loss of dilution and self-purification capacity of the river. As an example, in 2022 different European countries experienced a series of severe droughts, which were particularly evident in the Po basin, northern Italy [7]. The drought was exacerbated by a persistent sea level pressure anomaly dipole over north-western and south-eastern Europe from November 2021 to March 2022 which blocked the transit of Atlantic perturbations in the Mediterranean area and resulted in a negative rainfall anomaly in central and northern Italy. In general, in literature it is known that there is a relation between water quality in river segments and its dilution capacity especially in Mediterranean rivers [8–10], but the Alpine region was poorly investigated. Flow reduction essentially means a collapse or loss of wet surface and fast-flowing water environments, with the consequent depletion or disappearance of many habitats and the species that populate them [11]. Much more neglected is the aspect linked to the impact of water shortages on water quality. In this work, which is to be intended as a preliminary study, we hypothesised that water shortages may worsen the chemical and microbiological quality of rivers, leading to significant biodiversity loss. To test this hypothesis, we examined a river stretch affected by an anthropic discharge for one year, analysing changes in water quality and aquatic benthic communities both upstream and downstream of a WWTP. Our ultimate aim was to investigate, with an interdisciplinary approach, the modifications occurring in an alpine river during an important period of drought giving insights into the relationship between the chemical, microbiological, and ecological parameters and the flow conditions. To our knowledge, this is the first multidisciplinary study in which the impact of WWTP effluent discharge is evaluated in relation to water shortage induced by climate change in an alpine river. It must be underlined that, being carried out on a single river and in the time span of the year 2022 only, this study stands out as a circumscribed description of the response of a stream subjected to water stress conditions, and is meant to be a first step in the understanding of a broader phenomenon requiring more time and more extensive datasets to be fully assessed.

3.2.3. Materials and methods

3.2.3.1. Study area and hydrological description

2022 has been one of the driest and hottest years ever recorded in the western Alpine area, resulting in severe runoff deficit in most of the lotic systems [7]. The study was conducted in the Stura di Lanzo (ST) river, a 3rd order lotic system and a tributary of the Po river in Piedmont region, NW Italy. The river has a torrential pluvial-nival hydrological regime and, according to the Regional Agency for the Environment Protection (ARPA) 2021 data, its average annual flow rate at the mouth is 23.36 m^3/s. Like in many other Alpine basins, in the last years this stream experienced long periods of reduced flows alternating with short but often intense floods [12]. According to the data collected directly from ARPA Piemonte, in January the Stura di Lanzo showed a reduced runoff (6.17 m^3/s), and the situation worsened in February and March where flow rates resulted markedly below the average, reaching a deficit of − 60 % (4.88 m^3/s). This trend continued over the year and characterised not only this lotic system, but the entire hydrographic network. In December the average inflow (5.53 m^3/s) was far above the reference average conditions and the overall deficit of the largest river in this area, the Po, was 67 % lower than the historical average value. We focused on a ST river reach characterised by the presence of 12,000 p.e. (population equivalents) WWTP which employs biological processes and secondary sedimentation and treats mainly urban wastewater.

3.2.3.2. Sampling

From January to December 2022, twelve sampling campaigns were carried out monthly. On the studied section of the river (850 m long), three sites of sampling were defined (Fig. 1). The first one located 200 m upstream of the discharge (U), the second one located directly on the WWTP discharge (W) and the last one located 500 m downstream of the discharge (D).

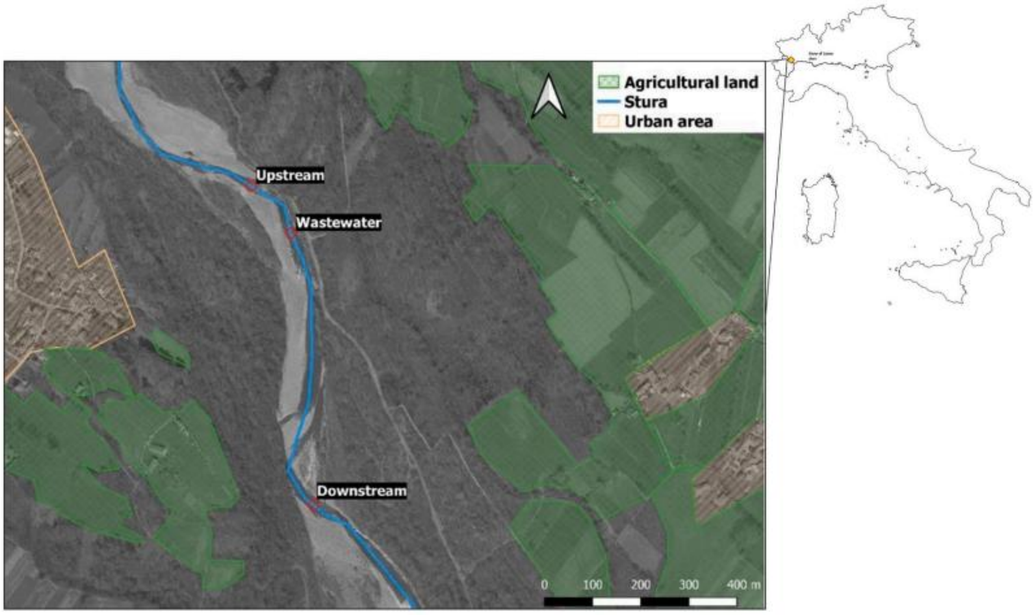

Fig. 1. Location of the Stura River (NW Italy) and position of the three sampling sites. Upstream (U - coordinates 45°13′23.0″ N, 7°33′42.2″ E), WWTP discharge (W - coordinates 45°13′18.6″ N, 7°33′43.7″ E) and downstream (D - coordinates 45°13′01.0″ N, 7°33′48.2″ E).

3.2.3.3. Determination of chemical parameters

The details of the reagents used for the analytical determination, as well as those regarding the treatment of the samples, are fully reported in paragraph S1 of the Supplementary Material (hereafter SM). Temperature (T, °C), electrical conductivity (EC, $\mu S\ cm^{-1}$), dissolved oxygen concentration (DO, $mg\ L^{-1}$), oxygen saturation (DO%) and pH were measured at each sampling point with a multiparametric probe (Scubla WTW Multi 3430). The concentrations of anions (Cl^-, PO_4^{3-}, SO_4^{2-}) and cations (Na^+, K^+, Mg^{2+}, Ca^{2+}) were determined by ion chromatography, while Total Carbon (TC), Inorganic Carbon (IC) and Total Nitrogen (TN) were determined using a TOC analyser. The instruments and the working conditions are listed in paragraph S2. The soluble reactive phosphorus (SRP) was measured spectrophotometrically, using the method described in a previous study [13]. Nitrites, ammonia, total phosphorus, silica and anionic surfactants were determined spectrophotometrically with a Cary 100 Scan spectrophotometer (quartz cells, pathlength 1 cm) according to the methods described in paragraph S3, and previously reported [14]. The volumetric contribution of the WWTP effluent to the stream flow (Y, in %) could not be directly measured, but it was estimated according to the calculations reported in paragraph S4.

3.2.3.4. Determination of microbiological parameters

For the microbiological analysis, 2 L from each sampling point were collected, transported refrigerated and processed within 24 h of sampling. All samples were evaluated for faecal indicator parameters (total coliform, enterococci, *Escherichia coli* and *Clostridium perfringens* spore counts) and pathogens (*Salmonella* spp. and verocytotoxin-producing *E. coli* - VTEC). Quanti-TrayTM 2000 (IDEXX Laboratories, Milan, Italy) was used for the quantification of coliforms, enterococci and *E. coli*, and the results were expressed as Log Most Probable Number (MPN)/100 mL. The enumeration of *C. perfringens* spores was performed using a membrane filtration method according to ISO 14189:2013 (International Standards

Organisation, 2013) [15], and the results were reported as Log Colony-Forming Unit (CFU)/100 mL. The presence/absence of *Salmonella* spp. and VTEC DNA in water samples was evaluated using a PCR method with a previously reported protocol [16,17].

3.2.3.5. Determination of benthic macroinvertebrates and diatoms

To assess biological diversity and richness we focused on two benthic communities, i.e. macroinvertebrates and diatoms, because of their importance in ecological functional processes and diffuse use in biomonitoring methods. Regarding macroinvertebrates, five samples were collected monthly with a Surber net (600 µm mesh size; 0.05 m^2 area) randomLy in riffle (i.e. erosive) habitats far away from each other approximately 5 m in both the U and D stretches, then preserved into plastic jars with 75 % ethanol until identification (see paragraph S5). For phytobenthos, samplings were carried out following the macrobenthic sampling schedule. Briefly, diatom samples were collected, treated and analysed following the standard procedures [18,19]. For each sample we calculated the Inter calibration Common Metric index (ICMi) [20], the diatom quality index adopted at national scale in the framework of the WFD application, by using the OMNIDIA 6.1.5 software. Ecological guilds (i.e. low profile, high profile, motile and planktonic) were assigned to each taxon basing on the classification proposed in a previous study [21]. Details concerning sampling, treatment and analyses of diatom samples are reported in the Supplementary Material (see paragraph S5).

3.2.3.6. Statistical analysis

For chemical parameters, linear and non-linear fits were carried out with the Origin(Pro) 8.5 software package (OriginLab Corporation, Northampton, MA, USA). The dataset was centred, autoscaled and then subjected to PCA to get insights into possible correlations among the measured chemical parameters in the U and in the D sites, the chemical-physical parameters (pH, T, EC, %DO, ppm DO) and the daily flow regimes (Qday). The Principal Components analysis (PCA) was carried out using the software Chemometric Agile Tool - CAT [22] and the significance of the correlations was assessed using the Pearson's test. For microbiological parameters, the presence/absence of pathogens and concentration of bacterial indicators (log conversion) were statistically analysed with IBM SPSS Statistics version 28.0 for Windows. The data distribution was evaluated using the Shapiro-Wilk's test. According to non-gaussian distribution, to analyse the differences in microbial indicators between U, W and D samples, a Kruskal Wallis' test was applied. The correlation between microbial indicators and river flow was analysed with Spearman's correlation. The relationship between presence/absence of pathogens and river flow was evaluated with binary logistic regression. Significance was evaluated with 95 % confidence intervals ($p < 0.05$). Regarding macroinvertebrates and diatoms, analyses of abundance data were conducted in the R Environment (R Development Core Team, 2020) [23]. Concerning benthic macroinvertebrates, each Surber sample was considered as a replicate, and prior to performing regression models, outliers were removed using the method for data exploration reported in a previous study [24]. Differences in the community composition between sites (U, D) were investigated by using non-metric multidimensional scaling (NMDS), multivariate analysis of variance (PERMANOVA) and PCoA (see details in the supplementary material). Statistical differences in community composition associated with variation of flow in different months (Q), site (U, D and also W for diatoms) and their interaction were tested via permutational multivariate analysis of variance

(PERMANOVA), with Bray-Curtis distance, using the 'ADONIS' function in the vegan package [25]. Analysis on specific taxa importance was quantified using the similarity percentage procedure (SIMPER) [26]. Moreover, a generalised additive model (GAMs) was used to assess the influence of flow variations on selected biological parameters. Further information on the statistics applied is reported in paragraph S5.

3.2.4. Results and discussion

3.2.4.1. Chemical parameters

The water temperatures ranged from 4–5 °C in winter to 21–26 °C in summer (see SM, Fig. S1). Stream flow (Q) varied with seasons: in spring months, the registered values were up to three times higher than in winter and two times higher than in summer. EC followed the inverse pattern of Q, falling to lower values in spring and early summer. As shown in Fig. S2, the concentrations of all the measured parameters in the downstream samples were generally higher than the concentrations in the upstream ones, pointing out the role of the inputs from the WWTP in delivering extra-nutrient loads in the stream. Concentrations in the effluents were remarkably higher for almost all the measured parameters, with the few exceptions of Ca^{2+}, Mg^{2+}, SiO_2 and for $N–NO_3$ in the period from January to June. In some cases, the differences in the nutrient loads U vs D were consistent (e.g. $N–NO_2$, $N–NO_3$, $N–NH_3$, Fig. 2a-b-c). Though seasonal trends could not be observed, the differences between U and D tended to become more pronounced in the months associated with lower flow regimes (January–February and July–September), as a consequence of the less dilution power of the water body. Exceptions to this were represented by Mg^{2+}, Ca^{2+}, SiO_2 and IC (Fig. 2d–e), whose difference between U and D (ΔUD) remained negligible through the year, and by species as SO_4^{2-} (Fig. 2f), K^+ and Cl^-, whose ΔUD kept constant month by month. Stream and effluent may differ in nutrient composition, for example in the case of nitrogen: the nitrogen content was mainly represented by $N–NO_3$ in the stream and by $N–NH_3$ in the effluent. This data is coherent with a previous study on a pool of streams in the region of Catalonia, Spain [27], which, being dominated by Mediterranean climate, are typically subjected to summer and winter low flows and even flow intermittency. Furthermore, it is worth noting that $N–NH_3$ concentration in the stream tends to fall in the spring months (April–June) until reaching 0 in summer (July–August); meanwhile, $N–NO_3$ remains well present in the waters. The contribution of the WWTP effluent to the flow of the receiving stream was variable, from 35 % (Fig. S3), with no strict correlation to the period of the year, contrarily to what was previously observed in other studies [28,29]. This may be due to the fact that this stream, regardless of the severe drought conditions of 2022 and though displaying a highly variable hydrologic regime, is not an intermittent water course. Yet, it is true that the highest % values are shown in the driest months, with an average of 25.3 %: this is coherent with the increased nutrient concentration observed in the same period at U and D. The 79 % of June is likely to be biased by an extra addition of water in the WWTP effluent from a side canal, observed during this sampling and not present in the previous and following ones. For this reason, the estimated value for that month has been excluded from Fig. S3. Thus, the WWTP inputs become more impactful during low flows as the dilution capacity of the stream decreases, while during higher flows a major role in shaping the final chemistry of the receiving stream may be played by the upstream conditions. In order to investigate possible correlations between the measured concentration data and Qday, we performed a PCA on the U and D data. The W samples were excluded from the analysis, since the highest concentration values flattened the informativity (i.e., the relative differences) of the upstream and downstream. Fig. 3a shows the scores and the loading plot for PC1 vs PC2, which explain the 39.07 % and the 19.6 % of the total variance respectively. PC1 displays positive loadings for the daily flow and negative loadings for all the nutrients, except for SiO_2: this suggests a negative correlation between the hydrological and the chemical variables for the stream, implying that lower values of Qday positively affect the concentration of the nutrients in water at the downstream. This is particularly evident in those months associated with lower Qday values, January–March and November–December. On the other hand, the grouping of spring months in the same position as the Qday loading is evident. Thus, the PC1 axis emphasises the role of the flow regimes (and, as a consequence, of the dilution capacity of the stream) in contrasting the effect of WWTP effluents as point sources of pollution and chemical discontinuity. As further evidence, most of the measured parameters appear to be negatively correlated with Qday in the loading plot. In the case of TP, TC, TOC, IC, $SO_4^{\ 2-}$, Cl^-, Na^+, Mg^{2+} and Ca^{2+} the linear negative correlations are statistically significant (Pearson's test at 95 % confidence level, see Fig. S4). This result is consistent with a previous study [29] assessing the influence of WWTPs over nutrient uptake in intermittent watercourses. Moreover, as reported in Fig. 3b, the plot of PC1 vs PC2 showed a clear differentiation between the upstream and the downstream samples, which are distinctly clustered for positive and negative scores of PC1, respectively. The sample scores on PC1 support the hypothesis of the contribution of the WWTP effluents to the water quality downstream, which display higher nutrient loads. A positive correlation between Qday and the sampling sites is observed in the high-flow seasons, when the difference between U and D tends to be levelled.

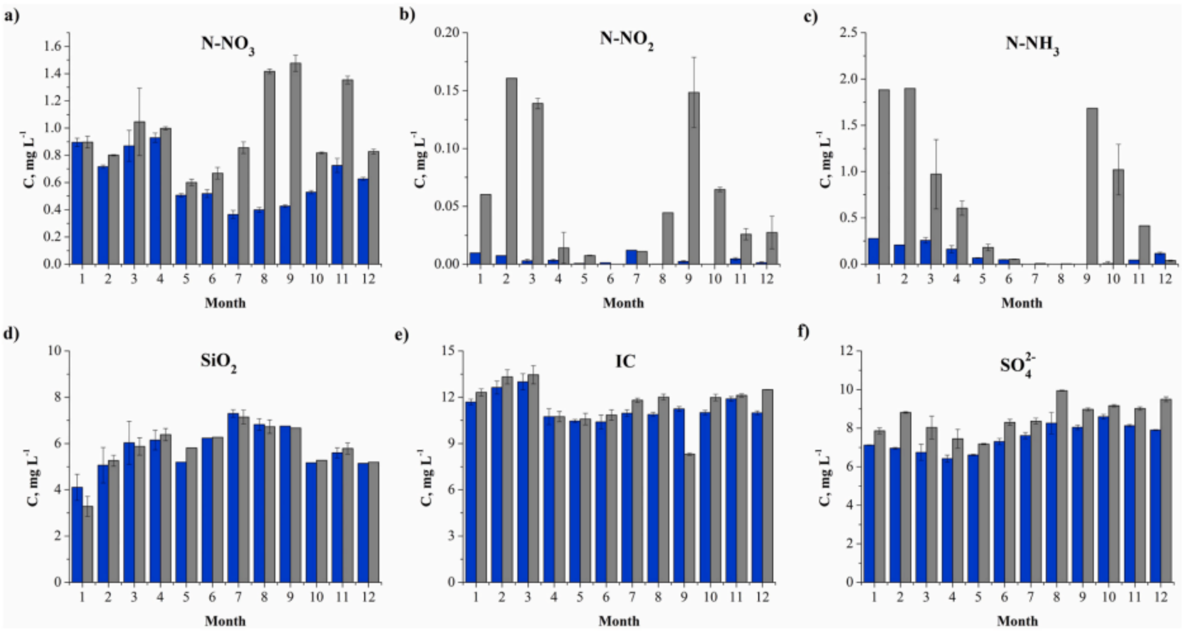

Fig. 2. Concentrations (C) of some of the chemical parameters measured in the Stura river through the year 2022. Upstream values are reported in blue, downstream values in grey. The waste values have been omitted to appreciate the marked or less marked differences between the U and D concentrations.

IC = inorganic carbon. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

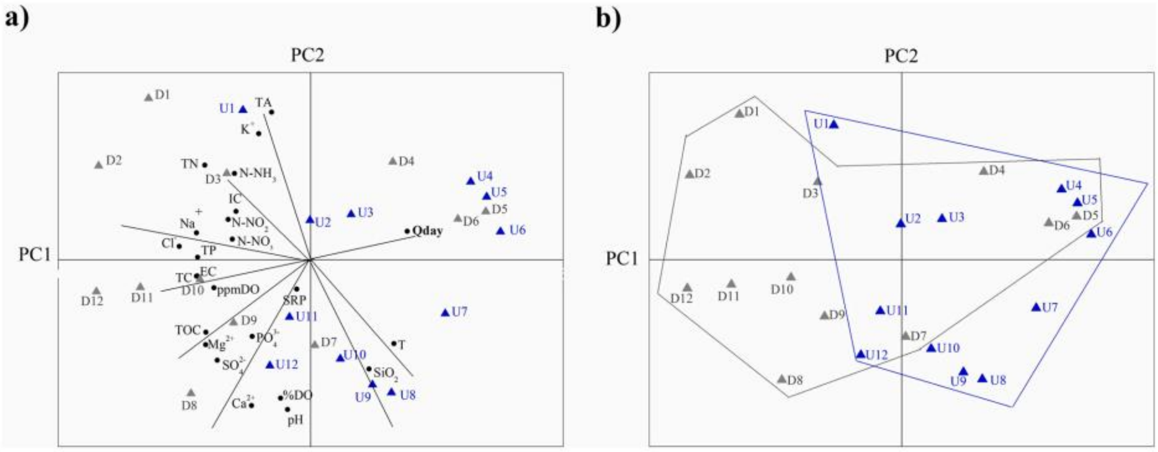

Fig. 3. Principal Component Analysis (PC1 vs PC2) of the data. a. Biplot with the scores of the samples U (in blue) and D (in grey) and loadings of the measured parameters. b. Scores plot of U samples and D samples with highlights on the two groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2.4.2. Microbiological parameters

The results of faecal indicator bacteria monitored in the different samplings are reported in Fig. 4. The concentration of coliforms (Fig. 4a), *E. coli* (Fig. 4b), enterococci (Fig. 4c) and *C. perfringens* spores (Fig. 4d) is strongly influenced by the discharge of the WWTP (W) since the concentration of all the microbial indicators increases significantly between the U and D sample (SM, Table S1). Moreover, there is also a statistically significant difference between U and W samples for all the microbial indicators analysed, indicating the ability of the WWTP to induce a detectable faecal contamination with the discharge of its sewage (SM, Table S1). Moreover, the concentration of indicators in the W sample was higher with respect to other studies [17,30,31] that analysed wastewater effluent. Furthermore, a statistically significant difference was observed for coliforms, *E. coli* and enterococci between W and D samples (SM, Table S1) even if with lower intensity with respect to the difference between U and D. These results highlight the possibility that the river is not able to restore completely the microbial indicator concentrations present before the discharge of the sewage. The same trend was observed in other studies [32,33], in which they observed an increase of coliforms, E. coli and enterococci counts just downstream of the WWTP discharge. The presence of faecal indicator bacteria in rivers is of great interest to public health authorities since their presence is associated with the presence of human pathogens [34] implicated in waterborne diseases, such as *Salmonella* spp., pathogenic *E. coli* strains and *Shigella* spp. [35]. The presence of faecal indicator bacteria is an indication of the status of the receiving water body since their presence in high quantities could indicate an alteration in the WWTP operation and/or a decrease of water flow in the receiving river. The results of the correlation analysis between the faecal indicator bacteria concentration and the stream flow data (used as an indicator of water shortage) are reported in Table 1 (graphical representation reported in Fig. S5). The upstream and downstream sample points were analysed separately, to determine the impact of WWTP in association with the water quantity variation. In particular, in the U sample no statistically significant correlation was observed but, interestingly, in the D sample the counts of faecal indicator bacteria (i.e., *E. coli*, enterococci and *C. perfringens*) were inversely correlated with stream flow variation, emphasising the negative impact of the WWTP as the flow rate decreases. In literature, it is well reported the assumption that the faecal indicator bacteria quantity in surface waters can be influenced by floods and drought conditions in relation to climate change [36,37]. As aforementioned, although different studies that considered rivers of different origin are published, no data on faecal contamination in alpine rivers were reported. Conversely to our findings, in other studies [38,39], MPN of *E. coli* in river (montane areas of South-East Asia and Brazil, respectively) was found higher during the wet season with respect to dry season. This could be explained not only by the different sources of contamination, e.g. animal and human dejections at the soil surface and the overflowing of latrines, but also - and mainly - by the different location and typology of the river. Moreover, in a river situated in Spain (a Mediterranean stream) [40], the authors found the highest stream recovery capacity during the dry season (the period with the highest temperature). In alpine rivers, it is possible to observe the opposite trend; i.e., the period with the highest stream flow should be the period with increasing temperature, as late spring/early summer, since it is due to the release of water from alpine glaciers and snow melting. To the best of our knowledge, this is the first study in which the impact of a WWTP on an Alpine river has been investigated in relation to climate change and especially during a prolonged severe drought period, so it is not possible to directly compare the results with previous reports. The different results reported here could be explained by the different origin of the river, in which the dilution factor of the stream flow plays a pivotal role in the dispersion of faecal indicator bacteria with respect to other factors, such as the temperature, and highlights the importance to preserve these natural environments from the water shortage, direct consequence of the climate change. The results of pathogen (i.e., *Salmonella* spp. and VTEC) detection performed with conventional PCR are shown in SM, Table S2. The presence of *Salmonella* spp. was detected in 50 % (6/12) of the U, in 83.3 % (10/12) of the W and in 75 % (9/12) of D, highlighting the impact of WWTP on the river. A similar result was obtained in another study (Morocco) [33] in which the presence of *Salmonella* was detected only in the downstream sample and only in the summer season, due also to the flow reduction that is characteristic of the hottest period. Conversely, the frequency of positive water samples for *Salmonella* reported in another study [16], in different rivers, was lower than the value revealed in the present study. As reported in Table S2, E. coli O157:H7 and Shiga-like toxin (I and II) were never found in the samples analysed. The only genes detected were the one encoding for the flagellar antigen H7 (U: 50 % (6/12); W: 100 % (12/12) and D: 75 % (9/12)) and the one encoding for intimin protein (U: 0 % (0/12); W: 8.3 % (1/12) and D: 8.3 % (1/12)), necessary for the infection since it is responsible for bacterial adhesion to the host intestinal epithelium. In this regard, it should be emphasised that the flagellar antigen H7 is not directly associated with the presence of pathogenic strains [41] and that its detection in the present study is not correlated with the presence of the pathogenic strain O157:H7, as no positivity for the somatic antigen O157 was ever detected for any of the samples analysed. The presence of pathogens in rivers poses a direct threat to human health, especially when the water is used for recreational and irrigation purposes. In our study, despite the absence of *E. coli* O157:H7 in all the sampling points, Salmonella spp. was found upstream and increased along with the sewage discharge highlighting the need to carefully monitor the WWTP activities and to develop mitigation strategies to reduce the effect of water shortage. Conversely to indicator bacteria, the pathogen presence, even if increased after the WWTP discharge, is not associated with stream flow variation.

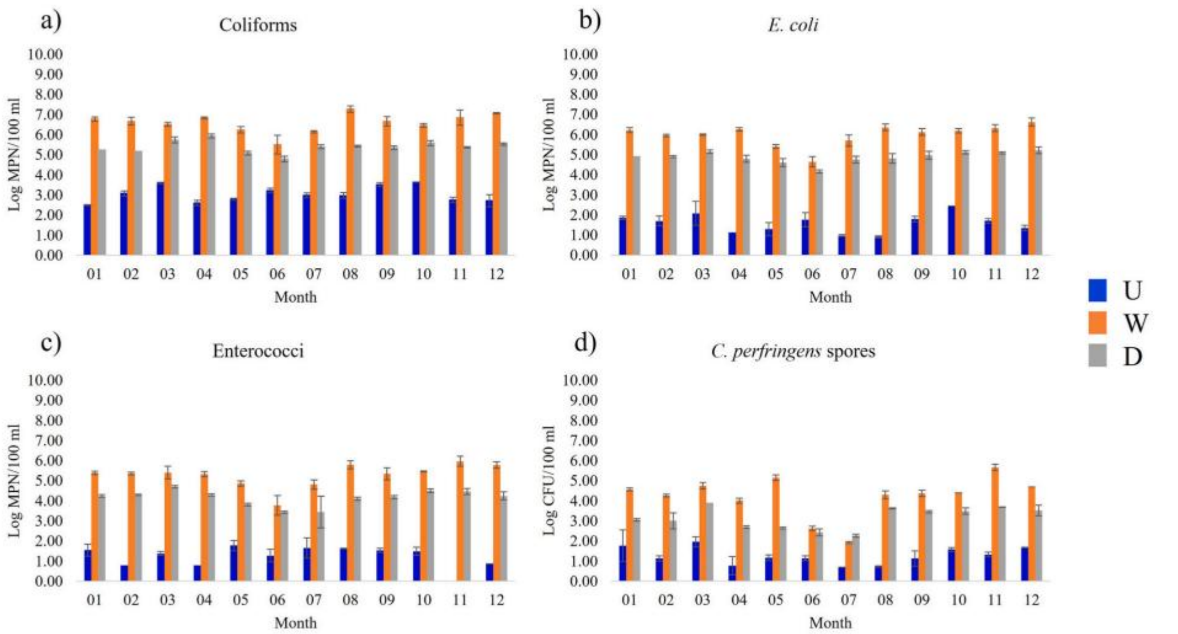

Fig. 4. Mean concentration (± standard deviation) (log CFU/100 mL or log MPN/100 mL) of microbial indicators in water samples of 12 samplings. U: upstream of WWTP discharge; W: WWTP discharge; D: downstream of WWTP discharge.

	Stream flow (m^3/s)
Log MPN Coliforms (U)	−0.291 p = 0.385
Log MPN *E. coli* (U)	−0.455 p = 0.160
Log MPN Enterococci (U)	0.456 p = 0.159
Log CFU *C. perfringens* spores (U)	−0.433 p = 0.184
Log MPN Coliforms (D)	−0.400 p = 0.223
Log MPN *E. coli* (D)	**−0.800 p = 0.003**
Log MPN Enterococci (D)	**−0.700 p = 0.016**
Log CFU *C. perfringens* spores (D)	**−0.664 p = 0.026**

Table 1. Spearman's correlation between faecal indicator bacteria from upstream (U) and downstream (D) sampling points and stream flow. Bold indicates statistically significant results.

3.2.4.3. Benthic macroinvertebrates

We collected 120 macroinvertebrate samples (12 months × 5 samples × two sites, U and D). A total of 62.465 individuals were counted and identified, out of which 45.424 upstream and 17.041 downstream. We identified 61 taxa upstream and 57 taxa downstream. The decrease of invertebrate densities in the downstream stretch suggests an evident impact of WWTP discharge, as the morphometry of stream didn't change between U and D and the flow velocity, shear stress, shading are the same on both sample sites. The upstream communities were primarily dominated by *Simuliidae* (56.58 %) and Baetis sp. (20.25 %), while downstream com munities were predominantly dominated by *Naididae* (31.54 %) and *Baetis* sp. (19.71 %). Taxa richness was higher upstream (mean 16.66 ± 3.19 SD) than downstream (mean 12.58 ± 2.84 SD). Ephemeroptera, Plecoptera, and Trichoptera (EPT) together amounted to 26527 individuals, with 18388 specimens collected upstream and 8139 downstream. The average taxonomic richness of EPT (EPT_S) per surber was higher upstream (10.8 ± 2.12 SD) compared to downstream (6.74 ± 1.16 SD). In the downstream section, a small number of pollutant-resistant groups (e.g., Oligochaeta and Chironomidae) became dominant, while sensitive taxa (represented by EPT) decreased in abundance and richness or disappeared. Regarding the Functional Feeding Groups, upstream samples were predominantly composed of filter feeders (53.15 %), in downstream ones, collectors dominated the communities, with an elevated presence of gatherers (81.99 %). In downstream sites, scrapers and filterers tended to decrease, while collector abundance increased probably because of the increased organic matter loads [42]. To depict differences in the taxonomic composition of benthic communities between sites, an NMDS ordination and a PERMANOVA (Fig. 5a) were performed, yielding significant differences ($F = 0.17$; $p = 0.001$) and the dispersion with beta dispersion. We observed that the downstream site community was more heterogeneous along the sampling period, compared to the upstream site community, indicative of a response to multiple stressors caused by the wastewater treatment plant and water scarcity. Most likely, during the months when the flow increased, differences between the two communities (U and D) were less evident. The SIMPER analysis highlighted those families contributing the most to the differences between sampling sites. In particular, D sites were characterised by a higher relative abundance (specimens/surber) of Naididae (D mean = 141 vs U mean = 7), Lumbriculidae (D mean = 27 vs U mean = 6) and Hydroptilidae (D mean = 12 vs U mean = 4) and lower relative abundance of Hydropsychidae (D mean = 45 vs U mean = 135), *Caenis* sp. (D mean = 31 vs U mean = 57) and *Habrophlebia* sp. (D mean = 0 vs U mean = 7). ADONIS showed a significant difference between sites, with a stress of 22 % (df = 1, SS = 3,29, $R2 = 0{,}16$, $F = 41{,}26$, $P < 0.001$) and a significant difference compared to flow conditions (df = 1, SS = 1,023, $R2 = 0{,}051$, $F = 12{,}83$, $P < 0.001$). We registered significant nonlinear relationships of the attributes of the benthic macroinvertebrate community in relation to the river flow. In particular, for ASPT (Average Score Per Taxon), the patterns suggested a hump-shaped relationship, with the flow as a good predictor of the community attributes of the macroinvertebrate community, especially in D sampling sites. In general, higher values of ASPT were recorded in the upstream site, which remained quite constant throughout the studied year, (Fig. 5b). On the contrary, in the downstream section we detected an evident variation in ASPT values, with the lowest values registered when the flow achieved the lowest range ($F = 24.14$; $R2 = 67$ %; $P < 0.001$, Fig. 5c) and an increase of ASPT values as the flow rate increases. The analysis of the difference in metrics, such as EPT_S, between the upstream and downstream sites revealed a significant relation ($F = 5.14$; $R2 = 65.5$ %; $P = 0.004$) and correlation (Spearman's p-value < 0.05) with the decrease in flow. Specifically, it was observed that at higher flow rates, the percentage similarity between EPT_S in U and D sites was highly evident whereas the opposite occurred at reduced flow rates, where the difference increased significantly (Fig. 6). With this study it has been possible to find some indicator taxa and community metrics for water quality-quantity assessment. For instance, most species of Chironomidae are very tolerant to water pollution [43]. Moreover, high amounts of

Oligochaeta and Gastropoda are often indicators of nutrient pollution [44]. However benthic macroinvertebrates are also susceptible to changes in flow regimes [45]. In particular, invertebrate community composition often changes in response to low or reduced discharge. These changes are probably a result of increased habitat suitability for some species and decreased suitability for others [46]. The responses of certain invertebrate taxa to flow reduction suggest that invertebrates might be useful indicators in assessments of reduced-flow impacts. It should be possible to identify taxa that are sensitive to flow reduction, such as those that drift when flows change, and are affected by sedimentation or have specific velocity requirements. This is underscored by the trend of certain indices, such as ASPT, which exhibited a negative trend, consequently indicating lower ecological quality, determined by the presence of high diversity also in terms of sensitive taxa such as EPT, as river discharge decreases. In the end, it was possible to highlight that the differences between macrobenthos communities, especially for sensitive taxa such as EPT, increase with low flow.

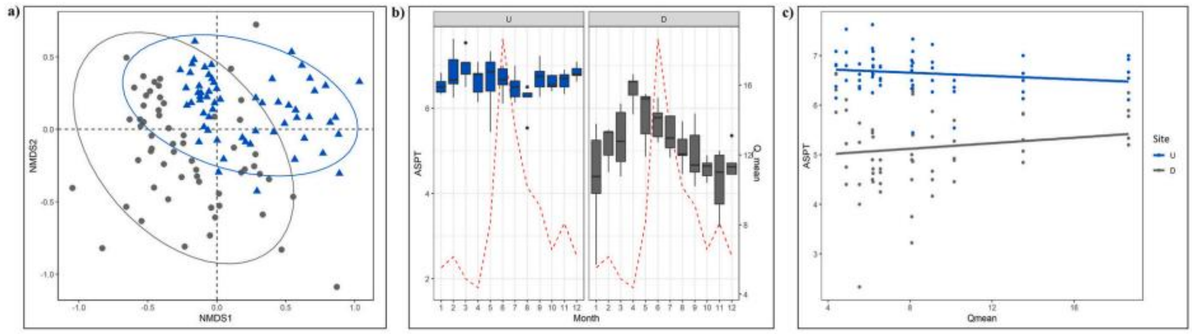

Fig. 5. Benthic macroinvertebrates. a) NMDS ordination plot depicting differences in the taxonomic composition of benthic invertebrate com munities between U (blue triangles) and D (grey points) sites. Ellipses represent standard deviations around the centroids of the two groups. b) time series (x axis, Month) of flow (Q_mean) and Average Score Per Taxon (ASPT, y axis) boxplot with box = 25th–75th percentiles; line = median; whiskers = 1.5 IQR (interquartile range), for U and D sites. c) Representation of the GAM smoother for flow (Q_mean) interacting with ASPT. The lines (blue for U site and grey for D site) represent the smoothed function, while the dots indicate the distribution of all samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

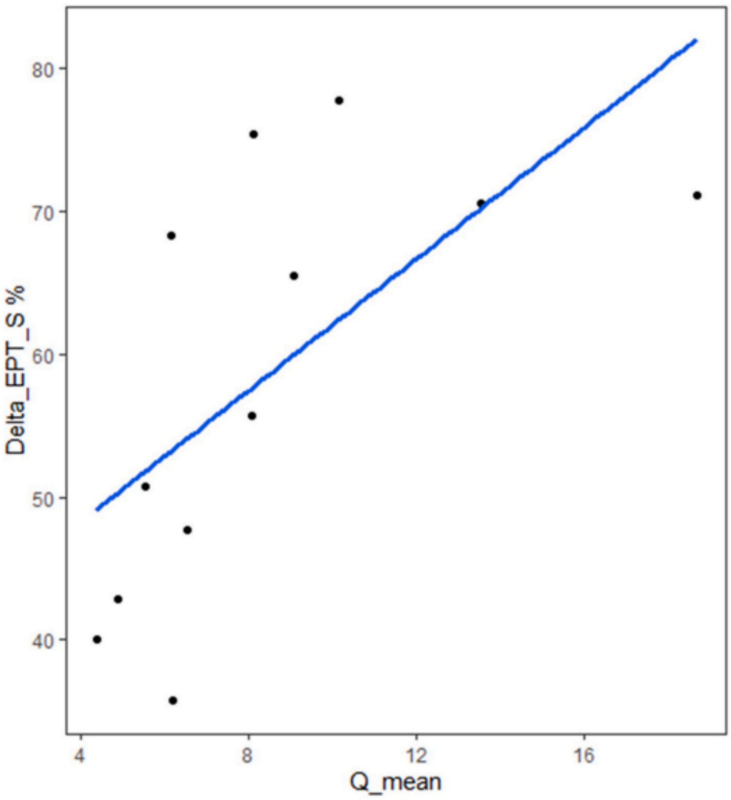

Fig. 6. Variation of EPT_S upstream and downstream the WWTP discharge in different flow conditions (Q_mean): Delta means the similarity percentage richness of Ephemeroptera, Plecoptera and Trichoperta in the D site comparing to the U site for each sampling date.

3.2.4.4. Diatoms

In total, we collected 36 diatom samples, 12 in the U site, 12 in the D site and 12 in the W site, and we identified 78 species, 49 upstream, 63 downstream and 43 in wastewater. Taxa richness was significantly higher in D compared to U and W (22.41 D±3.65, 17.33 U ± 4.51 and 16.16 W ± 3.51); the same pattern was observed in terms of Shannon diversity index (2.96 D, 2.07 U and 2.46 W) and Evenness (0.66 D, 0.50 U and 0.61 W). Our results are partially in agreement with those highlighted in another study [47] where authors found higher diatom diversity (Shannon index) in a site located downstream a WWTP in comparison to the upstream section, while lower diatom richness downstream in comparison to the upstream site. Likely, in our study, communities developing in the D section received a contribution of taxa from both U and W sites, which led to the formation of richer and more diversified assemblages. Concerning ecological guilds, we observed a prevalence of low-profile taxa (72.83 %) in the upstream site, according to the definition of this guild (i.e. resource-stressed but disturbance-free [48]) which is generally associated to nutrient-poor environments [49] and high-flow velocity conditions [21,48]. According to previous studies, downstream and wastewater assemblages were dominated by motile taxa (respectively 61.60 % and 66.10 %), generally considered as successful competitors for nutrients in nutrient-rich environments [48] thanks to their ability of synthesising extracellular enzymes to consume macromolecules [50]. Moreover, motile diatoms have a competitive advantage compared to sessile ones, since they can actively move towards more suitable conditions [51]. In our study, this guild was more abundant during the warmest months; in this context, the warm water temperatures which can be reached downstream of a WWTP could enhance the ability of motile taxa to move, through a decrease of the cytoplasm viscosity in the raphe slit [47,52]. To visually inspect for differences in terms of taxonomical composition among diatom assemblages collected in the three sampling sites, we performed a PCoA. The analysis highlighted a clear difference in terms of diatom species composition between sampling sites; Fig. 7a displays that samples of the U site are mostly placed on the right side of the diagram, while those collected in the W site are mainly distributed on the left side, with a clear separation of the two spider graphs. The communities in D site are positioned halfway between those upstream and those in the wastewater, once more confirming previous results published in literature [47]. Fig. 7a displays an apparent taxonomic homogenization of the diatom communities inhabiting W, whose ellipse ap pears strongly gathered around the centroid. Communities belonging to the W site appeared, indeed, simpler and less heterogeneous than those characterising U and D sections. This could be explained by the conjoint chemical and hydrological pressures affecting the assemblages, confirming the results of previous studies [53,54]. The SIMPER analysis highlighted the diatom taxa significantly contributing to differences between the site assemblages. In particular, downstream (D) assemblages were mainly characterised by α-meso polisaprobic taxa; for instance, *Fistulifera saprophila* (D mean = 68 vs U mean = 0 and W mean = 14), Nitzschia costei (D mean = 48 vs U mean = 0 and W mean = 8) and *Nitzschia fonticola* (D mean = 54 vs U mean = 28 and W mean = 3) displayed higher abundance means in D compared to U site and W site. On the contrary, a lower relative abundance of β-mesosaprobuos taxa, compared to the upstream site, such as *Achnanthidium delmontii* (D mean = 28 vs U mean = 110 and W = 49), *Achnanthidium pyrenaicum* (D mean = 42 vs U mean = 127), Achnanthidium minutissimum (D mean = 6 vs U mean = 23) was observed. In the wastewater (W) diatom communities were mainly defined by polysaprobic taxa as *Achnanthidium saprophilum* (W mean = 119 vs D mean = 13 and U mean = 0), *Mayamaea permitis* (W mean = 86 vs D mean = 21 and U mean = 1), *Nitzschia soratensis* (W mean = 73 vs D mean = 11 and U mean = 1), *Sellaphora nigri* (W mean = 43 vs D mean = 3 and U mean = 0). The taxonomic and functional changes observed in our communities are consistent with those detected in another study [47], highlighting a shift in diatom assemblages from oligosaprobic/oligotrophic groups belonging to the low- and high-profile guilds upstream of a WWTP, to polysaprobic motile groups in the impacted sites. ADONIS, with a stress of 17 %, furtherly confirmed a significant difference between sites (df = 1, SS = 0.89, $R2$ = 0.30, F = 15.03, P = 0.005) and with changes in river flow (df = 1, SS = 0.16, $R2$ = 0.41, F = 2.82, P = 0.005). Despite the importance of diatoms as bioindicators in lotic environments and the importance of their inclusion in the river monitoring programs [55], species composition and structural dynamics in WWTPs have rarely been documented [47,56]. In our study, diatom quality index ICMi displayed significantly ($p < 0.05$) higher values in U in comparison to D and W sampling sites (0.81 U, 0.57 D and 0.42 W; i.e., corresponding respectively to "good", "moderate" and "poor" ecological status), thus responding to this high anthropogenic pressure, as already observed in another study [47] for BDI index. We registered significant nonlinear influences of the attributes of the benthic diatom community in relation to the river-flow. In particular the flow resulted as a good predictor of the community attributes measured ICMi. In addition, the elevated values of R2 indicated that the benthic diatom attributes had a good fit with the flow. Higher values of ICMi were recorded in the upstream site, especially in sampling periods where the river flow reached the range 16 m^3/sec - 18 m^3/s (Fig. 7b). The lowest values of ICMi were registered in the downstream site when the flow achieved the less range 5 m^3/s - 7 m^3/s ($F = 5.96$; $R2 = 83$ %; $P < 0.001$, Fig. 7c). In both U and D sections, the ICMi increased as the flow rate increased (Fig. 7c).

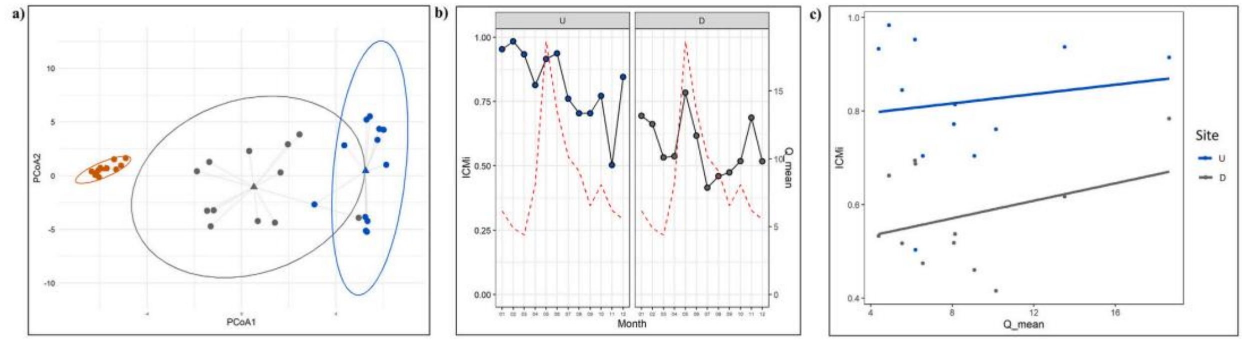

Fig. 7. Benthic diatoms. a) PcoA ordination plot based on Bray-Curtis dissimilarity matrices depicting differences in the taxonomic composition of benthic communities between U (blue points), W (orange points) and D (grey points) sites. Ellipses represent standard deviations around the centroids of the two groups. b) time series (x axis, Month) of flow (Q_mean) and Intercalibration Common Metric Index (ICMi, y axis) points, for U and D sites. c) Representation of the GAM smoother for flow (Q_mean) interacting with ICMi. The lines (blue for U site and grey for D site) represent the smoothed function, while the dots indicate the distribution of all samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2.5. Conclusions

The study highlights the complex interplay between climate change-induced water shortages, wastewater treatment plant effluents, and their impact on river ecosystems and water quality. The hydrological description revealed the severe runoff deficit in the western Alpine area during the whole 2022, with a significant reduction in flow rates. Normally, when people think about the impact of climate change on rivers, they always consider the *'quantity'* of water and not its *'quality'*. This study takes the case of one river to do a first evaluation of the impact of water shortage on water quality, attempting to provide some preliminary suggestions on how reduced flows may exacerbate the influence of anthropogenic discharges. In the case of Stura di Lanzo, these alterations had cascading effects on various water parameters, including nutrient concentrations and microbiological indicators. The analysis of the chemical parameters showed that, even if WWTP effluents have a low contribution in terms of final instream flow, they can heavily influence the final nutrient load in the downstream waters. The correlations found with the Qday values supported the hypothesis that the investigated Alpine river, already naturally subjected to seasonal flow regime shifts, became more vulnerable to WWTP effluents when drought conditions were pressing and its dilution capacity was threatened. To determine whether this is a characteristic pattern of rivers facing drought situations, the repetition of these analyses in the years to come and on com parable basins is essential. Following the same trend of nutrients, microbiological parameters, including faecal indicator bacteria, revealed a significant impact of the WWTP on downstream water quality. The presence of pathogens, such as Salmonella, raised concerns about potential health risks associated with water use during drought conditions. Macroinvertebrates and diatom communities responded sensitively to altered flow conditions, underscoring the vulnerability of the alpine river in the sampled year.

The decrease in invertebrate densities downstream indicated habitat loss and changes in food availability. The shift in diatom community composition, with the increase in motile taxa downstream, reflected the influence of wastewater treatment plant effluents and reduced flow. The study also explored the potential of using specific taxa and community metrics as indicators of water quality and quantity. For instance, the analysis of macroinvertebrates and diatoms suggested that certain taxa, particularly those associated with sensitive ecological conditions, could serve as indicators of reduced-flow impacts. Beside climate-related effects, waters are heavily subjected to local human pressure, which affects their features on multiple sides. Manipulation of channels, building or modification of drainage basins etc., alter the natural flows, while pollution and land use may overturn their quality. It is therefore fundamental to consider local factors, such as wastewater treatment plant discharges, in conjunction with climate-induced changes. At the same time, the role of flow regimes in shaping the response of chemical and bio logical components in the river ecosystems emphasises the need for integrated monitoring and management strategies that account for both climate change and local stressors. In conclusion, this research provides preliminary insights into the complex interactions between climate change-induced water shortage, local impacts and river ecosystem health in alpine regions. The findings underscore the importance of holistic approaches in water resource management, considering both quantity and quality aspects, especially in the context of changing climate conditions. Studies like the presented one may offer insights for policymakers, environmental managers, and researchers working towards sustainable river ecosystem conservation and management in the face of global environmental challenges. For this reason, the next steps may involve replicating the same experiment on other rivers with wastewater treatment plants of different origins, to assess whether a consistent response pattern of the examined variables can be observed.

3.2.6. References

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3.2.7. Supplementary materials

Paragraph S1.

Reagents. NaCl, $NaNO_3$ (>99%), NaOH (97%), Na_2SO_3 (98%), $Na_2S_2O_5$ (97%), Na_2CO_3 (99.5%), $K_2S_2O_8$ (>98%) and $CHCl_3$ (LiChrosolv gradient grade) were from Merck. Na_2SO_4 (99%), $NaNO_2$ (>97%), NH_4Cl (≥99.5%), KCl (≥99%), $CaCl_2$ (≥93%), $NaHCO_3$ (≥99.5%), L-ascorbic acid ($C_6H_8O_6$, 99%), oxalic acid dihydrate ($C_2H_2O_4\cdot 2H_2O$, 98%) and methylene blue ($C_{16}H_{18}ClN3_3S$, ≥82%) were from Sigma Aldrich. $Na_3PO_4\cdot 12H_2O$ (98%), $MgCl_2$ (99%), KI (99.7%), HgCl, ethylenediaminetetraacetic acid disodium salt (EDTA-$Na_2\cdot 2H_2O$, >99%), K_2CO_3 (≥99%) and methanesulfonic acid (CH_3SO_3H, >98%) were from Carlo Erba Reagents. Na_2SiO_3 and N-(1-Naphthyl)ethylenediamine dihydrochloride ($C_{12}H_{14}N_2\cdot 2HCl$, 96%) were from Alfa Aesar. HCl (37%), H_2SO_4 (95%), formic acid (CH_2O_2, 99%), 1-Amino-2-hydroxy-4-naphthalenesulfonic acid ($C_{10}H_9NO_4S$, 99%), sulfanilamide ($C_6H_8N_2O_2S$, >98%), ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 80%), antimony(III) potassium oxitartrate trihydrate ($C_8H_4K_2O_{12}Sb_2\cdot 3H_2O$, ≥99%) and ethanol ($C_2H_5OH$, ≥99.9%) were from VWR. Dodecylbenzenesulfonic acid (sodium salt, $C_{18}H_{29}NaO_3S$, 88%) was from Thermo Scientific. All the reagents were used as received, without further purification. Ultra-pure water was produced by a Milli-Q system (Millipore, 18.2 MΩ cm resistivity, ≤ 5 ppb TOC). The stock solutions to produce the standards were prepared weekly or monthly and stored according to the necessities in or outside the fridge. The solutions were stored in the dark.

Sample treatment. 1 dm^3 of water sample was collected monthly in each of the 3 sampling sites (U; W; D) using glass bottles. The samples for the chemical analysis were then stored under refrigeration and transported to the laboratory, where they were vacuum-filtered on Sartorius membrane filters (regenerated cellulose, 0.45 µm pore size), stored at 4 °C till further processing and analyzed as soon as possible.

Paragraph S2.

The concentrations of the anions (Cl^-, N-NO_3, PO_4^{3-}, SO_4^{2-}) were determined using a Dionex ion chromatograph equipped with a Dionex IonPac AS9-HC column, a Dionex IonPac AG24A RFIC guard column and an electrically regenerated suppressor Dionex AERS 500, 4 mm. The analysis was carried out in isocratic mode, with potassium carbonate 9 mM as eluent. The cations (Na^+, K^+, Mg^{2+}, Ca^{2+}) were determined with the same Dionex ion chromatograph, equipped with a Dionex IonPac CS12A column, a Dionex AG24A guard column and a dynamically regenerated suppressor Dionex CDRS 600, 4mm. The analysis was carried out in isocratic mode, with methanesulfonic acid 20 mM as eluent. Total Carbon (TC), Inorganic Carbon (IC) and Total Nitrogen (TN) were determined using a Shimadzu TOC-VCSH Total Organic Carbon (TOC) Analyzer. TOC was estimated as the difference between the measured TC and IC.

Paragraph S3.
For the determination of N-NO_2, 200 µL of SA (sulfanilamide, $C_6H_8N_2O_2S$, prepared in a solution of water and HCl 10%) 0.06 M were added to 10 mL of sample and let react for 2 minutes. After, 200 µL of NEDA (n-(1-naphthyl)ethylenediamine dihydrochloride, $C_{12}H_{14}N_2 \cdot 2HCl$, prepared in water solution) 0.004 M were added to the solution. After 15 minutes from the addition, the solution would develop a pink color and the absorbance at 543 nm was measured. For the calibration, standard solutions of 10 mL of $NaNO_2$ were prepared using a concentration range of 0-0.2 mg/L N-NO_2 and treated as samples.

For the determination of N-NH_3, 100 mL of Nessler's reagent were prepared according to the protocol suggested by [1]. During the determination procedure, 400 µL of Nessler's reagent were added to 10 mL of sample and let react for 15 minutes before measuring the absorbance at 420 nm. For the calibration, standard solutions of 10 mL of NH_4Cl were prepared using a concentration range of 0.00 – 4.00 mg/L N-NH_3 and treated as samples.

For the determination of the total phosphorus, 2 mL of $K_2S_2O_8$ (potassium persulfate, prepared in a solution of water and H_2SO_4 0.7 M) were added to 50 mL of sample and stored at 120°C for 3 hours. 1.5 mL of mixed reagent (ammonium heptamolybdate 0.08 M, H_2SO_4 4.5 M and antimony(III) potassium oxitartrate 0.05 M) and 1.5 mL of ascorbic acid 0.4 M were then added and the absorbance at 712 nm was measured after 20 minutes. For the calibration, standard solutions of 25 mL were prepared with KH_2PO_4 using a concentration range of 0.00-1.00 P-PO_4^{3-} . 750 µL of mixed reagent and 750 µL of ascorbic acid were added to the solutions and the absorbance at 712 nm was measured after 15 minutes from the last addition.

For the determination of SiO_2, 200 µL of a solution water:HCl 1:1 and 400 µL of ammonium heptamolybdate 0.08 M at pH 8 were added to 10 mL of sample. After 5 minutes, 300 µL of oxalic acid 0.8 M and 400 µL of a reducing solution (1-amino-2-hydroxy-4-naphthalenesulfonic acid 0.01 M, sodium sulfite 0.04 M and sodium metabisulfite 0.7 M) were added to the solution. The absorbance at 650 nm was measured after 15 minutes from the last addition. For the calibration, standard solutions of 10 mL were prepared from a stock solution of Na_2SiO_3 0.04 M using a concentration range of 0.00 – 5.00 SiO_2 mg/L and treated as samples.

For the determination of anionic surfactants, 100 mL of sample were put in a separatory funnel with the addition of 10 mL of buffer solution ($NaHCO_3$ 0.3 M + Na_2CO_3 0.25 M, pH = 10), 5 mL of methylene blue solution 0.001 M (prepared 24 hours before the analysis) and 15 mL of $CHCl_3$. The mixture was shaken and, after sedimentation and separation, transferred to a second separatory funnel containing 110 mL of MilliQ and 5 mL of acidified methylene blue solution

0.001 M. After stratification, the chloroform phase was filtered through hydrophile wadding treated with ethanol and $CHCl_3$ and collected. The extraction was repeated for three times and the final volume extracted was taken to 30 mL with $CHCl_3$ before measuring its absorbance at 650 nm. For the calibration, MBAS (dodecylbenzenesulfonic acid) volumes corresponding to concentrations from 0.5 to 4 mg/L (1 mL, 2 mL, 4 mL, 6 mL, 8 mL) were put in the separatory funnel with a such amount of MilliQ that the final volume in the funnel would be 100 mL, and then treated as samples. The values of anionic surfactants are reported as equivalent mg L^{-1} of MBAS.

Paragraph S4.

For the estimation of the volumetric contribution of the WWTP effluent to the stream flow (Y, in %), we considered the mass balance of some selected chemical species (i.e., TOC, TN, Cl^-, SO_4^{2-}, Na^+) before and after the contribution of the WWTP outflow (see eq. 1). This was possible being the 3 sampling sites (U, D and W) sufficiently close to be considered negligible the contribution of possible groundwater infiltration to the river flow.

$$C_{i,D} \cdot Q_D = C_{i,U} \cdot Q_U + C_{i,W} \cdot Q_W \text{ (eq. 1)}$$

where $C_{i,D}$, $C_{i,U}$ and $C_{i,W}$ are the concentrations of the i species in the upstream U, downstream D and waste W respectively, while Q_D, Q_U and Q_W are the related volumetric flows. Taking into account this equation, the WWTP effluent contribution ($Y= Q_W/Q_D$) was calculated for each of the considered chemical parameter i (eq. 2) and their average computed (eq. 3):

$$Y_i(\%) = (C_{i,D} - C_{i,U}) \,/\, (C_{i,W} \cdot C_{i,U}) \text{ (eq. 2)}$$

$$Y(\%) = \frac{\sum_{i=1}^{n} Yi(\%)}{n} \text{ (eq. 3)}$$

Paragraph S5.

Macroinvertebrates and diatoms methods

To assess biological diversity and richness we focused on two benthic communities, i.e. macroinvertebrates and diatoms, because of their importance in ecological functional processes and diffuse use in biomonitoring methods. Regarding macroinvertebrates, five samples were collected in riffle (i.e., erosive) habitats far away from each other approximately 5 meters were defined in the U and D stretches We selected riffles (velocity/depth ratios > 3.20) [2] because of their importance in alpine lotic systems both in terms of representativeness and biological richness. Macroinvertebrates samples were collected using a Surber sampler (600 µm mesh size; 0.05 m2 area), then preserved into plastic jars with 75% ethanol until identification. In the laboratory, all benthic invertebrates were identified according to previous studies [3, 4, 5] to the genus level for Ephemeroptera and Plecoptera and to the family level for the other taxa. Based on their trophic strategies, macroinvertebrates were classified into Functional Feeding Groups according to another study [5].

For phytobenthos, samplings were carried out following the macrobenthic sampling schedule. At each U, W and D sampling point, five pebbles were selected. Diatoms were then collected by means of a toothbrush, by scraping the upper surface of each cobble and samples were preserved in ethanol [6]. In the laboratory, samples were treated following the standard procedure by cleaning them with hydrogen peroxide (30%) and HCl (1N) [6]. Slides for light microscope analysis were mounted in Naphrax® resin. Diatom identification was performed according to the most common European diatom floras and monographs as well as recent taxonomic papers [7]. For each slide, we identified at least 400 valves at species level, according to the standard protocols [8]. Ecological guilds (i.e. low profile, high profile, motile and planktonic) were assigned to each taxon basing on the classification proposed in [9]. Finally, for each sample we calculated the Intercalibration Common Metric index (ICMi) [10], the diatom quality index adopted at national scale in the framework of the WFD application. The ICMi is defined as the mean of the Ecological Quality Ratio (EQR) of IPS and TI [11] and was automatically calculated by using the OMNIDIA 6.1.5 software [12]. The ICMi ranges from 0 (corresponding to the "bad" water quality status) to 1 (corresponding to the "high" water quality status). In detail, concerning the Alpine siliceous macrotype, boundaries for the ecological status assessment are the following: H/G= 0.85; G/M= 0.64; M/P= 0.54 and P/B= 0.27. In contrast to Stura river, the effluent of wastewater treatment plant maintains a constant flow and is not subject to the influence of climatic changes but rather to the operation of the facility, it has been preferred not to correlate the ICMi data with the flow data of the discharge.

Macroinvertebrates and diatoms statistical analysis

Taxonomic metrics of community abundance, taxa richness, richness of Ephemeroptera, Plecoptera and Trichoptera (EPT) and abundance of taxa characterised as sensitive to environmental alterations (Average Score Per Taxon), were calculated for each sample. Functional metrics such as the abundance of shredders, collectors, and scrapers, functional richness and functional evenness were also calculated. Functional feeding groups were assigned based on the fuzzy coded [5] database and as a result some taxa were coded in more than one group. For benthic diatoms, frequency, Shannon Diversity Index, Evenness, Intercalibration Common Metrics index (ICMi) and ecological guilds (i.e. low profile, high profile, motile and planktic) [9] were calculated.

Possible differences in the community composition of macrobenthos between sites (U, D) were visually and statistically examined by non-metric multidimensional scaling (NMDS) followed by a permutational multivariate analysis of variance (PERMANOVA). The Bray-Curtis dissimilarity was applied to macroinvertebrate abundances of each sampling occasion. The Tukey's honest significant difference test was used for the post hoc comparisons. To visually inspect for possible differences in terms of taxonomic composition of diatoms collected in the three sampling sites (U, W and D), we performed a Principal Coordinate Analysis (PCoA) based on Bray-Curtis distance. Possible dissimilarity in taxonomical composition of diatom communities was tested through a PERMANOVA [13] applied on distance matrices, by using the function "adonis" in the package vegan [14]. The taxonomic matrix, with the relative abundance of each recorded taxon in each sample, was converted into a site-by-site distance matrix using the Bray-Curtis distance with the function "vegdist" of the vegan package [14]. The distance of each site to its associated group multidimensional median was calculated and differences among such site distances were tested by means of multivariate analogue of the Tukey's test for homogeneity of variance ("betadisper" function) with 9999 permutations, to determine whether the dispersions between the groups were different.

Statistical differences in community composition associated with variation of flow in different month (Q), site (U, D and also W for diatoms) and their interaction were tested via permutational multivariate analysis of variance (PERMANOVA), with Bray-Curtis distance, using the 'ADONIS' function in the vegan package [14]. Where significant differences occurred by treatment, pairwise comparisons of differences were performed using the 'pairwise.adonis' function [15]. The influence of particular diatom and macroinvertebrate taxa in separating sampling sites was quantified using the similarity percentage procedure (SIMPER) [16], which computes the contribution of each species *i)* to the similarity within a pre-defined group (in the present paper U; W and D) and *ii)* to the dissimilarity between each pair of groups.
In this study, a generalized additive model (GAMs) was selected for the regression, since this model was shown to be effective in describing the site-flow-biota relationships for macroinvertebrate and diatom assemblages. All the GAMs were carried out using the "gam" function of the mgcv R package [17] and applying a Poisson distribution. A negative binomial distribution was then applied in case of overdispersion. For correlation tests were applied Spearman's test.

Fig. S1. Time trends of the pH, Temperature (T), Conductivity (EC), Oxygen Saturation (%DO) and Dissolved Oxygen (ppm DO) in the Stura river for the year 2022, measured at the three sampling points located upstream (U, labeled with empty triangles), at the discharge (W, labeled with stars) and downstream (D, labeled with squares) of the local WWTP. Months from January to December are reported in numeric format, from 1 to 12.

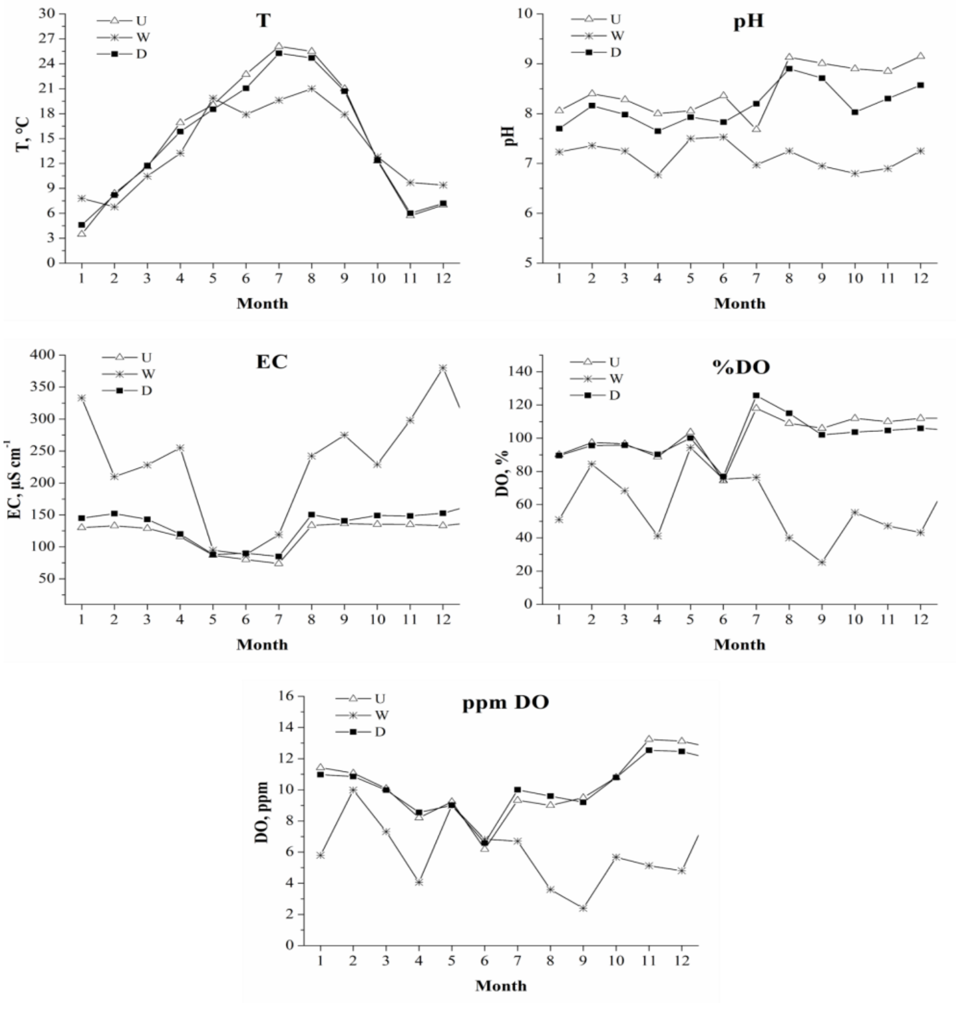
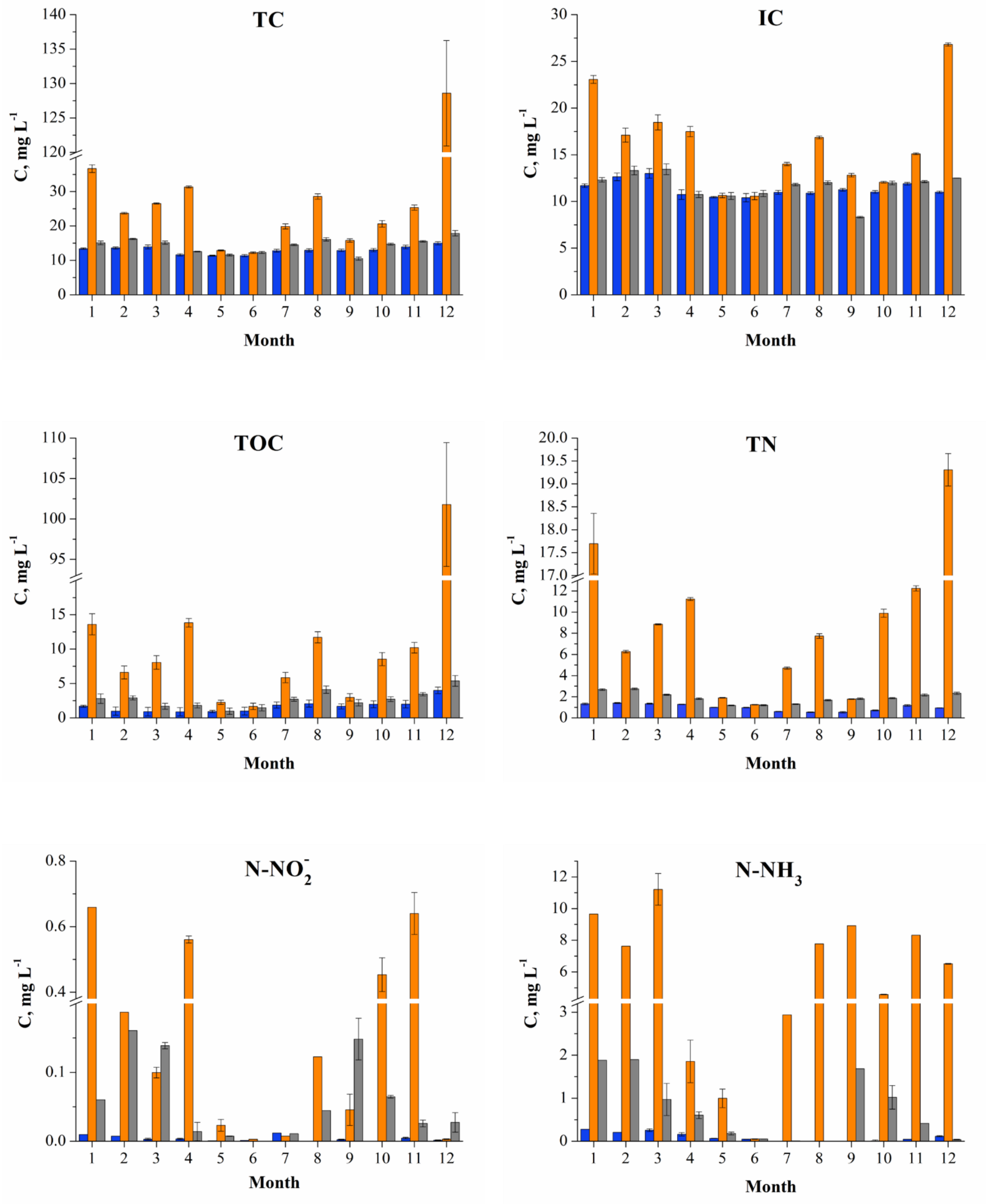
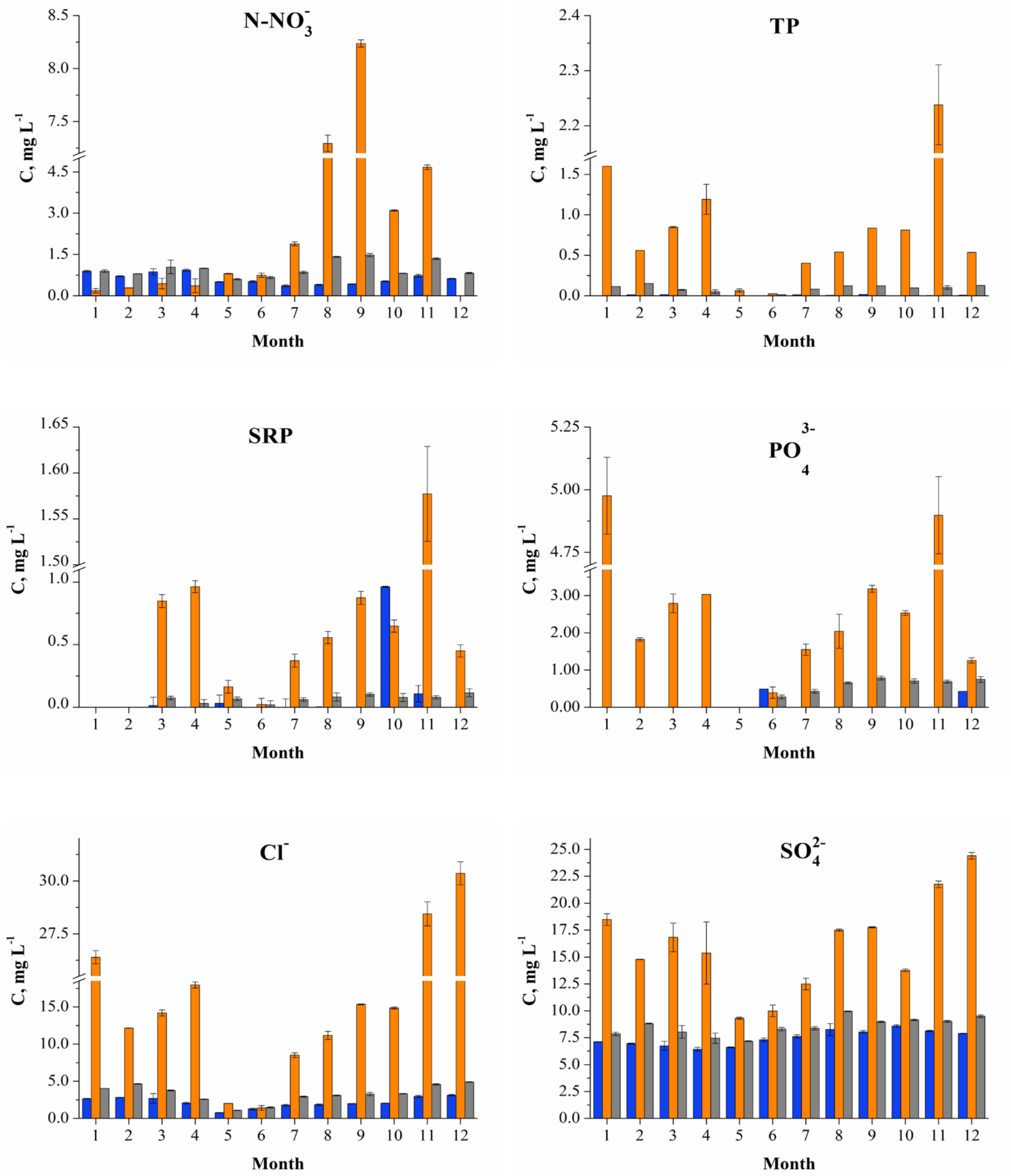


Fig. S2. Concentrations (C) of the chemical parameters measured in the Stura river through the year 2022. Upstream values are reported in blue, waste values in orange, downstream values in grey. TC = total carbon, IC = inorganic carbon, TOC = organic carbon, TN = total nitrogen, TP = total phosphorus, SRP = soluble reactive phosphorus.




N-NO$_3^-$
TP
SRP
PO$_4^{3-}$
Cl$^-$
SO$_4^{2-}$
C, mg L^{-1}
Month

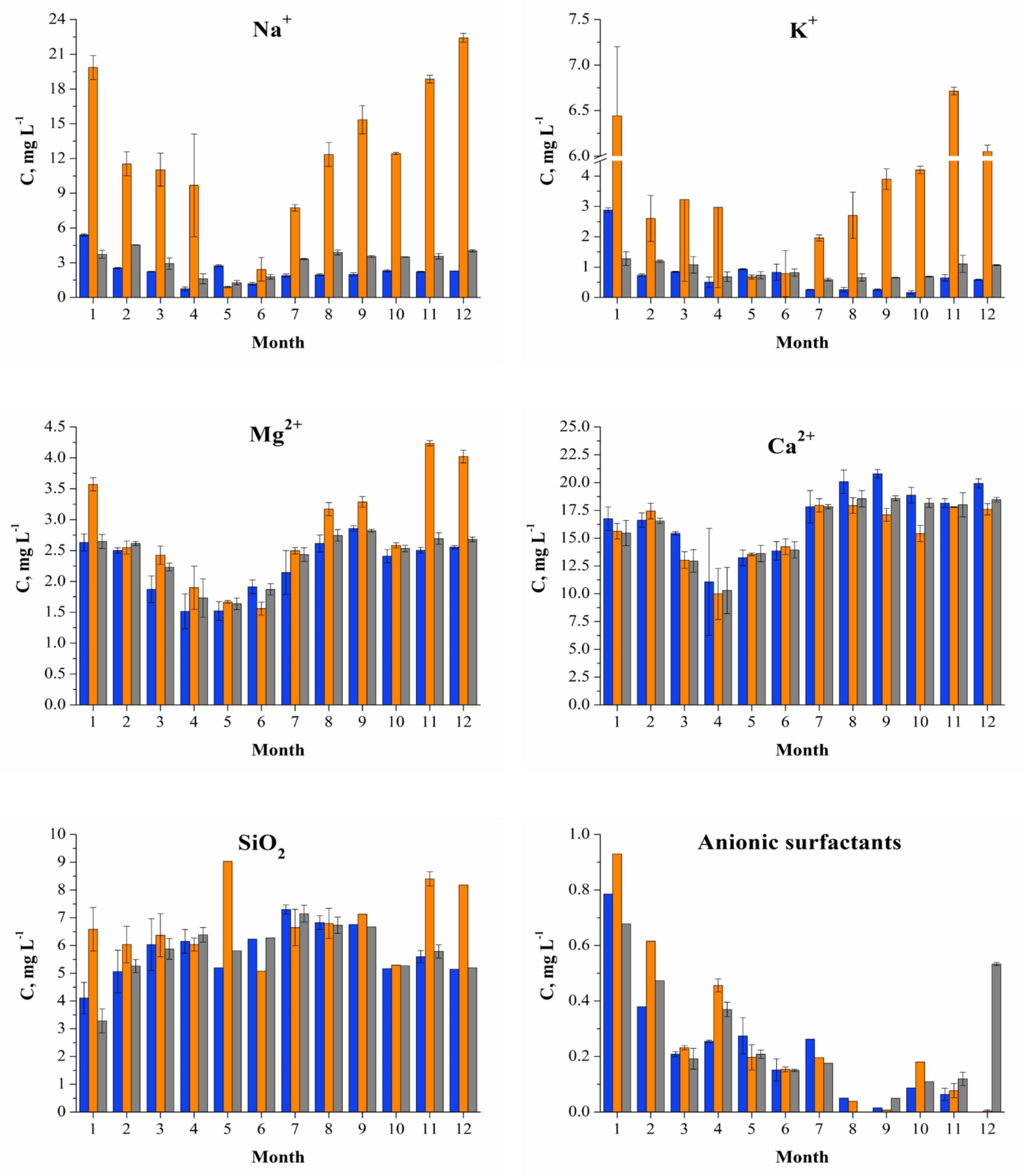

Na+
C, mg L-1
Month
K+
C, mg L-1
Month
Mg2+
C, mg L-1
Month
Ca2+
C, mg L-1
Month
SiO2
C, mg L-1
Month
Anionic surfactants
C, mg L-1
Month

Fig. S3. Volumetric contribution of the WWTP effluent to the stream flow (%), estimated by considering the mass balance of some chemical species before and after the contribution of the WWTP outflow. The value estimated for June (6) has been excluded from the graph because biased from by an extra addition of water in the WWTP effluent.

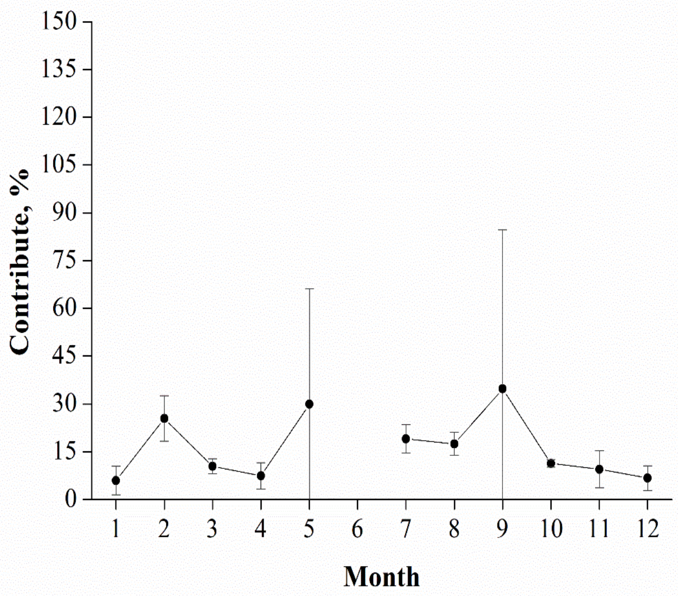


Fig. S4. Relation between IC, TOC, TC, TP and SO_4^{2-} concentration and Qday.

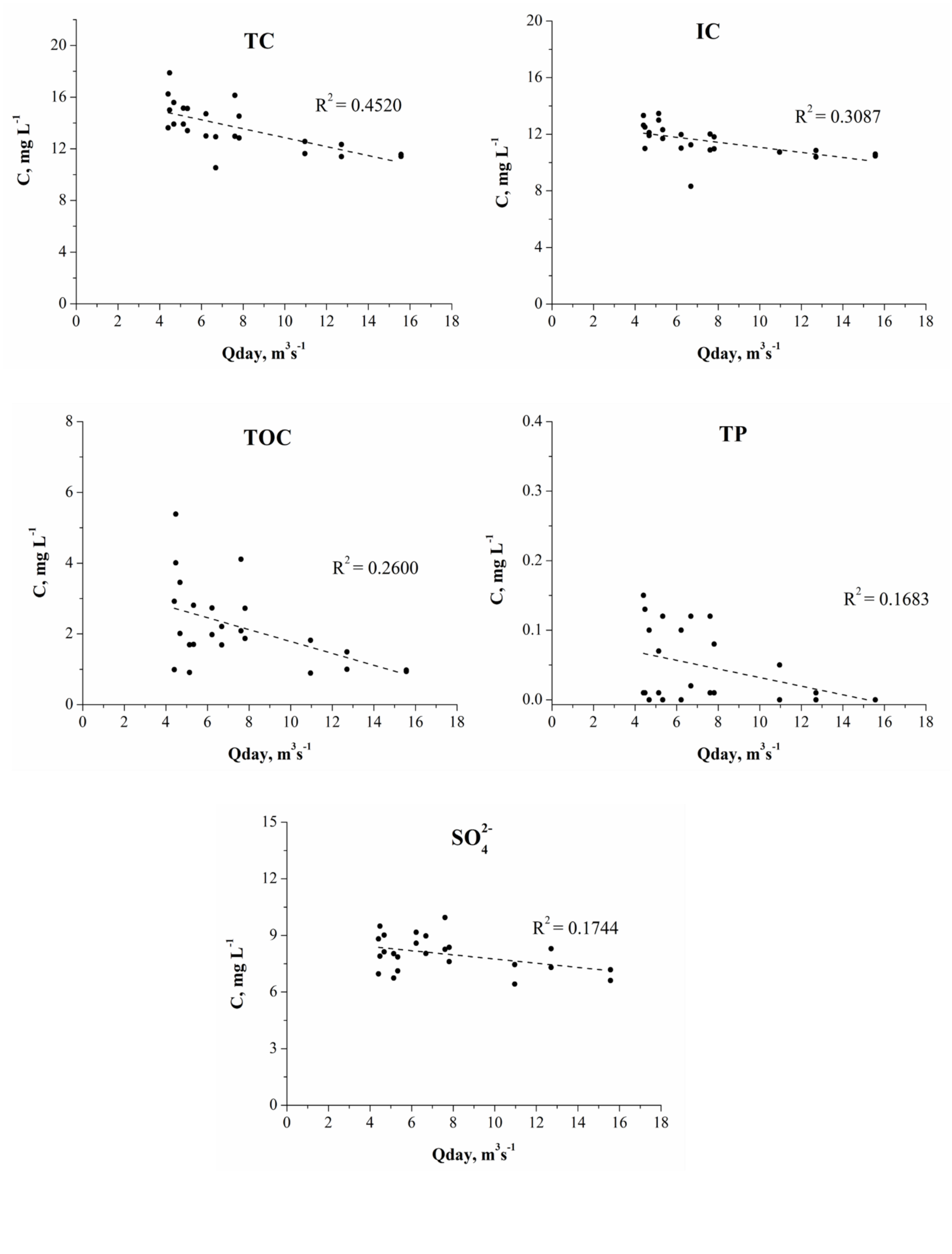


Table S1. P-values obtained with Kruskal Wallis' test for the analysis of microbial indicators (log transformed). U: upstream; W: waste; D: downstream; MPN: most probable number; CFU: colony-forming unit.

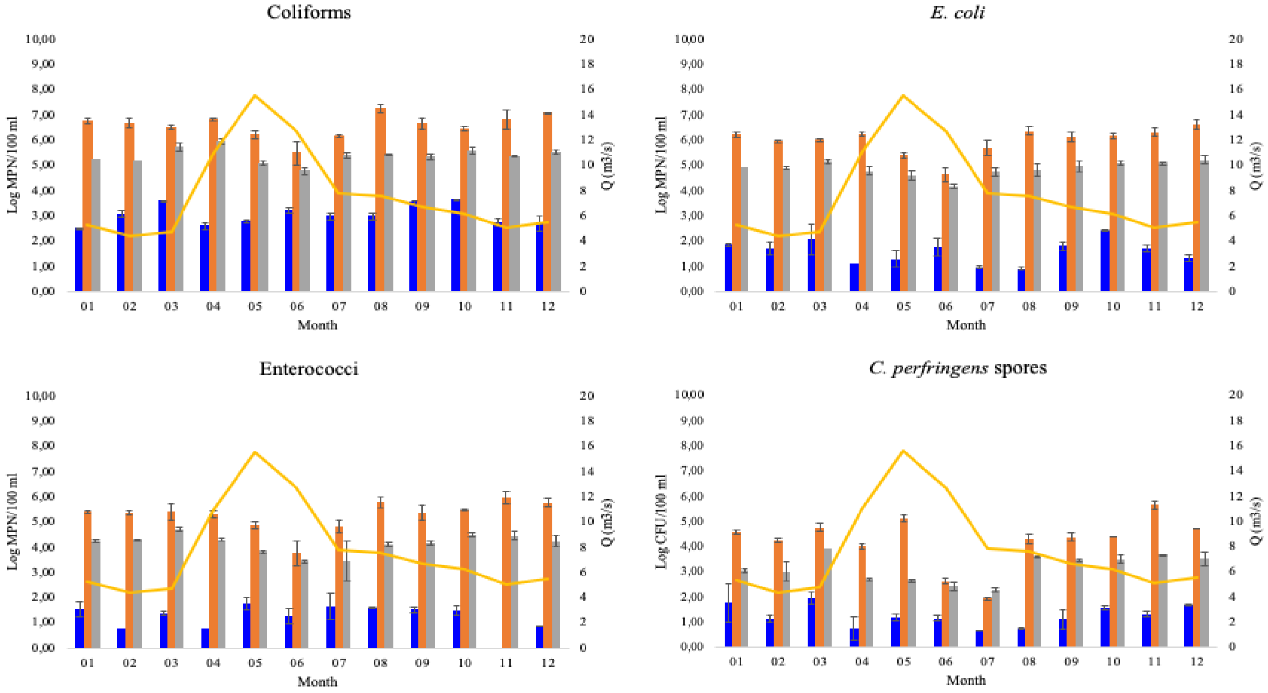
	Log MPN Coliforms	**Log MPN *E. coli***	**Log MPN Enterococci**	**Log CFU *C. perfringens* spores**
U *vs* W	< 0.001	< 0.001	< 0.001	< 0.001
U *vs* D	0.004	0.003	0.003	0.001
W *vs* D	0.008	0.016	0.016	0.055

Table S2. Results of detection with conventional PCR of *Salmonella* spp. and verocytotoxin-producing *E. coli*. U: upstream sample; W: wastewater discharge sample; D: downstream sample; +: presence; -: absence.

Sample	Month	*Salmonella* spp.	Verocytotoxin-producing *E. coli*				
		invA	O157	H7	Intimin	SLT-I	SLT-II
U	01	+	-	-	-	-	-
W	01	+	-	+	-	-	-
D	01	+	-	+	-	-	-
U	02	-	-	+	-	-	-
W	02	-	-	+	-	-	-
D	02	-	-	+	-	-	-
U	03	-	-	+	-	-	-
W	03	-	-	+	-	-	-
D	03	-	-	+	-	-	-
U	04	-	-	+	-	-	-
W	04	+	-	+	-	-	-
D	04	+	-	-	-	-	-
U	05	+	-	+	-	-	-
W	05	+	-	+	+	-	-
D	05	+	-	+	+	-	-
U	06	+	-	+	-	-	-
W	06	+	-	+	-	-	-
D	06	+	-	+	-	-	-
U	07	+	-	+	-	-	-
W	07	+	-	+	-	-	-
D	07	+	-	+	-	-	-
U	08	+	-	-	-	-	-
W	08	+	-	+	-	-	-
D	08	+	-	+	-	-	-
U	09	-	-	-	-	-	-
W	09	+	-	+	-	-	-
D	09	+	-	-	-	-	-
U	10	+	-	-	-	-	-
W	10	+	-	+	-	-	-
D	10	+	-	+	-	-	-
U	11	-	-	-	-	-	-
W	11	+	-	+	-	-	-

D	11	-	-	-	-	-	-
U	12	-	-	-	-	-	-
W	12	+	-	+	-	-	-
D	12	+	-	+	-	-	-

Fig. S5. Association between streamflow and faecal indicator concentrations. Upstream values are reported in blue, waste values in orange, downstream values in grey.



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4. *General conclusions*

The effects of climate change can manifest in various forms across different regions of the planet. While the direct loss of human lives is more easily linked to extreme events driven by climate change, the connection between the indirect effects of climate change and Public Health is more complex. Among the most significant indirect effects is the development of diseases resulting from the degraded quality of environmental matrices. Water is one of the environmental matrices most susceptible to climate change effects. As a resource closely linked to climate, any alteration can also induce changes in the hydrological cycle, resulting in seasonal changes in river flows and increased severity and frequency of floods and/or droughts in some regions. This trend is expected to lead to a decline in river flow and quality and an overall reduction in water availability in tropical and semi-arid regions. Exacerbating the issue of freshwater scarcity, driven by global reductions in polar and alpine glacier reserves and drought events, is the increased demand for water needed to meet the primary needs of a constantly growing global population. Water scarcity linked to climate change also necessitates the development of new sustainable strategies to address this shortage while safeguarding Public Health. According to the One Health concept, human, animal, and environmental health are deeply interconnected, necessitating a multidisciplinary approach to tackle global challenges, such as climate change.

A proposed approach in this context is the reuse of treated wastewater from wastewater treatment plants (WWTP). The research activity conducted during the Ph.D. 3-year activities was aimed at investigating the possible human health impact of this necessary practice in terms of microbiological risks (especially antibiotic resistance and pathogens).

The main achievements obtained can be summarised as follows:

- Wastewater treatment can significantly reduce pathogen and antibiotic resistance elements (antibiotics, bacteria, and genes). Despite this, these elements are still present in the effluent for reuse in all the WWTPs monitored in the present research activity (North and North-East Italy and South Spain).
- In a broader context of urban water cycle, the revision of the literature on antibiotic resistance in drinking tap water supports its potential role in environmental pathways contributing to antibiotic resistance.
- Not only waters are impacted by antibiotic resistance spreading. Antibiotic resistance genes were also detected in the air sampled in sites representative of Public Health (WWTP, farm, hospital, and urban area). WWTPs, in particular, can be recognised not only as reservoirs and sources of ARGs in treated effluents but also as potential emitters of ARGs into the air, raising additional concerns when the reclaimed water is intended for reuse applications.
- Besides antibiotic resistance elements, in the effluent for reuse, it was also possible to detect high quantities of *Legionella* spp. DNA, revealing the need to investigate routinely also the molecular aspect to avoid an underestimation of the risks.

- The WWTP effluents that are not reused are discharged into natural water bodies, such as rivers. The reduced river flow caused by climate change, in combination with WWTP discharge, is inversely correlated with faecal indicator bacteria concentration and pathogen presence. This indicates that the natural dilution capacity of rivers is strongly impacted by water shortage, increasing direct risks for human health.

The research findings emphasize the necessity for continuous monitoring of the microbiological risks associated with water reuse, particularly given the growing reliance on this practice due to climate change-driven water scarcity. The presence of antibiotic resistance genes and opportunistic pathogens in treated wastewater suggests that although current wastewater treatments are effective, they are not entirely sufficient to eliminate all potential health threats. Further improvements in treatment technologies, combined with stricter regulatory frameworks, are essential to ensure safe water reuse.

Wastewater is not the only matrix affected by this issue; tap drinking water is also impacted, highlighting its potential role as both a reservoir and a vehicle for antibiotic resistant bacteria and their genetic determinants.

Additionally, the detection of antibiotic resistance genes in air samples at various sites highlights the importance of considering airborne transmission as a potential route for the spread of antibiotic resistance. Moreover, the gene quantities detected also at WWTP site, especially considering the effluent reuse possibility, call for further interdisciplinary research to understand the role of air in the dissemination of antimicrobial resistance and the potential implications for Public Health.

The detection of pathogens in wastewater, such as *Legionella*, highlights its potential role in environmental dissemination and Public Health risk. Effective monitoring with a cultural and molecular combined approach is essential to mitigate its spread and reduce infection risks.

The interplay between reduced river flow, climate change, and wastewater discharge also highlights the importance of adopting integrated water resource management strategies. The correlation between lower river flow and higher concentrations of faecal indicator bacteria and pathogens suggests that climate change not only exacerbates water scarcity but also increases the burden of microbial contamination in natural water bodies. In this context, rivers often act as *de facto* recipients of treated wastewater, leading to indirect reuse scenarios where downstream communities may rely on these impacted water bodies for various purposes, including drinking water abstraction and irrigation. This underscores the urgent need for innovative approaches to mitigate these risks, such as advanced wastewater treatment processes, improved water monitoring systems, and the promotion of responsible water consumption behaviours.

In conclusion, the conducted Ph.D. research activity highlights the need to monitor the indirect effects of climate change on Public Health, with particular attention to the impact it may have on water in a One Health approach. Additionally, it underscores the necessity of assessing the risks associated with new strategies that can be employed to address water scarcity, such as water reuse, to safeguard Public Health. Future research should focus on refining wastewater treatment methods to further reduce the presence of antibiotic resistance elements and pathogens, as well as investigating the potential long-term health implications of exposure to these contaminants. Only through a multidisciplinary and proactive approach can we ensure that climate adaptation strategies not only address water shortages but also protect global health.

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